FINAL REPORT

ER-1319: Genetic and Biochemical Basis for the Transformation of Energetic Materials (RDX, TNT, DNTs) by Plants

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April 2007

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SERDP Project ER-1319

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Energetic Materials (RDX, TNT, DNTs) by Plants

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PROJECT TITLE

Genetic and Biochemical Basis for the Transformation of Energetic Materials (RDX, TNT, DNTs) by Plants

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(I). EXECUTIVE SUMMARY

The energetic materials (RDX, TNT, DNTs) are possible sources of groundwater and surface soil (<1 ft) contamination at DoD training and testing sites. RDX, in particular, is more mobile than the other compounds in groundwater. Phytoremediation is an inexpensive, self-sustaining treatment technology that may be suitable for prevention of contamination. Phytoremediation of energetic materials (RDX, TNT, DNTs) requires basic knowledge of the transformation pathways of the energetic materials for several purposes. Selection of high-performing native plants, engineering plants with enhanced transformation capabilities, identifying the fate of transformation products in the plants, and designing the external variables to operate a more effective phytoremediation process are all dependent on a knowledge base of the genetic structure, enzymatic structure, and biochemical reaction pathways.

This project used *Arabidopsis thaliana* as a model plant system to perform genetic and biochemical studies to assist in the identification of the genes, enzymes and pathway structure of metabolism of energetic materials. The strategy involved selection of mutants resistant to the energetic compound, and then genetic and metabolic characterization of the mutants. However, as a prerequisite to this strategy, two types of *Arabidopsis thaliana* mutant libraries, T-DNA insertion and Enhancer (4X)-trap, were generated and screening assays for resistance to TNT and RDX needed to be designed and implemented. Furthermore, uptake and metabolic fate studies of *Arabidopsis thaliana* exposed to RDX, TNT, and DNTs were necessary as controls to compare with the mutants. Thus, these prerequisite studies alone contributed to the tool and knowledge base on transformation of energetic materials in this genetically powerful plant system.

Knowledge of phytotoxicity effects of plants to TNT and RDX from prior literature was used as an aid to design. Although the NOAEL (no observed adverse effect level) concentration for phytotoxicity of TNT to plant seedlings is on the order of 5 mg/L in liquid media (reviewed in Burken et al., 2000), the toxicity of TNT for germination in liquid or solid media had not been studied previously. Toxicity of TNT to seed germination of 25–35 mg/L (level is dependent on seed concentration, among other variables) was determined for selection of *Arabidopsis* mutants for this project. The TNT germination screen was successful. From screening of approximately 250,000 seeds of T-DNA insertion mutant library and 300,000 seeds of the enhancer trap mutant library, 8 and 12 mutants, respectively, were selected for their greater TNT resistance.

RDX phytotoxicity is understood even less than for TNT. Since in poplar grown hydroponically, RDX is less phytotoxic (NOAEL ~ 20 mg/L) than TNT (Burken et al., 2000), this suggested apriori that the screening assay would be more difficult to develop for RDX. This indeed was the case. *Arabidopsis thaliana* seeds were still able to germinate at close to levels of 1400 mg/L RDX in solid agar media. Precipitation (crystals) was observed at 500 mg/L RDX in solid media and significant precipitation of RDX occurred at levels above 1000 mg/L RDX. Thus, the germination screen was not an effective screening method for RDX due to the limited toxic effects observed. A root length screening method (0 – 200 mg/L RDX) and an aerial screening method also indicated limited toxicity of RDX to plant growth.

The ability to screen for mutants resistant to TNT and not RDX suggested a difference in their ability to metabolize the parent compound and/or transformation products. TNT levels of 0.8 mg/L inhibited root growth in solid media and 1.0 mg/L reduced seedling growth yield in liquid media. [U-^{14}C] TNT mass balance studies and metabolite feeding studies of *Arabidopsis* seedlings in liquid culture showed the conversion of TNT to hydroxylamines, conjugates and unextractable-bound compounds, in agreement with the green-liver model and *Arabidopsis* transformed 4-substituted metabolites more efficiently than the 2-substituted metabolites. In contrast to TNT, RDX was not toxic to *Arabidopsis* at concentrations up to 35 mg/L RDX (close to the solubility limit) in liquid media. Furthermore, [U-^{14}C] RDX studies of axenic *Arabidopsis* seedlings grown in liquid media in the light indicated over 50% of the radioactivity being taken out of the media and over 11% of the ^{14}C label being completely mineralized to carbon dioxide after 35 days. The results of this study are consistent with the hypothesis developed by Jerry Schnoor and his collaborators (Van Aken, B. et al., 2004) that light exposed plant tissues can mineralize RDX.

Southern blot analysis of the T-DNA and Enhancer (4X)-trap mutants revealed that the Enhancer (4X)-trap mutants had fewer insertion sites. Thus the Enhancer (4X)-trap mutants were characterized by TAIL-PCR with subsequent sequencing and BLAST analysis of the PCR products. The majority of insertions occurred between two genes but in a few cases T-DNA insertion occurred in either an exon or an intron of a gene. About half of the genes have unknown function. Although the other half of the genes show similarity to the known genes from other organisms, none of the genes' functions have been experimentally identified in *Arabidopsis*. Most of the putative functions are involved in regulation of transcription and signal transduction pathways. Notably missing were genes for enzymes potentially involved in detoxification of TNT, such as nitroreductases, cytochrome P450s, and glutathione S-transferases. Salk T-DNA mutants, in which their insertion sites are similar to the sites of the T-DNA insertion in our Enhancer (4X)-trap lines, did not germinate better than the wild-type when challenged with 10 - 30 mg/L TNT. This implies that resistance phenotype of the activation-tagged mutant lines were not caused by an interruption of a gene, but possibly by an enhancement of a gene up-regulated by the 4X 35S enhancer element in the T-DNA.

The Enhancer (4X)-trap mutants were tested for their ability to transform TNT in liquid culture, when TNT was added at either exponential or stationary growth phase and from concentrations ranging from 6 mg/L to saturated levels, ~170 mg/L. A difference in TNT removal capacity between the wild-type and the mutants was observed when TNT was added to seedlings during their exponential growth phase at only very high TNT concentrations (120 mg/L – 170 mg/L). For the cultures that transformed TNT faster, the mutant plants appeared healthier than the wild-type plants at these levels. In TNT phytotoxicity studies, mutant and wild-type seedlings had similar growth rates and biomass yields at low levels of TNT (2.5–15 mg/L) in liquid media. Root assays at TNT levels from 0.2 to 0.8 mg/L showed the same degree of growth inhibition for the mutants and wild-type. These results in conjunction with genetic results suggest that the mutants do not possess any specific advantages in transforming TNT; for example, they do not possess upregulated genes such as nitroreductases that are directly involved in TNT transformation. Instead, the mutants appear to be more resistant to the phytotoxic effects of TNT at very high levels of TNT, which explains their better health. Since they are less affected by TNT than the wild-type, this manifests in a better rate of TNT

transformation. This situation, wherein the greater resistance to TNT by the mutants is not due to a specific over-expression of an enzyme directly involved in TNT transformation, is more difficult to analyze. Drawing out the precise nature of the mutation, and the phenotypic difference between the mutant and the wild-types upon exposure to TNT will require significantly more research.

In contrast to TNT and RDX, less is known about the metabolism and fate of DNTs in plants. The scarcity of the research on phytoremediation of dinitrotoluenes may come from the speculation that the application of phytoremediation of dinitrotoluenes can be based on TNT studies due to the similar chemical structures between TNT and DNTs. However, the nitroaromatic explosives showed different fates in bioremediation. For example, DNTs are mineralized by aerobic bacteria (Nishino, et al., 1999) while TNT is not (Burken et al, 2000). Thus, studies on the phytotoxicity and fate of the dinitrotoluenes, 2,4-DNT and 2,6-DNT, and gene expression in response to DNT exposure were performed in *Arabidopsis*.

From the results of biomass assays for toxicity, 2,4-DNT was more toxic than 2,6 DNT, which is more toxic than TNT. As a consequence, rates of uptake were slowest for 2,4 DNT, then 2,6 DNT, then TNT. Interestingly, in root growth assays, similar levels of growth inhibition was observed at 1 ppm for 2,4-DNT and TNT versus 20 ppm for 2,6-DNT. The results of the fate and distribution of [U-^{14}C]2,4-DNT or [U-^{14}C]2,6-DNT in *Arabidopsis* seedlings in liquid culture are similar to those of TNT, indicating that the green-liver model is followed. Most of the radiolabel is located in the root tissue and mineralization by the plants was not observed. Also, the intracellular bound portion accumulated with time after DNT amendment, suggesting transformed products of DNTs may be incorporated into plant tissues such as lignin and cellulose. Monoaminonitrotoluene isomers and unknown peaks with short retention times were detected as transformed products of 2,4-DNT and 2,6-DNT by the plants. These results show that the fate of the DNTs in plants after uptake is similar to that of TNT and although the kinetics of transformation are not quantitatively the same, phytoremediation strategies of the DNTs may be based from the studies on TNT.

One phytoremediation strategy is to design transgenic plants with enhanced transformation rates and reduced phytotoxicity, as first reported by Neil Bruce's laboratory (French et al, 1999; Hannick et al, 2001). In collaboration with Neil Bruce, high levels and rapid turnover of 4-hydroxylamino-2,6-dinitrotoluene were observed in transgenic tobacco containing nitroreductase from *E. cloacae*. The high transformation rate of TNT is likely due to an enhanced reductive activity of the bacterial nitroreductase over that of the endogenous tobacco enzymes. This enhanced activity may enable greater conjugation and sequestration of TNT, hence decreasing its phytotoxicity. Thus, if the same enzymes are used in the transformation of the DNTS as for TNT, one explanation possible for the difference in kinetics of transformation of the DNTs in comparison to TNT is a difference in substrate specificity for the enzymes involved in detoxification.

The monitoring of plant gene expression in response to exposure to explosives as a means to identify specific genes potentially involved in plant detoxification of explosives. Enhanced expression of glutathione *S*-transferase (GST) genes in *Arabidopsis* (Ekman, et al., 2003; Mezzari, et al., 2005), horseradish (Ekman, et al., 2003) and poplar (Schnoor et al, 2006)

exposed to TNT has been reported. In our study, glutathione levels and expression of related genes (*GSH1* and *GSH2*) in Arabidopsis plants exposed to 2,4-DNT were increased 1.4 fold and 1.7 fold, respectively, compared to untreated plants. Genes of a glutathione *S*-transferase (GST) and a cytochrome P450, which were induced by 2,4,6-trinitrotoluene exposure in previous studies, were upregulated by 10 and 8 fold, respectively, in response to 2,4 DNT and 4.7-fold and 14-fold, respectively, in response to 2,6 DNT. Thus, in addition to the similarities in metabolism and fate of the DNTs with TNT, similar expression patterns are observed of these candidate genes.

To further investigate the role of GST, a homozygous *gst* mutant line was isolated in the specific GST monitored in the gene expression studies ((At1g17170). Comparison of uptake rates and root growth between the wild-type and the *gst* mutant amended with 2,4-DNT, 2,6-DNT or with TNT was performed. The uptake rates and the tolerance at different concentrations of 2,4-DNT, 2,6-DNT and TNT were not significantly different between the wild-type and the *gst* mutant, implying that induction of this GST gene is not related to the detoxification of these compounds in vivo. Although a purified GST enzyme may be able to conjugate TNT in an in vitro enzyme assay, this enzyme may not be the one used in vivo. There may be other enzymes in vivo that have a higher affinity for the parent compound. Thus, genes that are induced in response to xenobiotics from a quantitative gene expression study, such as SAGE analysis and real-time PCR, does not necessarily warrant the involvement of these genes in the detoxification pathway or in their involvement in tolerance to the xenobiotic by plants. Despite this fact, genes that are induced are good targets to test for activity in vivo. With the current availability of Arabidopsis mutants, many gene targets can be tested for pathway activity or tolerance, and is the subject of future research.

(II). PROJECT BACKGROUND

The energetic materials (RDX, HMX, TNT, DNTs) are possible sources of groundwater and surface soil (<1 ft) contamination at DoD training and testing sites. RDX, in particular, is more mobile than the other compounds in groundwater. Phytoremediation is an inexpensive, self-sustaining treatment technology that may be suitable for prevention of contamination. Phytoremediation of energetic materials (RDX, TNT, DNTs) requires basic knowledge of the transformation pathways of the energetic materials for several purposes. Selection of high-performing native plants, engineering plants with enhanced transformation capabilities, identifying the fate of transformation products in the plants, and designing the external variables to operate a more effective phytoremediation process are all dependent on a knowledge base of the genetic structure, enzymatic structure, and biochemical reaction pathways.

(III). TECHNICAL OBJECTIVE

This project (ER-1319) responds directly to the SERDP statement of need (CUSON-02-03) to address the potential remediation of the energetic materials (RDX, TNT, DNTs) via plant processes. We combined our expertise in biochemical engineering and in the life sciences and engineering to assist in the construction of a genetic and biochemical knowledge base for transformation pathways of energetic materials (RDX, TNT, DNTs) in plants. We used

Arabidopsis thaliana as a model plant system to perform genetic and biochemical studies aid the identification of the genes, enzymes and pathway structure of TNT metabolism. The biochemical approach used for TNT studies was used in determining fates of RDX and the DNTs, and combined with a genetic approach to understanding energetic material metabolism, it is expected that a sound knowledge base on transformation of energetic materials will be obtained.

OVERALL OBJECTIVE:

To construct a genetic and biochemical knowledge base for the transformation pathways of energetic materials (RDX, TNT, DNTs) by exploiting the fact that these chemicals are phytotoxic ~5 ppm (TNT) to ~20 ppm (RDX).

Specifically:

1. to screen mutagenized populations of the model plant, *Arabidopsis thaliana*, to isolate mutants resistant to RDX, TNT, and the DNTs, due to under or over-expression of individual genes
2. to genetically analyze mutants
3. to use metabolite analyses to select final mutants and characterize function of mutants.

(IV). TECHNICAL APPROACH

Plants have the ability to act as important phytoremediation agents since they can remove and detoxify organic contaminants found in the soil and groundwater through the "green liver" concept. Studies with RDX, TNT, and the DNTs have shown the potential of phytoremediation of these materials, although to varying degrees. Basic knowledge of the transformation pathways (and of the enzymes that catalyze the transformation reactions) of the energetic materials will aid phytoremediation design and assessment.

Fate and product identification studies have established that plants can transform TNT and DNTS, and bioaccumulate RDX. Beginning frameworks for the transformation pathways have been established for TNT. At this juncture, however, biochemical studies alone will not help elucidate the transformation pathway. Genetic tools are needed as well. A holistic use of genetic and biochemical studies of these complex systems will enable us to identify the genes, enzymes and pathway structure of TNT, DNT and RDX metabolism.

Arabidopsis thaliana is the model plant system used to perform these studies. RDX, TNT, and DNTs are all phytotoxic to plant growth. Hence a screen (T-DNA and Enhancer-Trap libraries will be used) to select for mutants with resistance is possible, but techniques that probe expression of genes can also be included as an alternative approach by searching for genes induced from these chemicals. The availability of knockout mutants (T-DNA mutants in particular) from the *Arabidopsis* genome projects then enables one to obtain a putative mutant to test the hypothesis on whether or not a gene that shows an abundant transcript in plants exposed to an energetic material is involved in energetic material metabolism. The combination of these techniques increases the success rate of obtaining *Arabidopsis* mutants with which to characterize with metabolic analyses in order to determine function or to test hypotheses.

Once the mutant lines in *Arabidopsis* that are tolerant to energetic materials are identified, molecular techniques will be used to isolate and clone the gene. Basically, mutants obtained using T-DNA and Enhancer-Trap libraries will use PCR technology. When the gene of interest has been cloned, its role in causing the explosive-resistance phenotype will be probed via metabolic characterization.

Central to the work of characterizing the mutants will be to have a sound base on which to analyze the transformation of RDX, TNT, and DNT metabolism in *Arabidopsis* biochemically. Mass balances and metabolite analyses will be performed for wild-type *Arabidopsis* using previously established methods. A HPLC system and library from our previous work were used to carry out analysis of the particular energetic materials and their transformation products. These experiments were used in the metabolic screen to narrow selection to the most desirable mutants, and also characterizing the mutants in more detail metabolically once selected.

It is possible that a mutant may be resistant to energetic materials but not differ in the profile of transformation products observed. In other words, a change in plant metabolism not directly associated with the biochemical pathways of the energetic material is possible – the change is one that reduces phytotoxicity but not the transformation of the parent compound. This type of change has traditionally been very difficult for molecular biologists to study, since in this case, the metabolism of the whole plant needs to be analyzed to determine the change.

(V). PROJECT ACCOMPLISHMENTS

1. Generation of mutants

1.1 Library generation

Three libraries of mutant *Arabidopsis thaliana* plants were created to screen for plants with alterations in TNT, RDX, or DNT metabolism. The results are shown in Table 1. The largest library contains 1.5 x 10^6 different genetic lines each containing 1 to 3 T-DNA knockout mutations. This library was constructed using the plasmid pCB302 as the mutagen (Xiang et al. 1999). Insertion of this T-DNA construct in the promoter region or the coding region of the gene will usually disrupt expression of that gene. The second library, the enhancer-trap library, contains about 1.5 x 10^5 different lines and is constructed using the vector pSKI015 (Weigel et al. 2000). This is an activation-tagging vector. It will knockout a gene if it lands in the coding region but can enhance expression of the gene if it lands in the promoter region. The third library is 1.25 x 10^5 individuals that have been mutagenized by EMS. These are point mutations and they can increase or decrease expression levels of a gene and can even changer the substrate specificity of the resulting protein.

The pCB302 tagged genes are fairly straightforward to clone using PCR. The pSKI015 tagged genes are very easy to isolate using the plasmid rescue features of the insert. The EMS mutagenized genes are difficult to isolate and must be done by genetic mapping techniques.

Table 1. Libraries of mutant *Arabidopsis thaliana*

Library	Mutagen	Number of lines	Gene Identification
1	pCB302	1.5 x 10^6	PCR
2	pSKI015	1.5 x 10^5	Plasmid rescue
3	EMS	1.25 x 10^5	Mapping

1.2 RDX Screening Assays for Wild-Type Seedlings

1.2.1 RDX Screening Assay for in Petri-Dishes

An assay was developed in order to screen for *Arabidopsis thaliana* mutants resistant to RDX. Many factors are considered in the design of an assay to screen 300,000 lines for which the end result is the indication of a clear toxicity effect to the wild-type plant for a reasonable cost of the assay. The amount of chemical, the form in which the chemical is administered, the number of seeds/seedlings per plate, media and culture conditions for the plant and the timing of amendment of the chemical are all design variables. Knowledge of phytotoxicity effects can be gleaned from the literature as an aid to the design. The TNT screening assay that was developed for this project involved a fair number of iterations. Although the NOAEL (no observed adverse effect level) concentration for phytotoxicity of TNT to plant seedlings is on the order of 5 ppm in liquid media (reviewed in Burken et al., 2000), the toxicity of TNT for germination in liquid or

solid media had not been studied previously. Toxicity of TNT to seed germination of 25-35 mg/L (level is dependent on seed concentration, among other variables) was determined for selection of *Arabidopsis* mutants for this project. Since RDX toxicity is understood even less than for TNT, and RDX is less toxic than TNT, this suggested that the screening assay would be more difficult to develop.

A limited number of studies (reviewed in Burken et al., 2000) have indicated that the NOAEL in maize and wheat grown hydroponically is estimated to be 13 mg/L, with toxic levels at 21 mg/L. Similar results were observed in sorghum and soybean plants and in hybrid poplars- no toxic effects for RDX concentrations up to 21 mg/L and after 30-days of exposure and 14 days of exposure, respectively for hydroponically grown plants. The saturation level of RDX in water is approximately 40 ppm at room temperature, but the saturation level of RDX in complex growth media is unknown. Thus, a wide range of RDX levels were tested for this assay. In addition, the source of RDX (RDX in a given solvent) for successful dissolution in complex solid media without significant toxic effects from the solvent was a critical factor in the design.

Arabidopsis thaliana wild-type seeds were grown in solid media containing various concentrations of RDX in order to determine the toxicity of RDX on germination. Medium consisted of 2.2 g of Murashige and Skoog (MS) salt mixture (Gibco BRL Life Technologies), 3 ml of 6% KH_2PO_4, 1 ml Gamborg B5 vitamin solution, 20 g of sucrose (Sigma), and 5 g of 99% MES hydrate (Acros Organics) added to 1 L of water. The pH was adjusted to 5.8 with NaOH. Phytogel was then added to the media (2.0 g/L media), the media was autoclaved, then cooled in a 65°C water bath. RDX and acetone were added to the medium which was then poured into sterile petri dishes. RDX was obtained in a solution of acetone (50 mg/ml) from AccuStandard. Acetone concentrations were kept the same throughout a screening assay in order to compare the effect of the RDX only. The petri dishes were then cooled to let the media solidify.

Seeds were sterilized by one of two methods. The first method consists of applying 20% bleach to seeds for 15 minutes and then rinsing with sterile water three times. The second method sterilizes seeds by exposing seeds to chlorine gas for three hours. The chlorine gas is formed from combining 200 ml bleach with 20 ml concentrated HCl in a closed container. Sterilized seeds were plated on the solid media plates. The petri dishes were then sealed with Parafilm to prevent evaporation and reduce the risk of contamination. Plates were then incubated in a cold room at 4°C for 3 days, then moved to the growth room, 22°C with constant light, to germinate. Seeds were monitored to record the time of germination and the size of the plant. Some plants were then transferred to clean media, with no acetone or RDX, or to soil to observe if they would be able to fully mature after exposure to high concentrations of RDX.

Wild-type *Arabidopsis thaliana* seeds were used to determine conditions to be used for screening. Concentrations of RDX in the media ranged from 0 mg/L to 2000 mg/L. Acetone concentrations remained at 30 mg/L. Seed concentrations ranged from 150 seeds per plate to 2000 seeds per plate. Two controls were used for the screening; one control consisted of media with no acetone or RDX added, while the other control consisted of media with acetone only.

No toxicity effects were observed below saturation levels RDX for any seed concentration. Precipitation was observed at 500 mg/L RDX and significant precipitation of RDX occurred at levels above 1000 mg/L RDX. Figure 1 shows a media plate at 1500 mg/L TNT. The precipitated crystals are visible at the bottom of the plate.

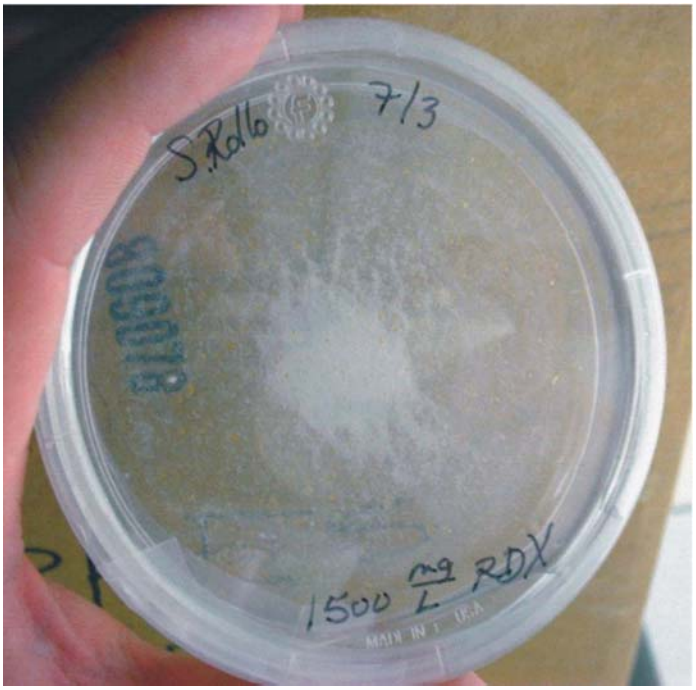


Figure 1. The medium was supersaturated with RDX at 1500 mg/L RDX.

Seed concentration was an important variable, as higher numbers of seeds screened per plate would lessen the time to obtain mutants considerably. Figure 2 shows a comparison of 1000 versus 2000 seeds per plate at RDX concentrations from 1200 – 1500 mg/l. Decreasing the concentration of seeds at a given concentration of RDX and acetone increased the toxic effects. In this concentration of RDX from 1300 mg/l – 1500 mg/l, the 2000 seeds/plate showed the same qualitative trends as 1000 seeds/plate relative to the 1200 mg/l RDX – but it was less toxic.

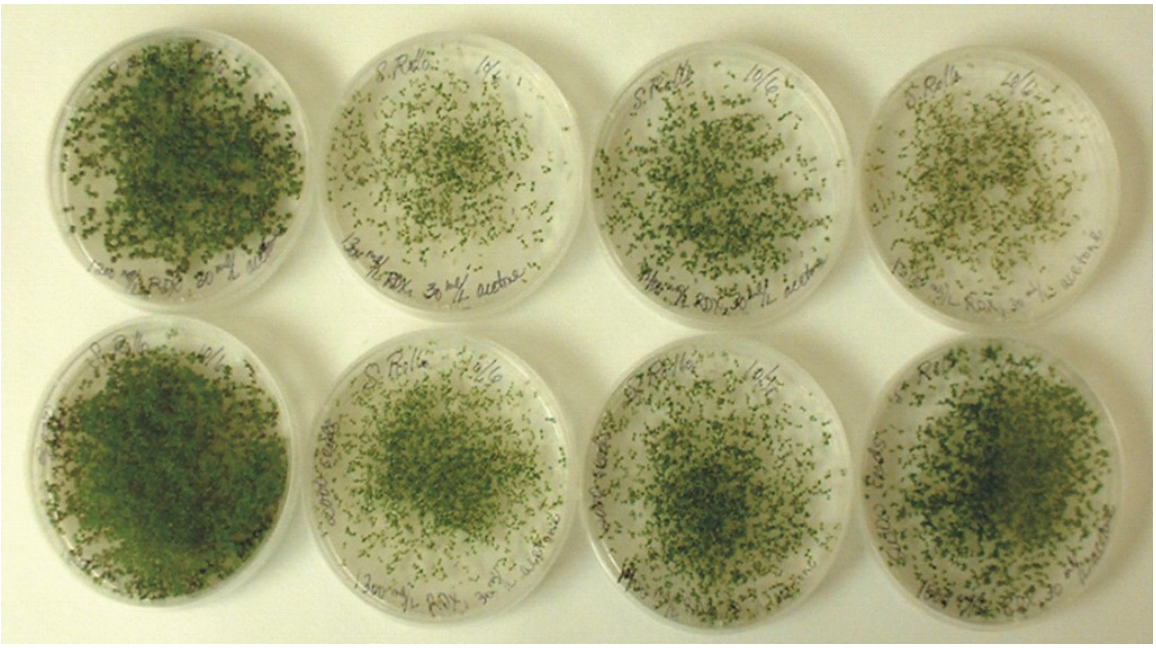


Figure 2. Ten day old plants. Concentrations of RDX, acetone and seeds are shown in the Table 2.

Table 2. Concentrations of RDX, acetone and seeds for plants in Figure 2

Plate Number	Concentration of RDX	Concentration of seeds
1	1200 mg/L	1000 seeds
2	1300 mg/L	1000 seeds
3	1400 mg/L	1000 seeds
4	1500 mg/L	1000 seeds
5	1200 mg/L	2000 seeds
6	1300 mg/L	2000 seeds
7	1400 mg/L	2000 seeds
8	1500 mg/L	2000 seeds

All plates have 30 mg/L acetone.

Shipping requirements for pure RDX require dissolution of RDX in a solvent. RDX is available in acetonitrile or in acetone as a solvent. Acetone is considerably less toxic to plants than acetonitrile (data not shown), thus acetone controls were necessary. The effect of acetone toxicity can be seen by comparing plates 1 and 2 in Figure 3. The effect of RDX toxicity can be seen by comparing plates 2 and 3 in Figure 3. Acetone has toxic effects, but the combination of RDX and acetone is more toxic.

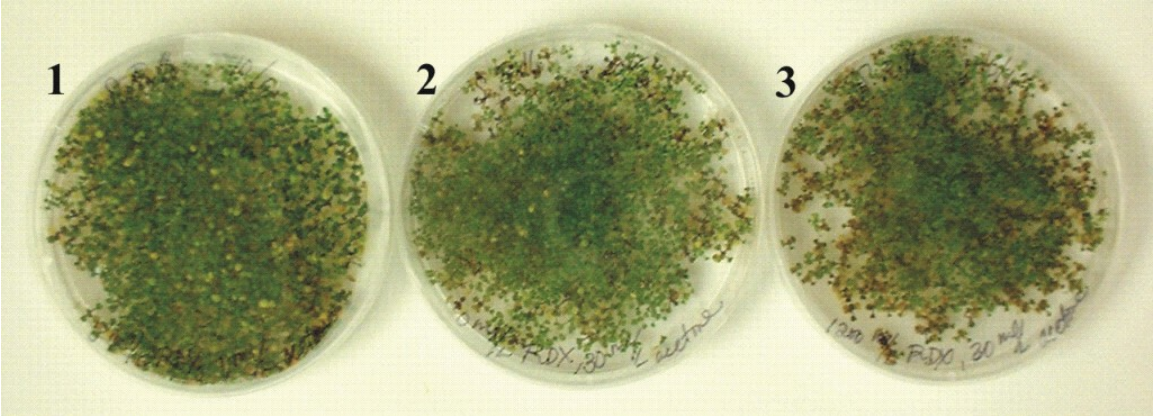


Figure 3. Plate 1 is a control with 0 mg/L RDX and 0 mg/L acetone. Plate 2 is a control with 0 mg/L RDX and 30 mg/L acetone. Plate 3 has 1200 mg/L RDX and 30 mg/L acetone. All three plates have 1000 seeds and are twelve days old.

The toxicity of RDX on germination and the development of seedling growth is indicated in Figures 4 through 7. Concentrations of RDX, acetone and seeds for these figures are shown in Table 3. Seed germination was delayed for RDX concentrations at 1300 mg/L and higher - at five days, these seeds had not yet germinated, as shown for in Figure 4. By 10 days, seeds at 1300 mg/L and higher had germinated (Figure 5), but are less vigorous than in the controls and at 1200 mg/L. The seeds that had germinated first also reached maturation first, as shown by the progression of browning at 12 days and 18 days in the controls and at 1200 mg/L RDX (Figures 6 and 7).

Table 3. Concentrations of RDX, acetone and seeds for Figures 4 – 7.

Plate Number	Concentration of RDX	Concentration of acetone
1	0 mg/L	0 mg/L
2	0 mg/L	30 mg/L
3	1200 mg/L	30 mg/L
4	1300 mg/L	30 mg/L
5	1400 mg/L	30 mg/L
6	1500 mg/L	30 mg/L

All plates have 1000 seeds.

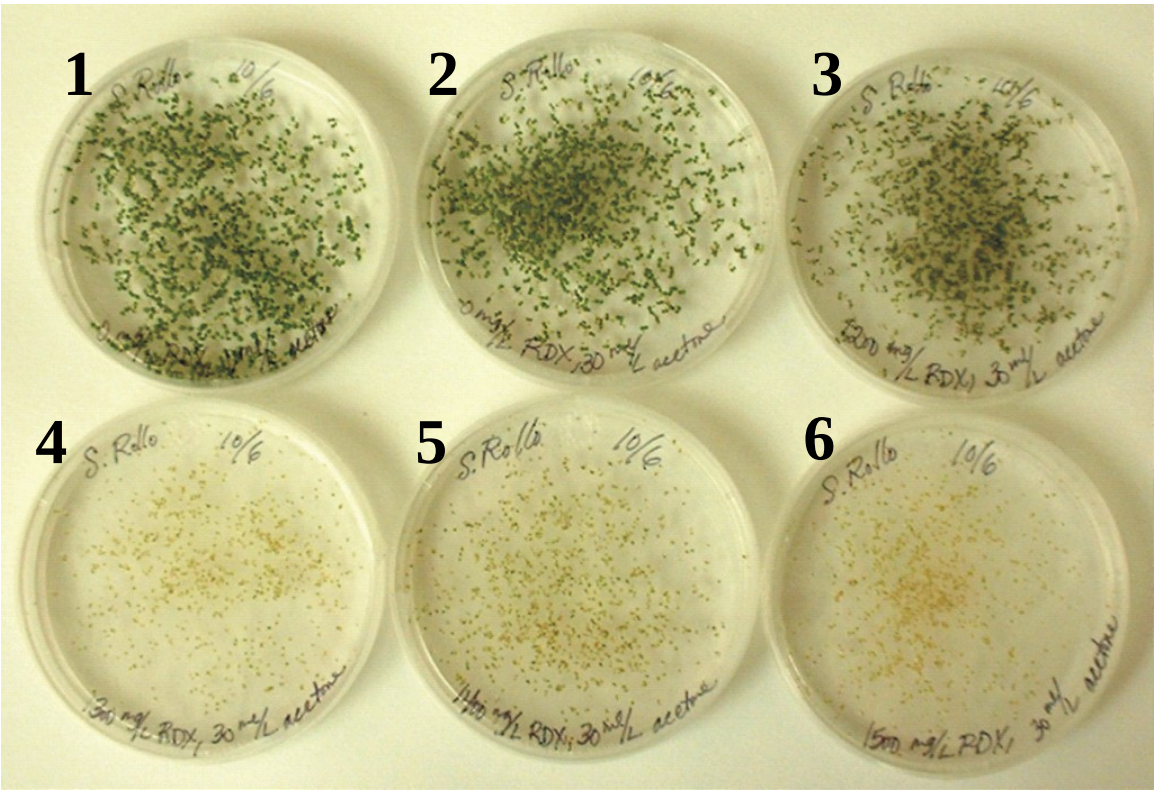


Figure 4. Five day old plants. RDX and acetone levels are given in Table 3.

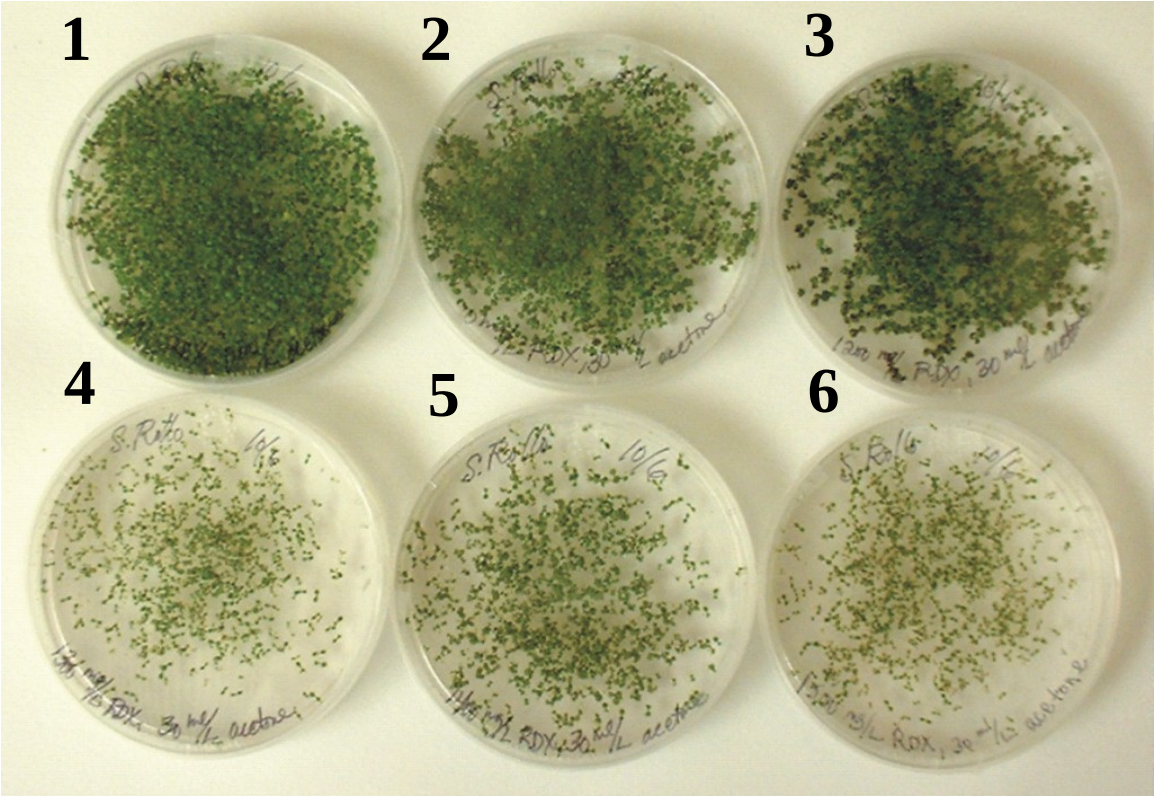


Figure 5. Ten day old plants. RDX and acetone levels are given in Table 3.

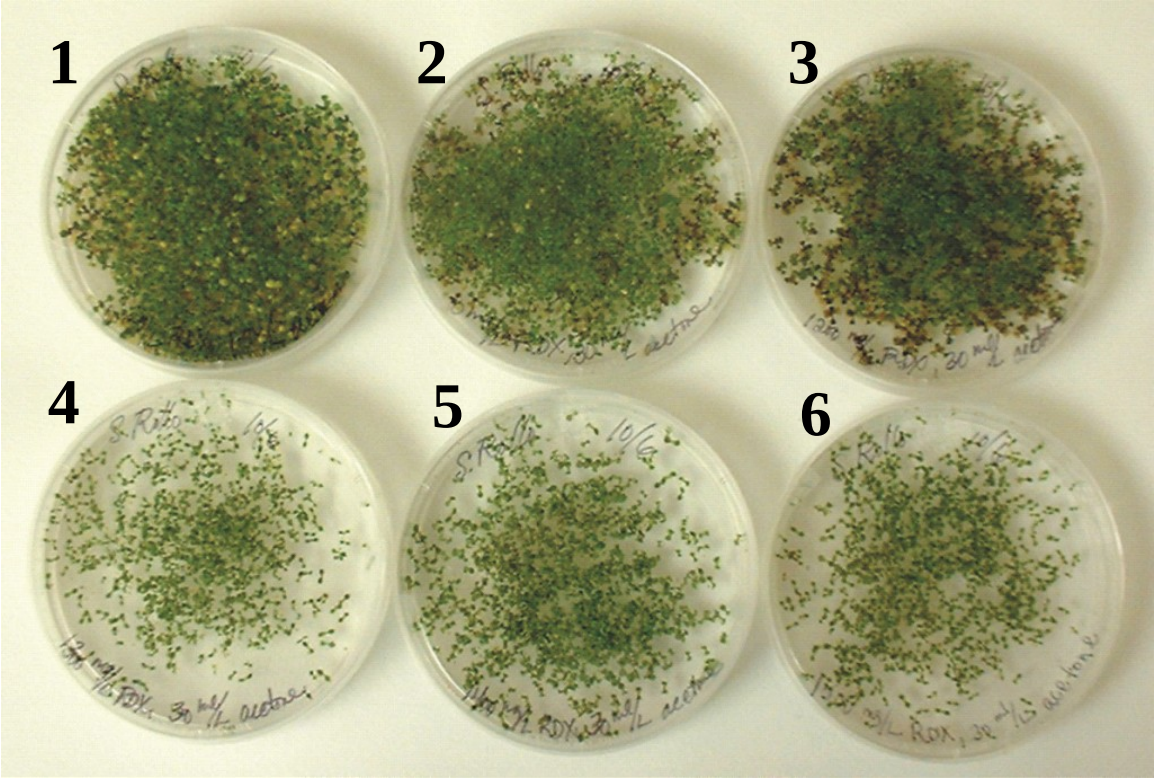


Figure 6. Twelve day old plants. RDX and acetone levels are given in Table 3.

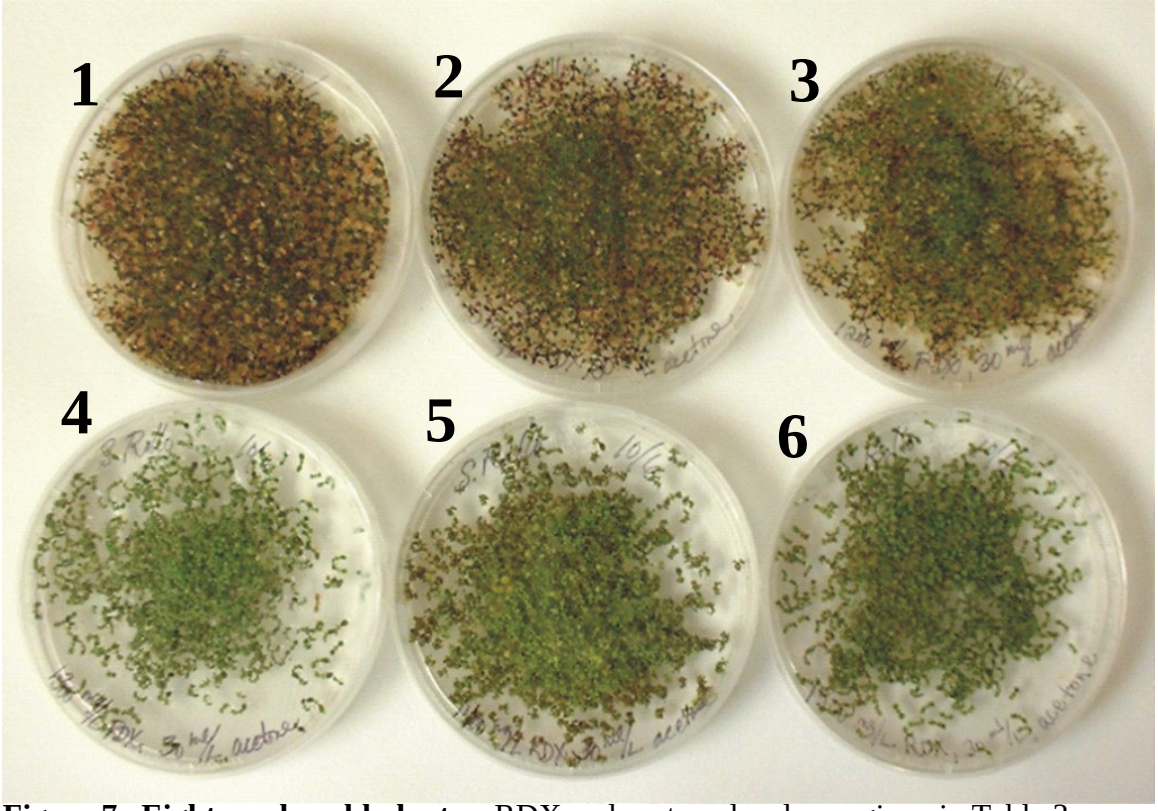


Figure 7. Eighteen day old plants. RDX and acetone levels are given in Table 3.

Upon further observation, it was determined that wild-type seeds are very stunted at 1400 mg/L and grow slowly. Furthermore, they have such severely damaged roots that the majority of the seedlings do not survive after being transplanted to clean soil at 24 days old. This result is in contrast to wild-type seeds grown at 1200 mg/L, for which the seedlings grew in soil when transplanted. The additional step of growth of putative mutants in soil further refines the screening of mutants. Any mutants that grow better than the wild-type will be selected for further screening.

Based on all the screening and soil transplantation experiments, the initial screening conditions were set at 1400 mg/L RDX and 28 ml/L acetone, and 2000 seeds per plate. Over 100,000 mutant seeds (25% of the mutant library) were screened for germination effects and no mutant candidates were selected.

Further details on these experiments are given in the Appendix in Rollo et al (2004a,b) and Rollo (2005).

1.2.2 RDX Screening Based on Root Growth

An alternative screen was developed based on Arabidopsis root growth upon their exposure to RDX. When *Arabidopsis* seedlings are exposed to RDX, their root growth may be severely stunted. Hence, an experiment which identifies RDX concentrations at which root growth is impeded was performed. When 4-day old wild type *Arabidopsis* seedlings were exposed to less than 50 mg RDX/L for 7 days, there were no significant differences in the root growth (Figure 8).

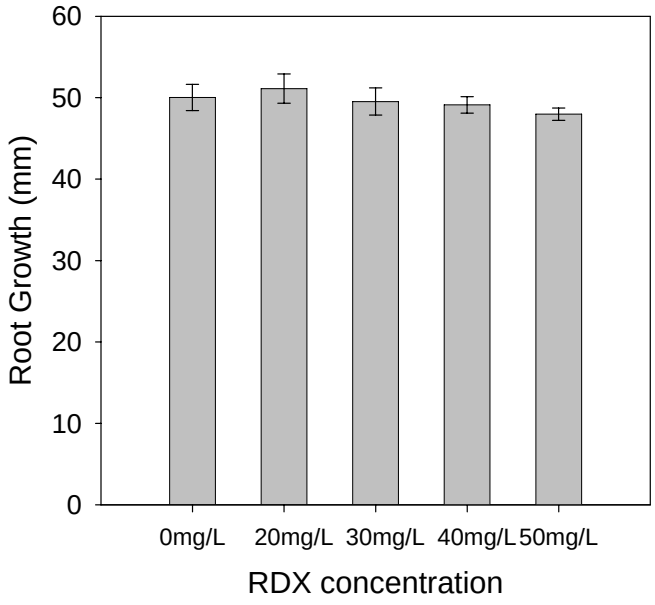


Figure 8. Root growth of the wild type seedlings exposed to different RDX concentrations. Error bars represent standard errors.

When the initial concentrations of RDX exposed to 4 day old *Arabidopsis* seedlings was increased to up to 200 mg/L, differences in root growth were observed. After 7 days of exposure to RDX, the root growth of wild type seedlings were inhibited significantly when they were exposed to 100 mg/L of RDX (Figures 9 and 10). The root lengths were seen to be reduced by over 50% when compared to the controls. However, it was observed that when seedlings were exposed to RDX concentrations of 150 and 200 mg/L, the toxicity effects declined and the root lengths began to increase. This observation could be because 100 mg/L RDX represents a super-saturated solution, whereas in the 150 and 200 mg/L systems, crystallization of RDX could have occurred thereby lowering the actual amount of RDX in solution.

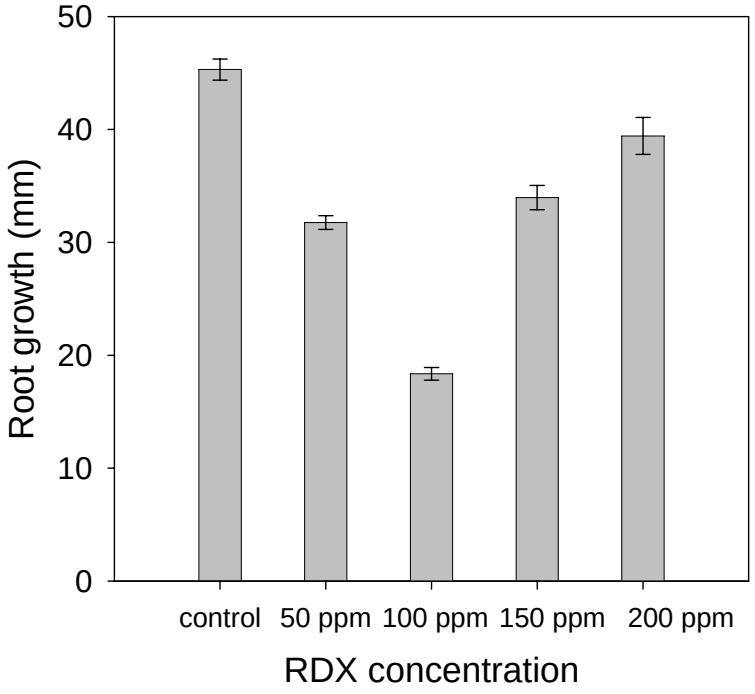


Figure 9. Root growth of the wild type seedlings exposed to different RDX concentrations. Error bars represent standard errors.

Further details on these experiments are given in the Appendix in Rollo (2005).

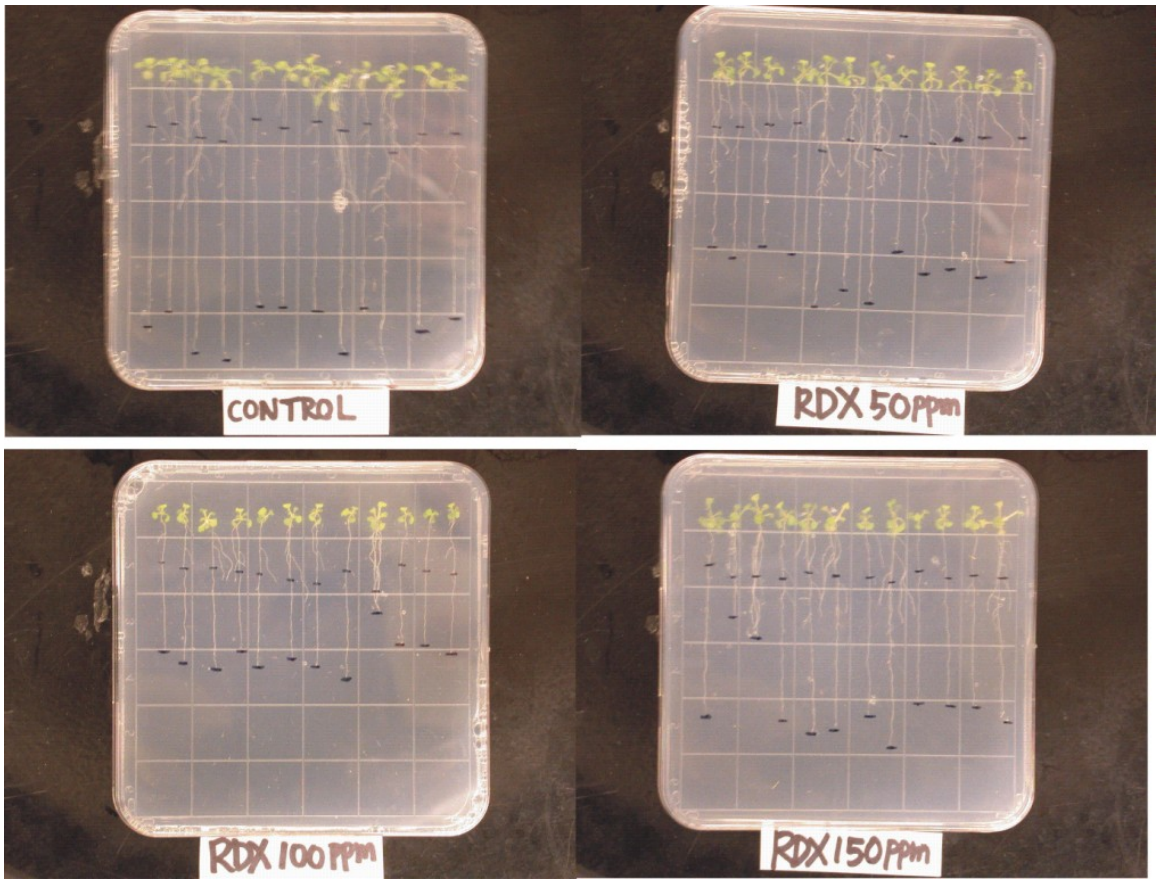


Figure 10. Photos of wild type seedlings after 7 day exposure to RDX.

1.2.3 RDX Screening Based on Aerial Application

As an alternative screening test, we sprayed 11-day-old wild-type seedlings with various concentrations of RDX stock solutions (50, 100, 200 mg/L RDX) in water with 1% acetone. The stock solutions were amended with 0.05 % Silwet detergent to assist the uptake of RDX through leaves, and were sprayed every two day for 10 days. RDX did not have a toxic effect on the growth of the seedlings as shown in Figure 11.

Figure 11. Photo of 11-day-old seedlings (left) and the seedlings treated with RDX (0, 50, 100, 200 mg/L from left to right in the tray) five times for 10 days (right). RDX did not show a toxic effect on the growth of the seedlings.

1.3 Screening Arabidopsis Mutants for RDX Resistance

Preliminary studies on screening *Arabidopsis* mutants using the determined initial conditions (1400 mg/L RDX with 2000 seeds/plate) have been commenced to isolate mutants with enhanced tolerance to RDX. The T-DNA seed pools were ordered from Arabidopsis Biological Resource Center (ABRC); over 62,000 seed lines were distributed among the 208 pools. The sterile cultured mutant seeds showed high levels of contamination, hence more stringent sterilization protocols have been developed. These include use of ethanol and a fungicide to pre-treat the seeds, and the use of longer sterilization times with higher bleach concentrations. These harsher sterilization conditions however retard seed germination and growth. Based on the various sterilization conditions, it was determined that using 70% ethanol for 10 minutes followed by 50% bleach for 25 minutes with fungicide in the media, can reduce contamination from 100% to 25%. Hence, these sterilization conditions will be used for the further screening studies.

Further details on these experiments are given in the Appendix in Rollo et al (2004a,b) and Rollo (2005).

1.4 Screening of mutants for resistance to TNT

1.4.1 Screening of T-DNA insertion mutants

Approximately 250,000 T-DNA insertion mutant seeds were screened for TNT resistance. The T-DNA insertion mutant lines were generated using a binary vector containing *NPTII* and *BAR* genes, which confer kanamycin and Basta resistance, respectively, to the transgenic plants. The seeds were surface-sterilized in a 20% bleach solution for 15 minutes with shaking and washed with sterile water three times. The sterilized seeds were plated on Whatman filter paper with 4 mL of *Arabidopsis* growth medium (half-strength MS salts, 0.018%

KH_2PO_4, B5 vitamins, 2% sucrose, 0.05% MES, pH 5.8) containing 20 mg/L TNT. After a cold treatment at 4°C for 3 days, the plates were incubated at 22°C under approximately 40 μmol/m^2/s continuous light from cool white fluorescent bulbs. The surviving seedlings were transferred to soil and grown to maturity and seeds were harvested from individual plants. From the primary screening, 221 mutant lines were selected from 22 superpools. In the secondary screening, about 200 seeds from individual mutant lines were plated on solid *Arabidopsis* growth medium (same as the liquid medium but contains 2 g/L Phytagel) containing 20 mg/L TNT. One hundred and twenty seven lines showed germination rates better than wild-type. Seeds of the 127 mutant lines were challenged again with higher TNT concentrations in the solid Arabidopsis growth medium. Thirty-nine mutant lines survived 23 mg/L TNT, whereas wild-type seeds showed no germination (Figure 12).

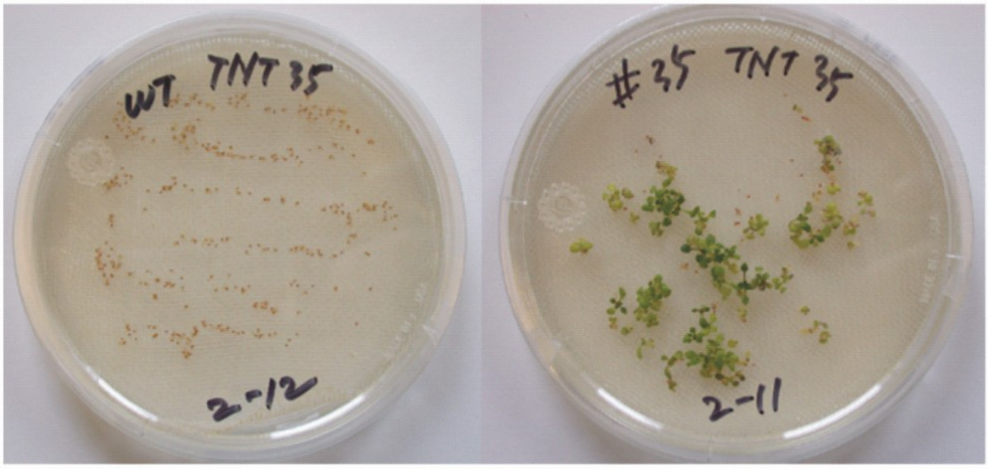


Figure 12. Comparison of germination between wild-type and a mutant line in the presence of 25 mg/L TNT 3 weeks after plating.

Eight final T-DNA insertional lines were chosen for further study based on their resistance to Basta treatment (Figure 13) and following a final challenge with 25 mg/L TNT.

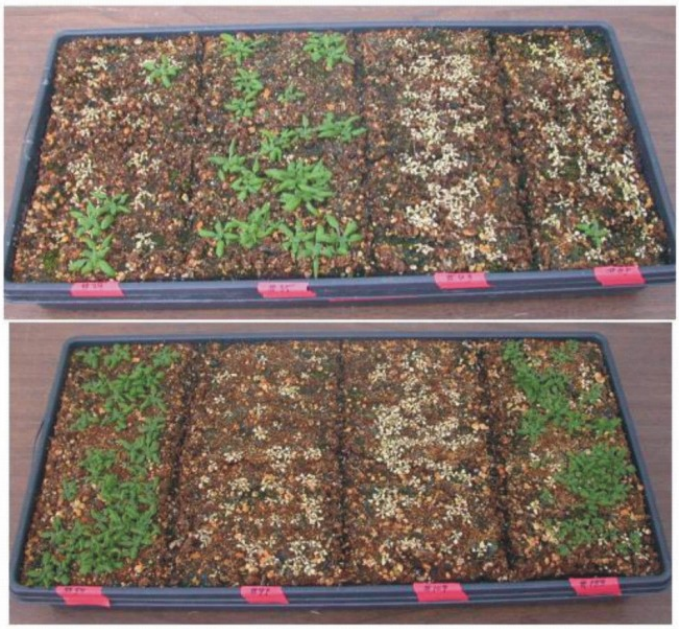


Figure 13. Basta treatment to the T-DNA insertion mutant lines. Two weeks-old seedlings were sprayed with Basta killing plants that are not resistant to the herbicide.

1.4.2 Screening of enhancer trap mutants

The enhancer trap lines were produced by the T-DNA vector, pSKI015, which contains the *BAR* gene for a selectable marker and 4x CaMV 35S enhancers which are designed to activate expression of genes near the T-DNA insertion (Weigel et al., 2000). 0.22 g of seeds from each of 25 superpools of enhancer trap mutant lines, approximately 300,000 seeds in total, were screened in the solid *Arabidopsis* growth medium containing 25 mg/L TNT. The density of seeds in a plate for the primary screening was about 2,000 seeds per 10 cm Petri dish. Two hundred and thirty five lines were selected and transferred to soil. They were grown to maturity and the seeds were harvested from individual plants. A secondary screening was carried out with approximately 200 seeds from each mutant line in a plate containing 25 mg/L TNT. Twenty-four lines showed germination significantly better than wild-type. They were challenged with 25 mg/L TNT again and their seedlings were sprayed with Basta. From the tertiary screening, 12 lines were selected for further characterization.

2 Metabolic and kinetic studies on wild-type *Arabidopsis thaliana*

2.1 Response of *Arabidopsis* to TNT

2.1.1 Toxicity of TNT to wild-type *Arabidopsis thaliana* seedlings

Wild-type *Arabidopsis thaliana* plants show toxicity at low levels of TNT when grown in liquid cultures under photoheterotrophic conditions, even though transformation activity is evident. One-week-old *Arabidopsis* seedlings were amended with TNT in the concentrations from 0–70 mg/L. For plants amended with 1 or 5 mg/L TNT, plant growth was retarded three days after TNT treatment. However, plants treated with 1 mg/L TNT remained healthy and caught up with the growth of the control plants after a week, whereas 5 mg/L treated plants showed signs of stress including some necrotic leaves and root growth significantly affected. One week after amendment with 10 mg/L TNT, *Arabidopsis* growth was significantly affected with roots turned brown and its growth retarded. At higher concentration levels, plants turned brown and/or bleached and their growth was severely reduced. Plant biomass is reduced by 40% at 40 mg/L and by 80% at 70 mg/L one week after amendment.

2.1.2 TNT transformation by wild-type *Arabidopsis thaliana*

TNT was added to 14-day-old hydroponic *Arabidopsis* seedling and media samples taken periodically until 120 hours. Initial TNT concentrations ranged from 20 to 125 mg/L (0.09 to 0.55 mM) per flask. The flasks were constantly shaken at 100 rpm, and were placed under light. A control with heat-killed biomass and a biomass free control were also amended with similar TNT concentrations. The biomass-killed control was to isolate the effect of absorption of TNT by the biomass, while the biomass-free control was used to identify the photodegradative and evaporative effects on TNT. A solely evaporative control was maintained by placing biomass-free TNT spiked media at 100 rpm in the dark. All controls had an initial TNT concentration of 50 mg/L (0.22 mM). Plants were sacrificed at 12 hours and at the end of the experiments (120 hours) and analyzed for their intracellular metabolites. Concentrations of TNT, 4HADNT, 2HADNT, 2ADNT and 4ADNT were determined from this analysis. In addition, presence of conjugates from the TNT metabolism by plant molecules was ascertained. Kinetic analyses of TNT disappearance from the medium were determined through linear regression of data assuming first-order kinetics with respect to TNT removal.

Rapid TNT removal was observed in the extracellular media at low and medium concentrations (7.5 mg/L – 58 mg/L). TNT was almost completely removed by 40 hours from the extracellular media. In contrast, the higher concentrations tested, 110 mg/L and 125 mg/L showed poor TNT uptake and removal, with a significant amount of TNT remaining in the media at the end of the experiment. At concentrations up to 58 mg/L the first-order TNT removal rate was $k = 4 \times 10^{-4}$ L/g FW*h, while at 110 mg/L and higher, the rate constant fell to $k = 2\text{-}4 \times 10^{-5}$ L/g FW*h, a loss in one order of magnitude.

In a separate experiment, wherein duplicates of 105 mg/L (0.46 mM) of TNT were added to one-week old axenic *Arabidopsis* seedlings, the metabolites 2HADNT, 4HADNT, 4,4'-Azoxy, 2ADNT and 4ADNT were detected. In addition, the previously identified

conjugates 4A-1, TNT-1, TNT-2 and 2A-1 were also observed (Figure 14). The hydroxylamines comprised greater than 20% of the initial TNT, while 4ADNT was formed between 10 to 15% (fraction of initial), while the 2ADNT was formed in amounts below 5% of the TNT added. 4HADNT was formed in higher levels than 2HADNT; 4ADNT was formed in levels higher than 2ADNT and the conjugate 4A-1 was the only conjugate observed in the system after 60 hours. A key difference between the response of *Arabidopsis* and *C. roseus* to TNT was the low levels of 2-substituted conjugates (2A-1 and TNT-1) and preponderance of 4-substituted conjugates (4A-1 and TNT-2) formed in *Arabidopsis*. The observation of the 4,4'-Azoxy metabolite was unique in an axenic TNT phytotransformation system. This metabolite, formed by the abiotic condensation of hydroxylamines (Wang *et al.*, 2003), was observed briefly at one time step at 4 hours and subsequently disappeared. No previous *C. roseus* studies have shown the formation of this metabolite.

Complete details on the TNT removal characteristics are given in Moon et al. (2004), Subramanian (2004), and Subramanian et al (2006).

2.1.3 Radiolabeled TNT Mass Balance Studies

Two-week old *Arabidopsis* seedlings were separately fed [ring-U ^{14}C] TNT at an initial concentration of 15mg/L (0.07 mM) and 50 mg/L (0.22 mM). The radioactivities were 0.06 μCi/ml and 0.2 μCi/ml for each system, respectively. Radiolabeled TNT was diluted with cold TNT to give the final concentration and activity. Complete mass balances were performed at every time step. Media samples were taken and volumes measured to calculate the extracellular radioactivity. Seedlings were sacrificed at every time step too; the sacrificed seedlings were subjected to the intracellular extraction process. The solvent thus obtained was used to quantify radioactivity and concentrations of the intracellular-extractable fractions. The remainder biomass was incinerated in a bio-oxidizer, as described, to determine the intracellular-bound fraction radioactivity. The experiment was continued until 168 hours with periodic removal of samples.

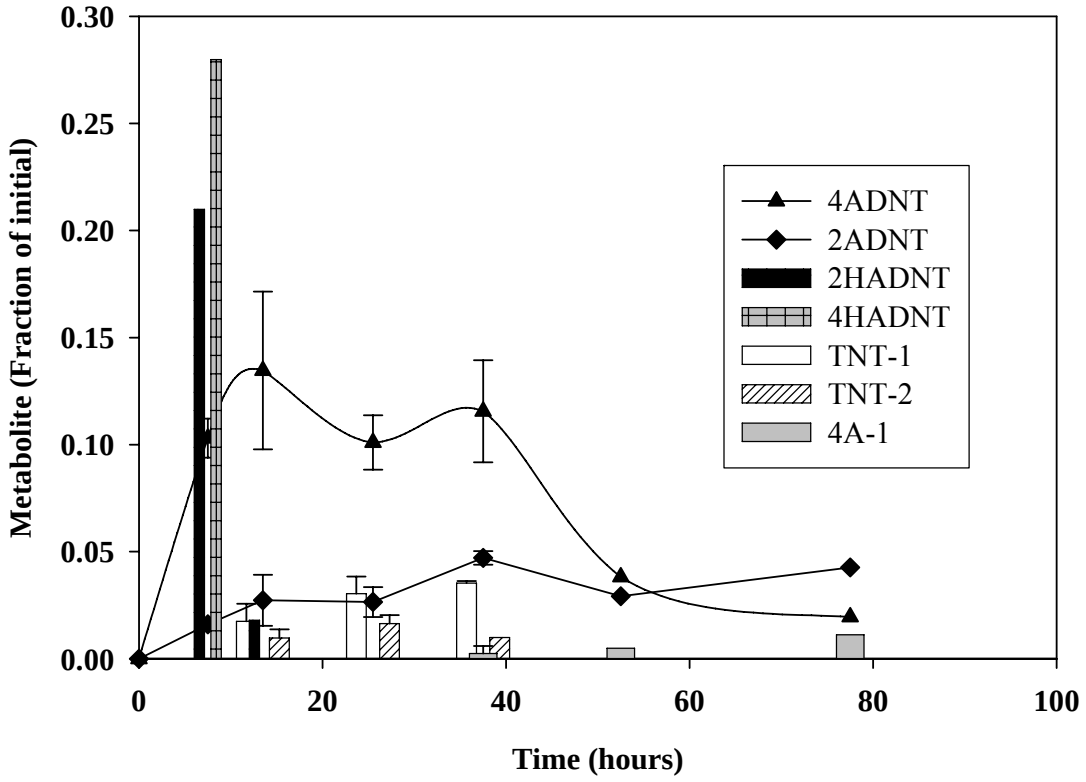
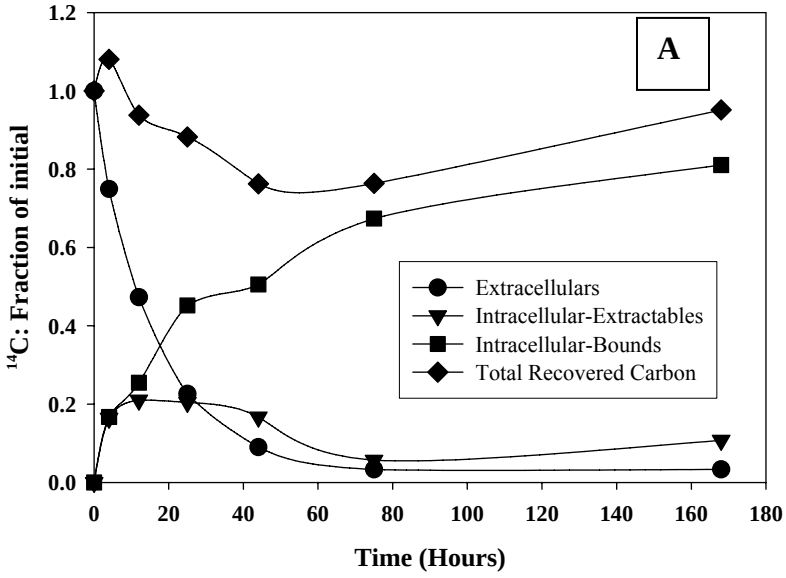


Figure 14: Transient Metabolite Concentrations: Extracellular, transient concentrations of metabolites and conjugates when 7-day-old axenic *Arabidopsis* seedlings were amended with 105 mg/L (0.46 mM) of TNT. Values of 2ADNT, 4ADNT, TNT-1 and TNT-2 are averages and standard deviations of duplicates, while the HADNT levels represent single measurements. An initial spike in HADNT levels is followed by the rapid decline; the ADNT concentrations follow a flatter profile, while three of the four previously identified conjugates are observed.

Quantification of radioactivity under the HPLC peaks was accomplished by having a Packard 505 Flow Scintillation Counter in serial attachment to the PDA detector. A ratio of 1:1.5 of mobile phase to Ultima Flo* M scintillation cocktail was used. Hence, overlaying the two traces helps identify peaks derived from the xenobiotic. Quantification of the radioactivity of extracellular, intracellular-extractable and intracellular-bounds fractions were done in a Packard 2900 TR Scintillation counter. A ratio of 1:5 of sample to Ultima Gold MV cocktail was used for aqueous samples, while a ratio of 1:5 of sample to Insta Fluor was used for organic samples. Residual radioactivity in the plant biomass was measured by combusting a portion of the biomass in an OX700 Harvey Biological Oxidizer. Oxygen was used to complete combustion, while Nitrogen was used to flush out residual radioactivity. 15ml CarboSorb was used to collect the radioactive CO_2 from the combustion chamber, which was combined with PermaFluor E^+ in a ratio of 1:5 and analyzed under the scintillation counter.

^{14}C radiolabeled TNT was taken up and transformed efficiently by the *Arabidopsis* seedlings. Initially, carbon from the TNT was observed completely in the extracellular portion, but is quickly taken up by the seedlings. In the low concentration system (15 mg/L , 0.07 mM of initial TNT), the extracellular radioactivity fell to less than 5% by the end of the experiment (168 hours), while the intracellular fraction peaked at around 20% within 20 hours of TNT addition, and then steadily declined to around 10% by 168 hours (Figure 14A). The amount of intracellular-bounds, the portion of the radiolabel that could not be extracted by sonication in methanol, rose steadily to more than 80% by 168 hours. A similar trend was observed in the high concentration system (50 mg/L, 0.22 mM of initial TNT), wherein the extracellular radioactivity (consisting predominantly of TNT) fell to less than 20% within 40 hours and was steady at less than 10% at 168 hours. The intracellular-extractable radioactivity rose sharply to 20% within 5 hours, and stayed at that level until 40 hours, from where it steadily declined to 10% within 168 hours (Figure 14B). The intracellular-bounds fraction was nearly 40% of the initial label in 40 hours, and increased to greater than 60% by 168 hours. The intracellular-bounds represent the final fate of the carbon from TNT, and its increasing number signifies complete TNT transformation. The complete mass balance for both the systems is also shown in Figures 15A and B. Complete recovery of the label is observed at the end point (168 hours) in both systems, which indicates that all the carbon from the TNT is shared between the extracellular, intracellular-extractable and intracellular-bound phases, and mineralization (conversion of TNT to CO_2) does not occur. In the 15 mg/L (0.07 mM) system, greater than 80% of the label is shown to be recovered at all times, while for the 50 mg/L (0.22 mM) system that number is 65%.

Figures 16A and B show the progression of the transformation pathway at 44 and 168 hours for the 50 mg/L (0.22 mM) TNT system. The proportion of intracellular-bounds is seen to increase from 35 to 69%, while the levels of all other fractions decreased correspondingly. The fraction termed “unknowns” represents the total quantified label minus TNT, ADNTs, conjugates and bounds; the amount of unknown label decreased from 35.9 to27%.



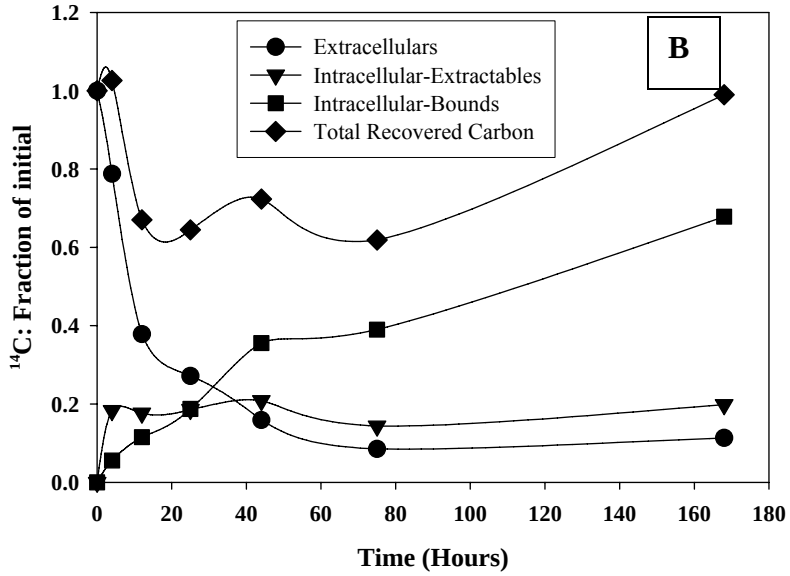


Figure 15: Radiolabeled TNT Mass Balances: Complete mass balance on 2-week-old axenic *Arabidopsis* seedlings exposed to [ring-U ^{14}C] TNT at an initial concentration of (1) 15 mg/L (0.07 mM) and initial radioactivity of 0.06 µCi/ml (Figure 6.2A) and (2) 50 mg/L (0.22 mM) and initial radioactivity of 0.2 µCi/ml (Figure 6.2B). Nearly 100% recovery of the C-14 label was seen by the end of the both experiments at 160 hours. Greater than 80% and 60% recovery of the label was observed in the 15 mg/L and 50 mg/L system at all times, respectively.

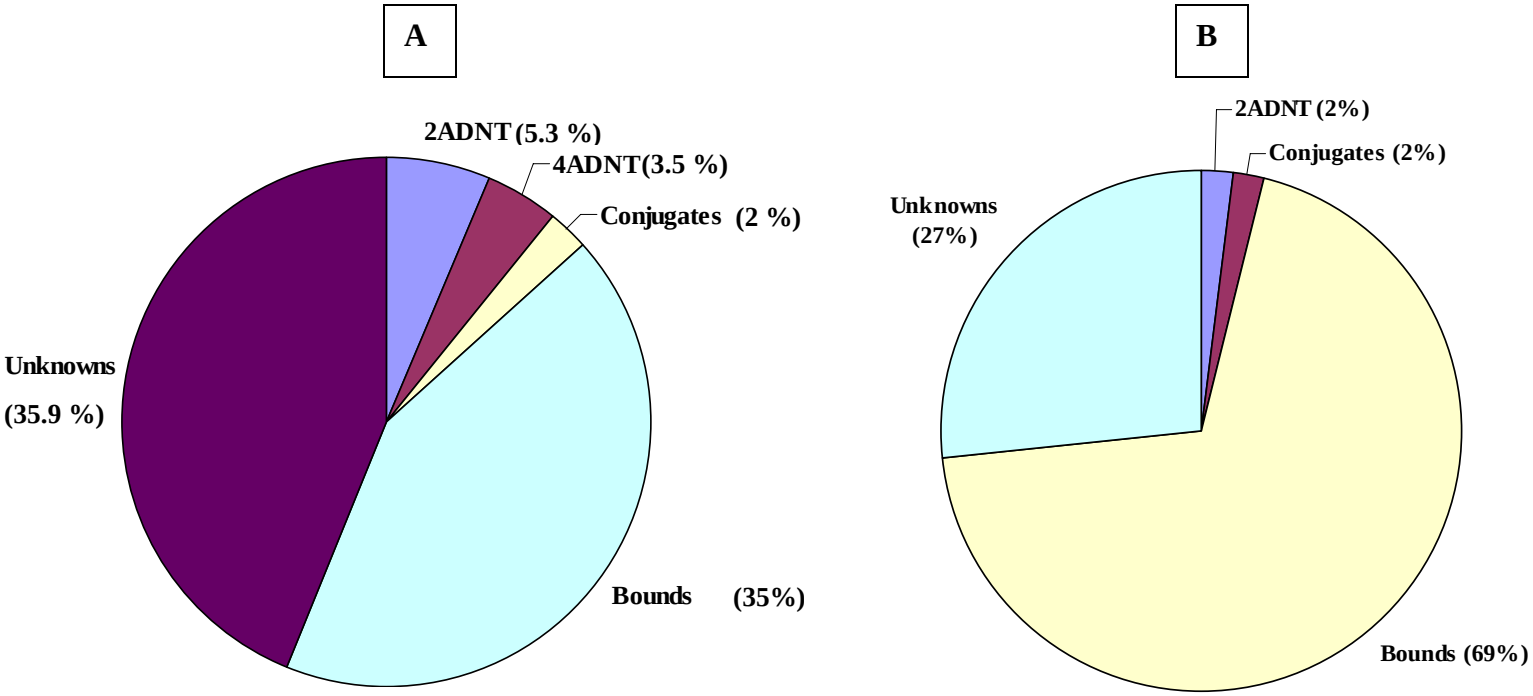


Figure 16: Snapshots of Metabolite Fractions: Metabolite fractions in *Arabidopsis* 44 hours (A) and 168 hours (B) after amendment with [ring-U ^{14}C] TNT at an initial concentration of 50 mg/L (0.22 mM) and initial radioactivity of 0.2 µCi/ml. The progression of the pathway is observed, as the proportion of bounds increases over time, with the amount of all other metabolites declining. The bounds represent a final form of the initial TNT.

2.1.4 Metabolite Feeding Studies

TNT, 2HADNT, 4HADNT, 2ADNT, 4ADNT and 4,4'-Azoxy were fed to one-week old axenic *Arabidopsis* seedlings, grown from 25 seeds in 25 ml of media at a concentration of 5 mg/L (0.02 mM). The uptake of the parent compound and formation of metabolites were measured for 55 hours.

When *Arabidopsis thaliana* seedlings were fed 4ADNT, they produced only the conjugate 4A-1 in the extracellular fraction; when they were fed with 2HADNT, they formed the conjugates 4A-1 and TNT-2, both in the extracellular fraction. Since 4HADNT is upstream of 4ADNT in the TNT transformation pathway, it follows that a portion of 4HADNT is being reduced to 4ADNT that is subsequently being conjugated to 4A-1; simultaneously a parallel branch of 4HADNT is being directly conjugated to form TNT-2. When 2HADNT was fed to the *Arabidopsis thaliana* seedlings no apparent metabolites in either the extracellular or the intracellular-extractable fraction were observed. Hence, no conclusive evidence on 2HADNT conjugation was seen in *Arabidopsis thaliana*. However, similar experiments performed on *C. roseus* roots demonstrated the conjugation of 2HADNT to TNT-1. Based on previously published feeding studies (Bhadra, Spanggord et al., 1999; Bhadra, Wayment et al., 1999) we postulated that hydroxylamines can be directly conjugated (Subramanian and Shanks, 2003). However, thus far, no confirmatory studies had been performed for the same. The hydroxylamine feeding studies performed here provide a confirmation that hydroxylamines can indeed be directly conjugated without a further reduction of their hydroxylamine moiety.

4,4'-Azoxy was also added to one-week old seedlings, but was taken up completely by the seedlings within two hours. No additional metabolite formation was observed. Hence, while the azoxies were taken up efficiently by the seedlings, their fate remains unknown.

These feeding studies were also used to observe toxic effects exerted by the metabolites on the seedlings. Figure 17 shows the fresh (wet) weight of the seedlings 55 hours after being exposed to the metabolite. As seen in the figure, 2HADNT, 4HADNT and 4,4'-Azoxy exert a significantly higher toxic effect than TNT and the ADNTs. The weight of the seedlings exposed to these metabolites is around 60% of the weights of the control seedlings not exposed to any foreign compounds. An ANOVA analysis gave a null hypothesis probability, P= 0.002, which indicated that the variation in wet weights was due to the different toxicities exerted by the xenobiotics and not due to random variations.

2.1.5 TNT Pathway Kinetics

First order kinetic rate of transformation for various metabolites in the pathway were calculated to provide relative rates between branches of the pathway. While the first

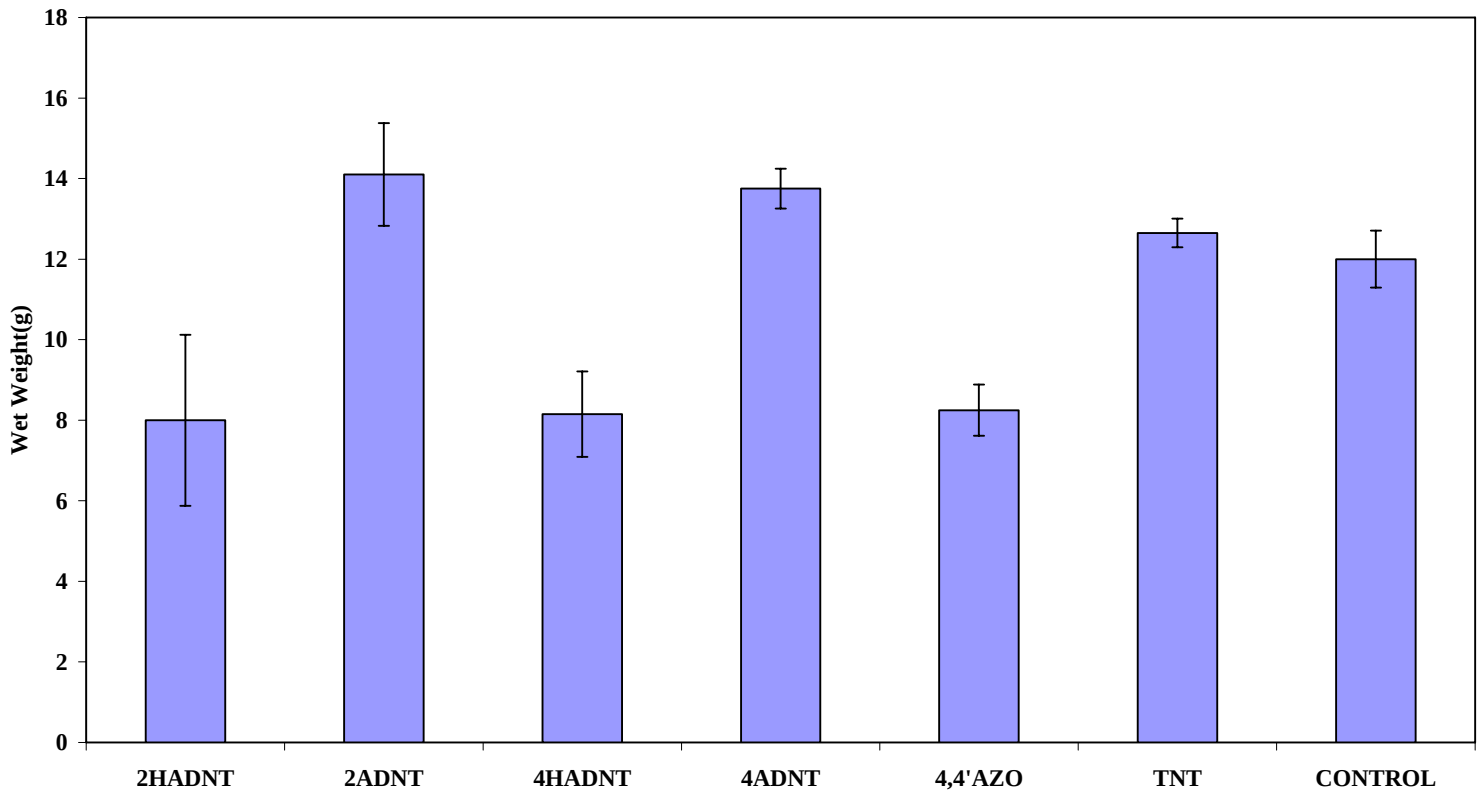


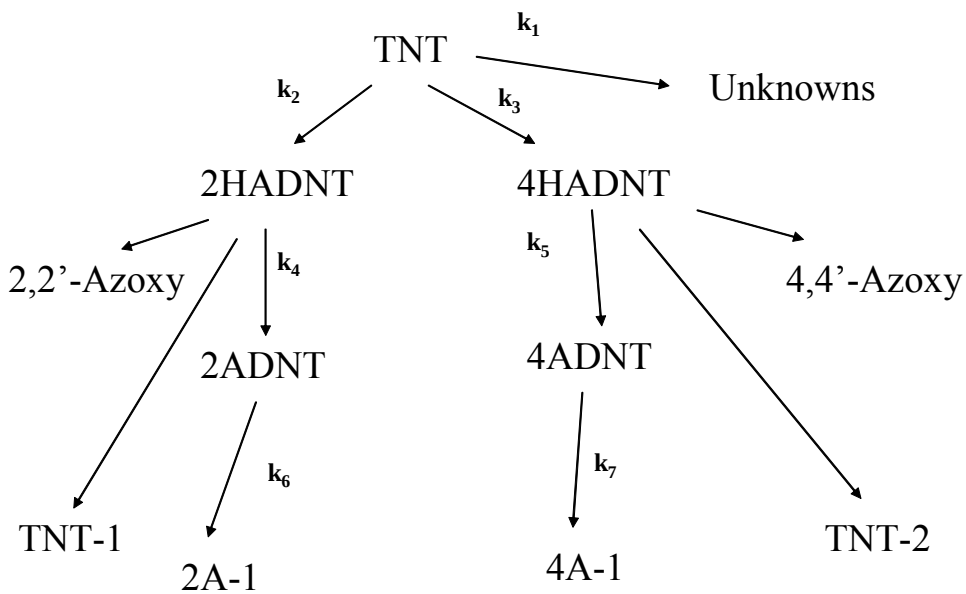
Figure 17: Comparison of Metabolite Toxicity. Wet weights of *Arabidopsis* seedlings exposed to various TNT transformation metabolites. The weights are reported 55 hours after exposure of the one-week old seedlings. The weights are averages and standard deviations of duplicates. As observed, 2HADNT, 4HADNT and 4,4'-Azo metabolites appear to detract TNT growth, while TNT, 2ADNT and 4ADNT do not have a noticeable toxic effect. The initial concentration of all the metabolites was 5 mg/L, except TNT which was fed at 75 mg/L, and the control which did not have any metabolites added to it. An ANOVA analysis of all the wet weight means gave P= 0.002, which indicates the difference in wet weights are due to toxic differences

order expression implicitly assumes a constant biomass, since early exponential phase *Arabidopsis* seedlings were used this assumption does not hold true. However, since the rate of biomass increase is the same for all metabolites, this approximate first order model provides relative rates between various transformations occurring in the pathway. The rate constant thus derived is referred to as Pseudo-first order rate constant. Data from the TNT feed experiments, metabolite feeding experiment and radiolabeled TNT feeding experiments were used to calculate the rate constants. Figure 18 shows the calculated rate constants for the various branches of the pathway. It was determined that the TNT to hydroxylamine step and the monoamine to conjugate step were the rate limiting sections of the transformation pathway. It was also determined that *Arabidopsis* seedlings are more efficient in the formation and removal of 4-susbstituted metabolites (4HADNT, 4ADNT, TNT-2 and 4A-1) compared to 2-substituted metabolites (2HADNT, 2ADNT, TNT-1 and 2A-1).

2.1.6 *Arabidopsis* Growth Curve Studies

Determination of the growth characteristics of hydroponic *Arabidopsis* seedlings was accomplished by cultivating them from seeds as described in the previous section. Seedlings were grown at 25ºC under light at 100 rpm for seven days, following which three samples were sacrificed for dry weight measurements. The dry weights were obtained by freeze drying the biomass for 48 hours. Triplicate samples were subsequently sacrificed at periodic time intervals to allow for the determination of dry weights at various time steps, until 35 days. Figure 19 shows the measured dry weights at various time steps, and it is observed that stationary phase was achieved 20 days after culturing. Exponential phase was observed to last from day 10 to day 20. In addition, variability between triplicates was high during the exponential phase, as seen in the standard deviations at day 15. However, by the 20th day after culturing of the seeds, variability between samples was less than 4% and biomass levels had reached a plateau, signifying the arrival of the stationary phase.

Complete details on the radiolabeled mass balance studies, the metabolite feeding experiments, pathway kinetic analyses and growth curve studies are given in Subramanian (2004) and Subramanian et al (2006).



Pseudo-1st Order Rate Constant	**(hr^{-1})**
k_1	0.04
k_2	0.03
k_3	0.04
k_4	0.41
k_5	1.97
k_6	0.05
k_7	0.16

Figure 18: Kinetics of TNT Transformation. Schematic of the TNT transformation pathway showing the various kinetic rate constants and their values in *Arabidopsis*.

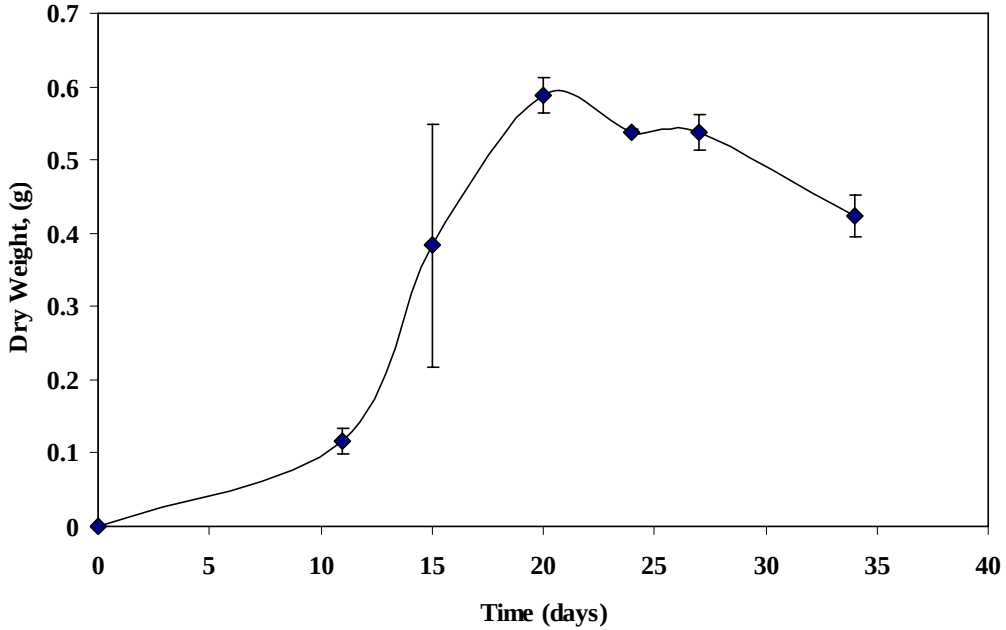


Figure 19: *Arabidopsis* Growth Curve. Growth curve for *Arabidopsis* seedlings, showing the dry weight of the seedlings at various time steps. High variability in the dry weights is observed during the exponential phase from day 10 to 20, following which deviation between samples falls to below 4%, and biomass levels reach a plateau. The stationary phase is seen to be reached by 20 days.

2.2 RDX Studies on wild-type *Arabidopsis*

2.2.1 RDX Toxicity and Removal

Preliminary studies were conducted on uptake of RDX and its effect on *Arabidopsis* seedling growth. From the limited literature data available, a toxic level of 21 mg/L RDX for hydroponically grown plants was estimated for wheat, sorghum, maize and poplar. Since the solubility limit of RDX in water is approximately 40 mg/L, two levels were chosen to span the solubility range and previous toxicity estimates. One-week-old *Arabidopsis* seedlings grown in liquid cultures under photoheterotrophic conditions were amended with 0 mg/L, 15 mg/L and 30 mg/L of RDX. Seedlings were sacrificed at 4 and 10 days, and dried at 60°C for 4 days, and the biomass weighed. Extracellular samples were taken at 4, 10 and 14 days and analyzed for RDX removal and metabolite production, via reverse-phased HPLC.

No dramatic effect of RDX toxicity was observed at either concentration tested, as shown in Figure 20. These results could either indicate the lack of RDX toxicity in this time frame, or that the number of repetitions was insufficient to observe finer differences. RDX uptake was observed in both systems, with nearly 80% removal in the 15 mg/L system and 60 % removal in the 30 mg/L system (Figure 21). The lower removal rate at the higher concentration level may be an indication of early signs of toxicity; a second round of experiments were performed to determine if this indeed was the case.

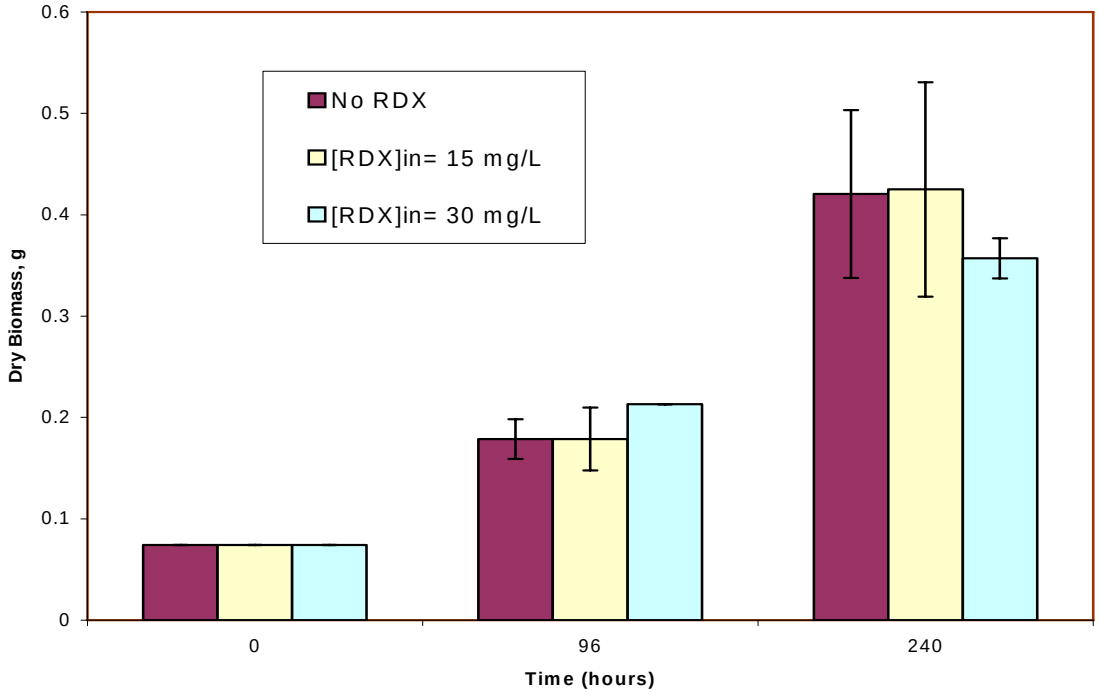


Figure 20. Effect of RDX on growth of *Arabidopsis* seedlings.

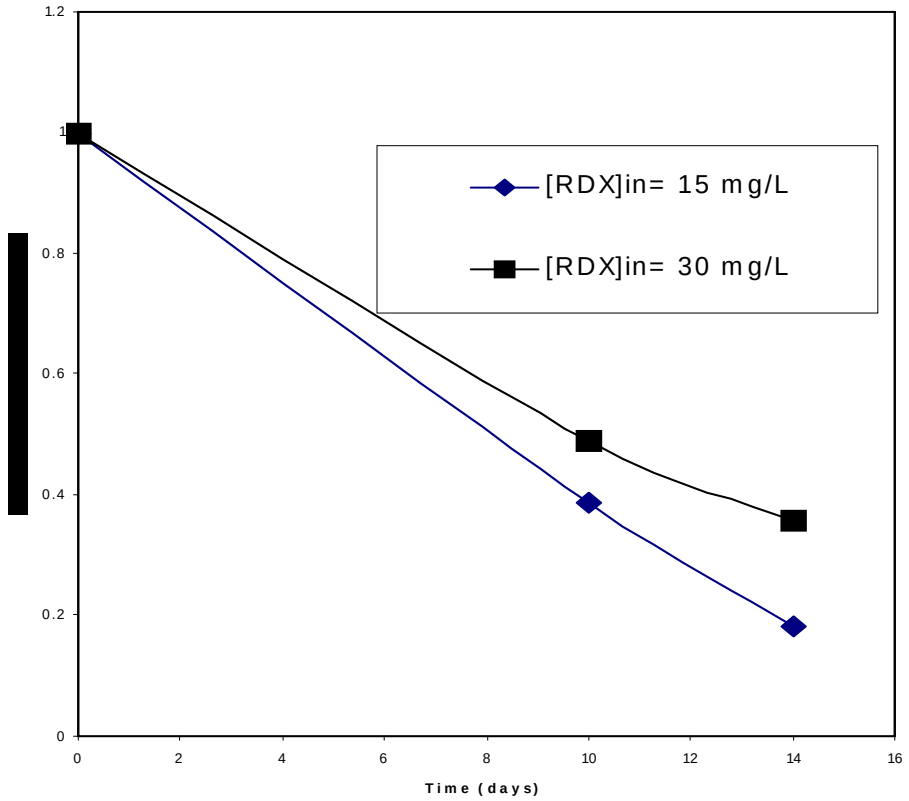


Figure 21. RDX removal from *Arabidopsis* seedlings. Extracellular RDX is shown to be taken up gradually by *Arabidopsis* seedlings, under sterile conditions.

In the second round of experiments, seeds were cultured for one week and then 0 mg/L and 35 mg/L RDX were added to the cultures. Seedlings were sacrificed at 0, 7, 10, 14 and 21 days and the dried biomass was weighed. Extracellular liquid samples were taken at 7, 10, 14 and 21 days and analyzed with HPLC. No significant RDX toxic effect was observed at this concentration. It appears that either the RDX was not significantly toxic at this level, or the RDX was not toxic in the duration of the study. A growth curve for this study and the previous study is given in Figure 22. HPLC analysis of the concentrations revealed the uptake of RDX by the *Arabidopsis* seedlings. After 21 days, the plants reduced the RDX concentration from 35 mg/L to 10 mg/L; at 35 mg/L RDX, 70% of the RDX was removed from the system (Figure 23). The RDX metabolites (MNX, DNX, TNX, methylene-dinitramine, and 4-nitro-2,4-diazabutanal) have been documented on bacteria studies by Jalal Hawari and his collaborators. Standards for these RDX metabolites have been purchased from Ron Spanggord at SRI International. Extracellular liquid samples and extractable plant tissue samples can now be analyzed for presence of the RDX metabolites.

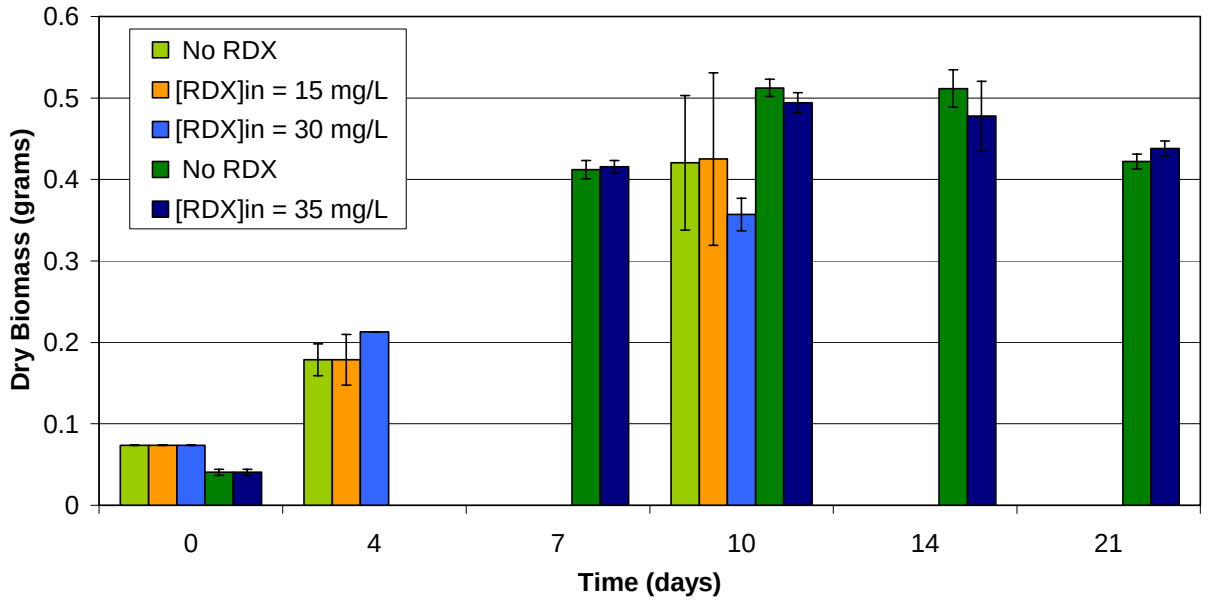


Figure 22. Effect of RDX on growth of *Arabidopsis* seedlings. Effect of several RDX concentrations on the growth of *Arabidopsis* seedlings. Different amounts of RDX were added to one week old seedlings. Biomass was collected at several time points, and then the biomass was dried and weighed. The system was maintained under sterile conditions. At the concentrations shown, RDX does not appear to have an effect on the growth of the seedlings.

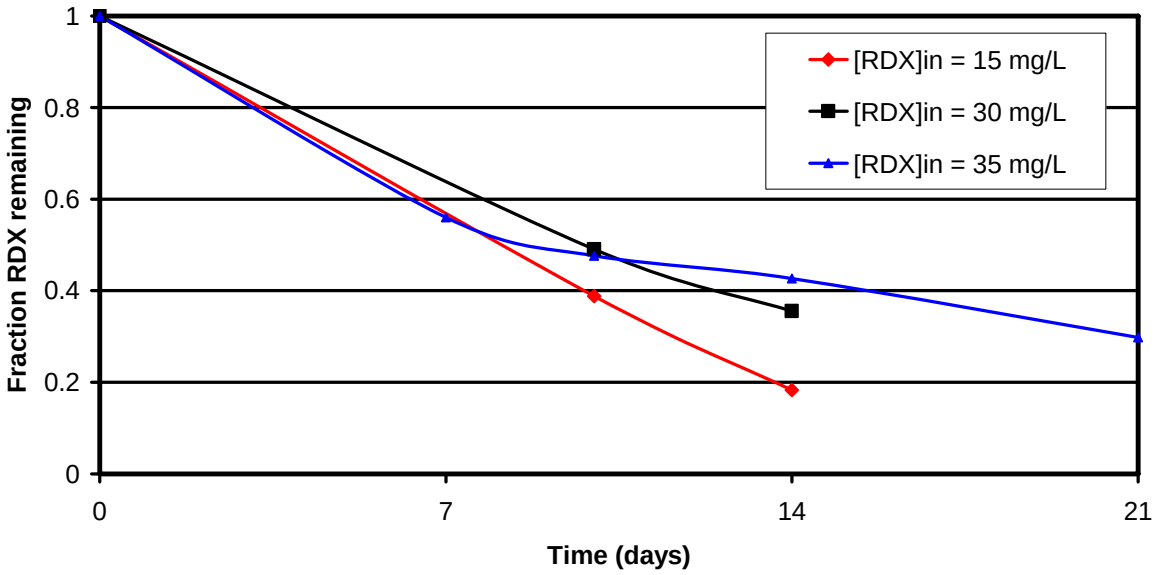


Figure 23. Fractional RDX removal from *Arabidopsis* seedlings. Extracellular RDX is taken up by *Arabidopsis* seedlings. The plants were one week old when RDX was added to them.

A separate set of RDX toxicity experiments were performed on hydroponic *Arabidopsis* seedlings. 25 wild type seedlings grown on the solid media for 4 days were transferred to each flask containing liquid media with different RDX concentrations (35, 50, 100, 150 mg/L) to simulate even germinations. As shown in Figure 24, there was no significant difference in the dry weights, fresh weights and transpiration amounts between the controls and various concentrations. Hence, higher concentrations are required to elicit RDX toxic response to seedlings.

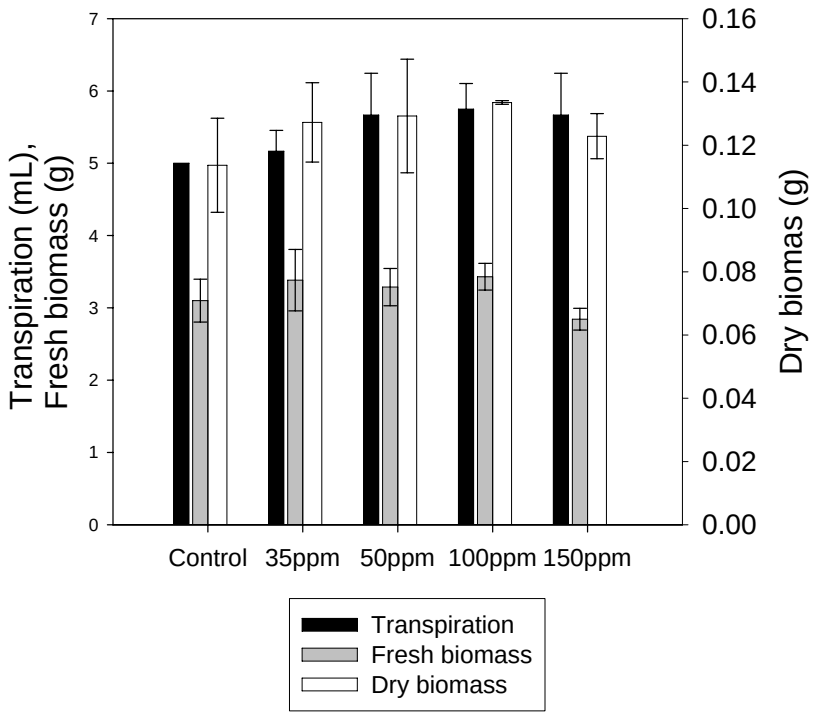


Figure 24. Dry biomass, transpiration, and fresh biomass of wild type seedlings after 8 days of exposure to various concentration of RDX. Error bars represent standard deviations.

2.2.2. [U-14C] RDX Mass Balance Study

A radiolabeled RDX feed study in wild-type *Arabidopsis* seedlings. 1 Molar potassium hydroxide traps will be used to detect carbon dioxide levels, while metabolite concentrations were determined by a combination of HPLC and Floscintillation detectors. Intracellular extractables were measured by a process of sonication and solvent extraction, while the intracellular bounds were detected using a bio-oxidizer. The initial study was carried out at 35 mg/L RDX and 40 μCi/L. The seeds were grown in liquid media for 10 days before being amended with ^{14}C-RDX. Liquid media samples were taken immediately after adding RDX in order to obtain initial concentrations of RDX and radioactivity for each flask. Liquid media samples and plant samples were taken 4 days and 7 days after the RDX was added. Additional samples were taken throughout the experiment. Figure 25A shows the new experimental equipment designed and constructed for use in the study. The erlenmeyer flasks bear two ports to

draw out sample and hold a potassium hydroxide solution. All ports are completely air tight and sterilized prior to use. Needles will be used to sample the potassium hydroxide and the media through the serum stoppers. This allows samples to be taken without opening the flasks. This helped maintain an air-tight environment throughout the experiment and ensure that a complete mass balance can be performed. Needles remained in the flasks throughout the experiment to help reduce contamination that may occur from repeated needle injections. The tops of the needles are sealed with Parafilm to prevent contamination from entering through the needles.

Figure 25A: Experimental set up showing the various ports in the sterile flasks to draw out samples and trap carbon dioxide.

Several controls are being used in this experiment. Heat kill controls consisting of plants that were autoclaved to ensure that no enzymatic activity is occurring are being used to account for adsorption of RDX to plant tissue. Light controls consisting of media with no plants are being used to account for photodegradation of RDX. Dark controls consist of a flask containing only media and then the flask is covered in foil. The dark controls are being used to account for evaporation of water from the media.

Initial data has been obtained from media samples taken throughout the experiment. The samples were then analyzed with the liquid scintillation counter to obtain the total extracellular

radioactivity in the flasks. This data shows that the plants are removing the radiolabeled RDX from the media (Figure 254B).

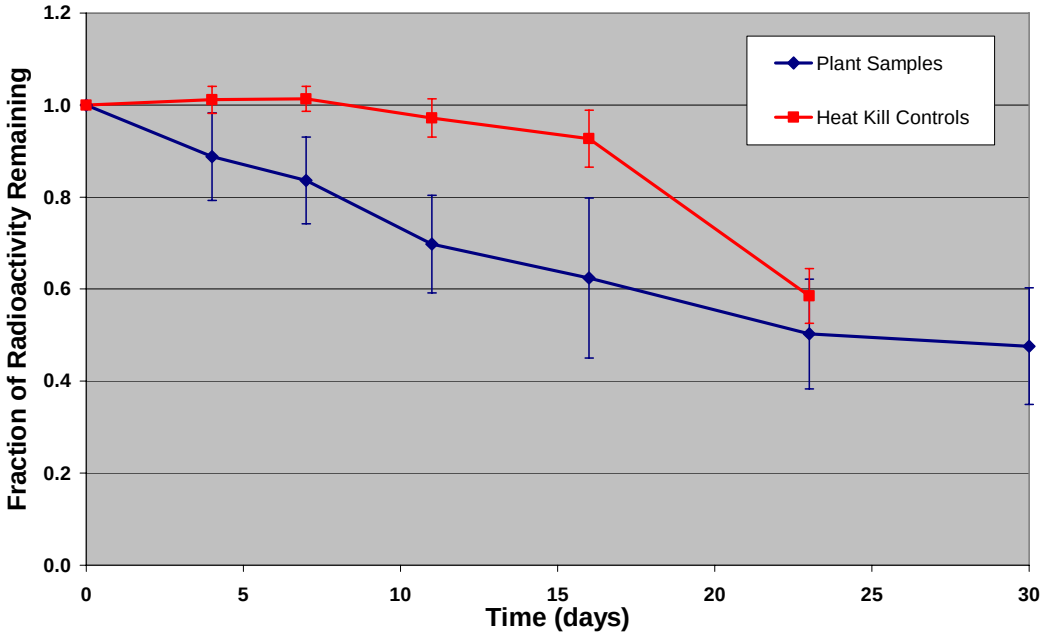


Figure 25B: The total radioactivity in the media decreases much more quickly in flasks containing living plants. The living plants remove the radioactivity at a higher rate until the shaker reaches a higher temperature. The rate of removal for all controls greatly increases after the temperature is raised.

From the second trial, living plants removed radioactivity from the media at a faster rate than all of the controls (heat-killed controls, light media controls, and dark media controls). Over 58% of the initial radioactivity was removed from the media by living plants after 35 days, while the controls removed less than 20% (Figure 25C). The media samples were also analyzed with HPLC to determine if there were any known metabolites (MNX, DNX, TNX, NDAB, and MEDINA) present in the media, but the media samples did not appear to have any of these metabolites.

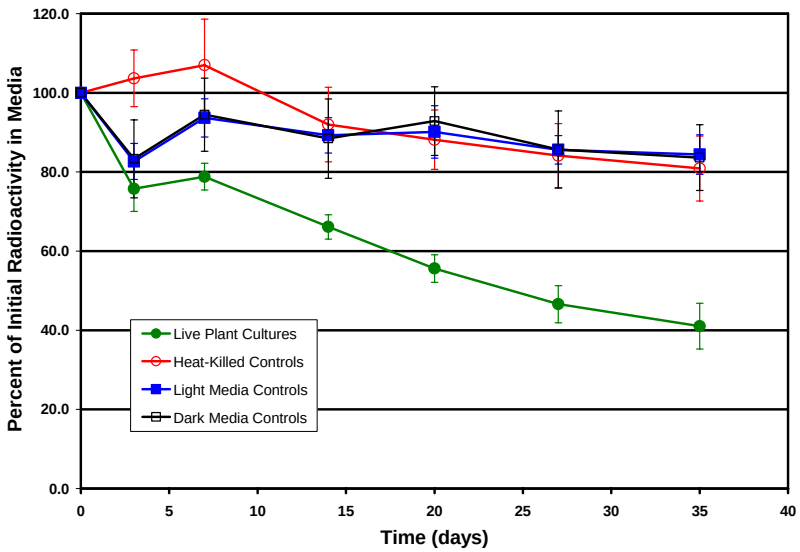


Figure 25C. The figure above shows that the percent of total initial radioactivity in the media decreases in all flasks during the radio-labeled experiment. Radioactivity decreases much more rapidly in flasks with living plants. Over 58% of the radioactivity was removed from the media by the living plants after 35 days, while all of the controls removed less than 20%.

The living plants mineralized RDX to form significant amounts of radioactive carbon dioxide, which can be seen in Figure 25D. 10% of the initial radioactivity was mineralized by living plants after 35 days while 0.5% mineralization was observed in all of the control flasks.

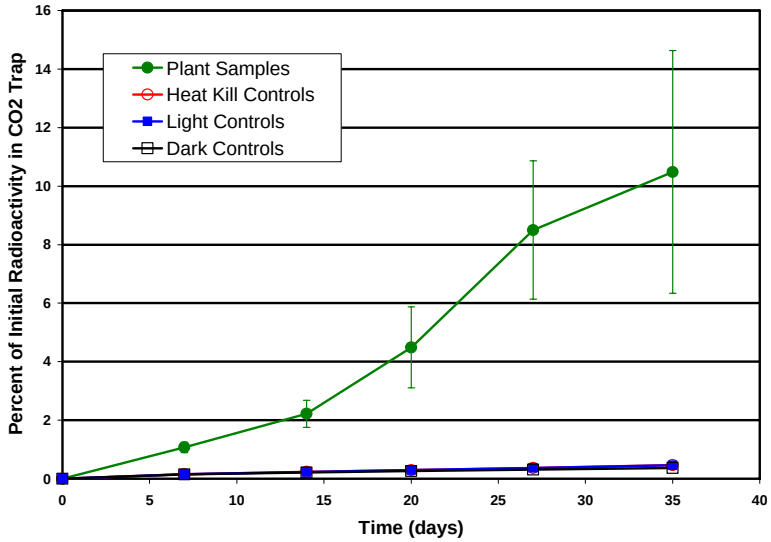


Figure 25D. The figure above shows the percent of initial radioactivity in the carbon dioxide traps increase in all flasks during the radio-labeled experiment. Flasks containing living plants convert RDX to carbon dioxide much quicker than controls, with over 10% of the initial radioactivity converted after 35 days. This is significantly more mineralization than the 0.5% that was observed in all of the control flasks.

The recovery of ^{14}C label for living plants and controls was over 80%. The overall mass balance for living plants was shown in Figure 25E. The living plant cultures have over 20% of the initial radioactivity in the extractable portion of the biomass.

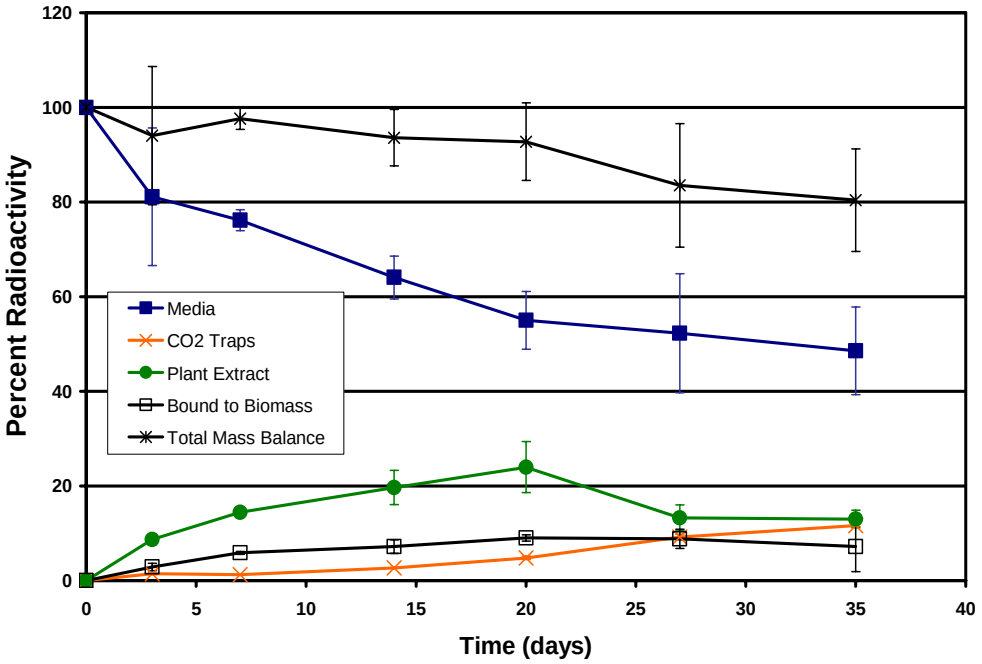


Figure 25E. The figure above shows the total radioactivity from the mass balances for the living plant cultures in the radio-labeled experiment. The plants are taking the RDX from the media, and converting some of the RDX to CO_2. The remaining radioactivity is either in the extractable portions of the plant or incorporated into the plant biomass.

For additional details, see Rollo (2005) reference.

3 Characterization of mutants

3.1 Genetic Characterization of TNT mutants

3.1.1 Southern blot analysis

When T-DNA insertion mutant library was generated by transforming *Arabidopsis* with *Agrobacterium tumefaciens* harboring a binary vector, two or more insertion events can occur in a single mutant line. This may make it more complicated to demonstrate which gene is responsible for giving a particular plant TNT resistance. To determine the number of insertions in the mutant lines, genomic Southern blot analyses were performed. Approximately 100 mg of rosette leaves were pulverized in liquid nitrogen and genomic DNA was extracted in an extraction buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 mM NaCl, 1% SDS, 10 mM β-mercaptoethanol). After treatment with 5 M potassium acetate, DNA was precipitated with isopropanol, washed with 70% ethanol and then dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Genomic DNA was digested with *Hind*III, DNA fragments were fractionated in an agarose gel, transferred to a blotting membrane, and probed with the *BAR* gene.

The number of insertion varied. Most of the T-DNA insertion mutant lines tested showed 2 or more bands with maximum of 6 (Figure 26). Only a few showed a single band.

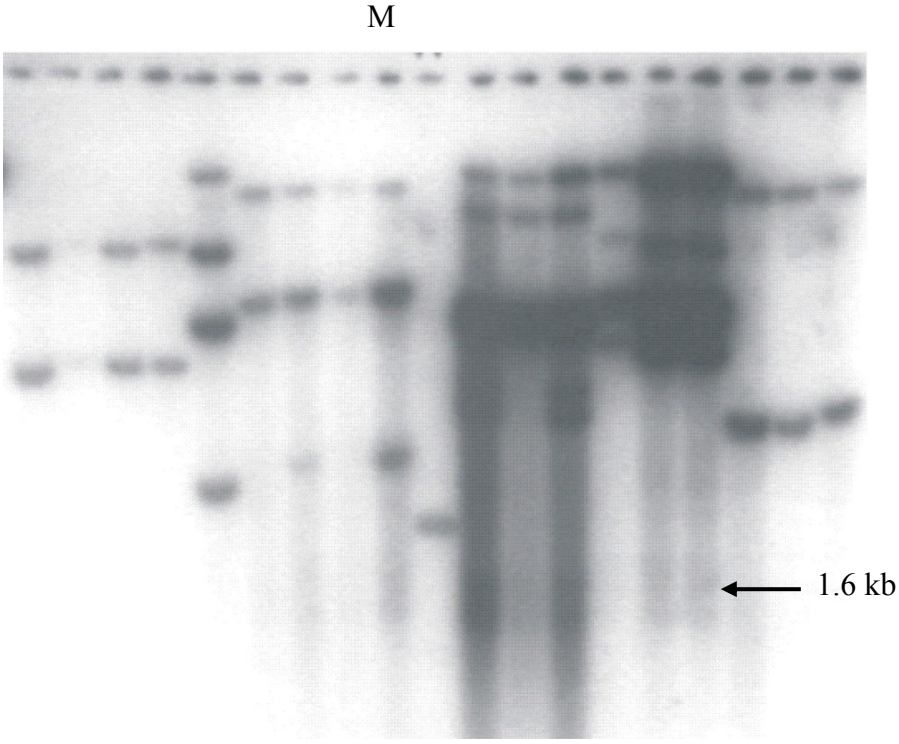


Figure 26. An example of Southern blot analyses for T-DNA insertion mutant lines. The number of bands indicates the number of insertion events in the plant. M, molecular size marker.

3.1.2 Cloning and Sequencing of Tagged Genes

Thermal asymmetric interlaced PCR (TAIL-PCR) was performed using genomic DNA of the enhancer trap mutants as templates to clone DNA fragments flanking the left border of the T-DNA insert in the selected mutant lines. The primers and PCR conditions were essentially the same as described by Liu et al. (1995) except that the annealing temperature for the high stringency cycles was 56°C and the concentrations of the degenerated primers and specific primers were the same. The specific primers, designed to anneal to near the left border, were LB1 (5'-ATACGACGGATCGTAATTTGTC-3'), LB2 (5'-TTATAATAACGCTGCGGACATCTAC-3'), and LB3 (5'-TTGACCATC-ATACTCATTGCTG-3'). The PCR products were gel purified and DNA sequencing reactions were carried out using the LB3 primer. The resulting sequences were analyzed using the program BLAST.

All 12 mutant lines selected from the enhancer trap mutant library were subjected to TAIL-PCR. Ten lines produced PCR products after three successive PCR reactions. Sequencing and BLAST searches of the PCR products revealed the sites of insertion of the T-DNA segment in those mutant lines. The number of insertion in the individual lines revealed by TAIL-PCR ranged from one to three. The majority of insertions occurred between two genes (Figure 27A), but in a few cases T-DNA insertion occurred in either an exon or an intron of a gene (Figure 27B). The results are summarized in Table 4. Although some of the genes show similarity to the known genes from other organisms, none of the genes' functions have been experimentally identified. Table 5 summarizes the gene functions as determined from the ABRC database.

ET32-3 and ET226-3 were seen to have a single T-DNA insertion as the Southern blot shows a single band (Figure 28), while ET40-6 and ET231-1 were seen to have three and four bands, respectively (Figure 28). More DNA blot analyses with different enzyme digestions or different probe revealed that ET231-1 had two T-DNA insertion sites and each site had a double T-DNA insertion, which gave rise to 4 bands. ET40-6 appeared to have two insertion sites, with one site having two T-DNA insertions and the other a single T-DNA insertion.

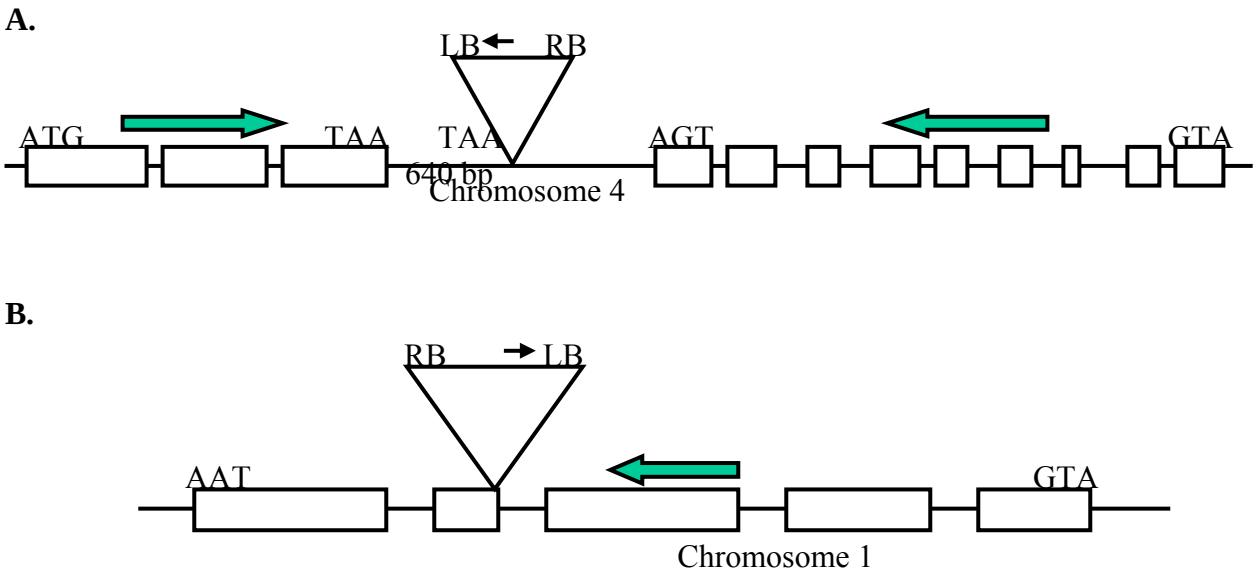


Figure 27. Examples of the map of the T-DNA insertion in enhancer trap mutant lines. The T-DNA was inserted either between two genes (A) or in the coding sequence of a gene (B). The arrow indicates the orientation of the genes. ATG is the start codon, and TAA and TGA are the stop codons of the genes. RB and LB are the right and left border of T-DNA, respectively.

Table 4. Genes flanked by T-DNA inserts in the enhancer trap mutants.

Mutant lines	Number of insertions revealed by TAIL-PCR	Insertion sites
ET25	1	Between At4g24750 and At4g24760
ET30	2	Between At4g35940 and At4g35950
		Between At4g12890 and At4g12900
ET32	2	Between At5g28590 and At5g28610
		Between At4g35940 and At4g35950
ET40	1	In the 3rd exon of a pseudo gene, At5g38192
ET148	1	Between At3g61630 and At3g61640
ET183	1	Between At4g24880 and At4g24890
ET212	3	Between At1g09940 and At1g09950
		In the 4th exon of At1g49560
		Between At1g25330 and At1g25340
ET218	2	Between At4g28720 and At4g28730
		In the 2nd intron of At5g41780
ET226	1	Between At3g04570 and At3g04580
ET231	2	In the 3rd intron of At5g53740
		Between At3g60930 and At3g60940

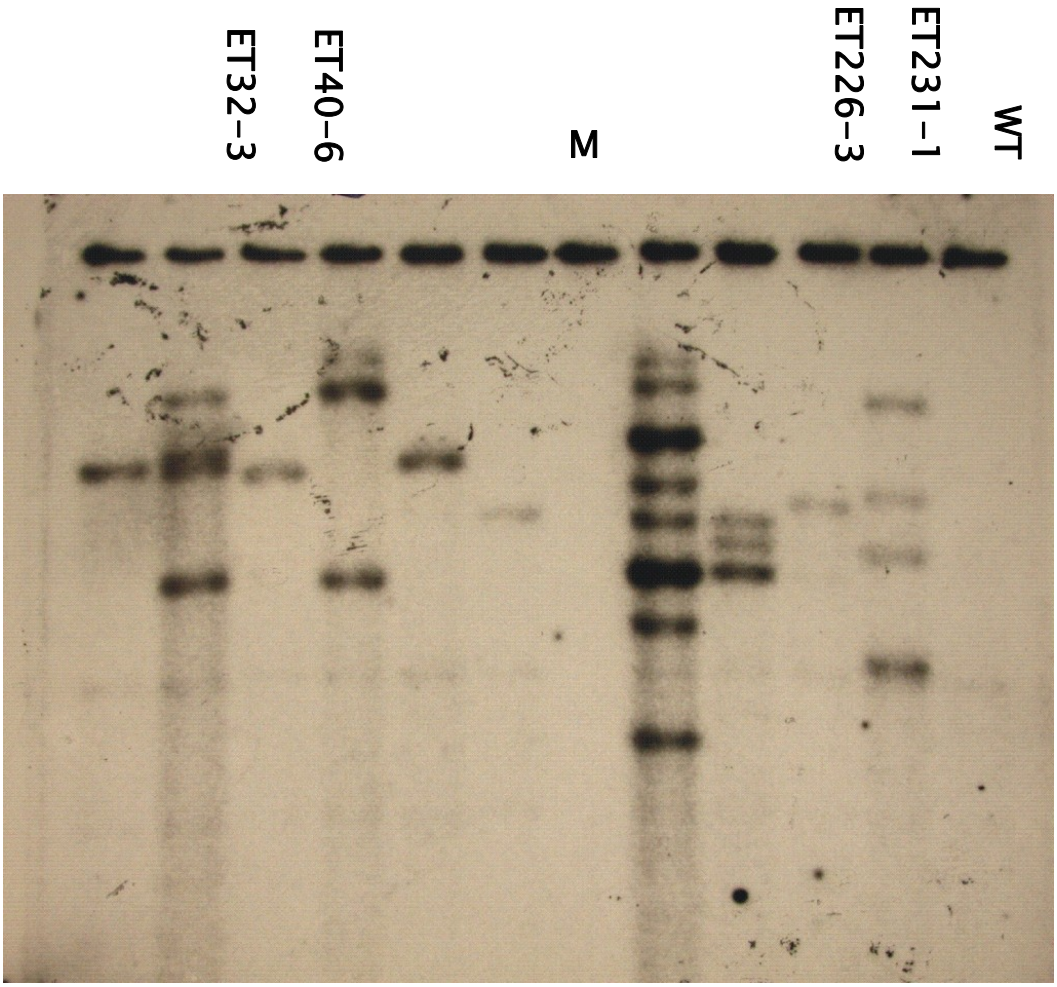


Figure 28. DNA blot analysis for the activation-tagged mutants. The number of band may indicate number of T-DNA insertion events in the mutant.

Table 5: Gene Functions: Summary of genes upstream or downstream of the T-DNA insertions in the enhancer trap mutants, with the function of the gene, as determined from The *Arabidopsis* Information Resource website (www.arabidopsis.org). Transcription factors appear to be the most widely affected genes.

Gene Name/ Locus	Mutant	Function
At4g24750	ET-25	Unknown
At4g24760	ET-25	Unknown
At4g35940	ET-30	Unknown
At4g35950	ET-30	small GTPase mediated signal transduction, GTP binding
At4g12890	ET-30	Unknown, catalytic activity
At4g12900	ET-30	proteolysis and peptidolysis, carboxypeptidase A activity
At5g28590	ET-32	Unknown
At5g28610	ET-32	Unknown
At4g35940	ET-32	Unknown
At4g35950	ET-32	small GTPase mediated signal transduction, GTP binding
At5g38192	ET-40	DNA recombination, RNA-dependent DNA replication
At3g61630	ET-148	regulation of transcription, DNA-dependent, transcription factor activity, DNA binding
At3g61640	ET-148	Unknown
At4g24880	ET-183	Unknown
At4g24890	ET-183	protein serine/threonine phosphatase activity
At1g09940	ET-212	porphyrin biosynthesis, glutamyl-tRNA reductase activity
At1g09950	ET-212	Unknown
At1g49560	ET-212	regulation of transcription, DNA binding
At1g25330	ET-212	regulation of transcription, DNA binding
At1g25340	ET-212	regulation of transcription, DNA binding
At4g28720	ET-218	electron transport, removal of superoxide radicals, dimethylaniline monooxygenase (N-oxide-forming) activity, disulfide oxidoreductase activity, ferredoxin hydrogenase activity, monooxygenase activity, oxidoreductase activity
At4g28730	ET-218	electron transporter activity, thiol-disulfide exchange intermediate activity
At5g41780	ET-218	copper ion transport, ATP binding, copper-exporting ATPase activity
At3g04570	ET-226	regulation of transcription, DNA-dependent, DNA binding
At3g04580	ET-226	signal transduction, regulation of transcription,
At5g53740	ET-231	Unknown
At3g60930	ET-231	phenylalanyl-tRNA aminoacylation, ATP binding, phenylalanine-tRNA ligase activity
At3g60940	ET-231	Unknown

3.1.3 Testing of Salk T-DNA insertion mutants

Eighteen T-DNA insertion mutants were obtained from ABRC, in which their insertion sites are similar to the sites of the T-DNA insertion in our Enhancer (4X)-trap lines (Table 6). When challenged with 10, 15, 20, 25, or 30 mg/L TNT, none of them showed germination better than wild type. This implies that resistance phenotype of the activation-tagged mutant lines were not caused by interruption of a gene, but possibly by an enhancement of a gene up-regulated by the 4X 35S enhancer element in the T-DNA.

Table 6. Some of the Salk T-DNA mutant lines that was tested for TNT resistance.

T-DNA Mutant	Location of T-DNA insertion	Related mutants in our study
Salk 039048	3' downstream of At5g28610	ET32
Salk037177	In the ORF of At3g04570	ET226
Salk 066894	In the ORF of At5g53740	ET231
Salk 014234	5' upstream of At3g60940	ET231
Salk 013802	In the ORF of At4g35950	ET30
Salk 029283	In the ORF of At4g35950	ET30
Salk 059595	In the ORF of At4g12890	ET30
Salk 119098	Between At4g12890 and At4g12900	ET30
Salk 119615	In the ORF of At4g24880	ET183

3.2 Toxic and Metabolic Analyses of TNT-Resistant Mutants

3.2.1 TNT toxicity to the growth of the mutants

The ten enhancer trap mutant lines listed in Table 4 were selected for their resistance to TNT based on their seeds germination capacity in the solid medium containing TNT. To determine if seedlings of those mutants can detoxify TNT in the medium better than wild-type, the effects of TNT on growth of the mutants in a liquid culture were investigated. A hundred seeds were surface-sterilized and cultured in 50 mL of *Arabidopsis* growth medium at 22°C with shaking at 120 rpm under approximately 40 μmol/m^2/s continuous light from cool white fluorescent bulbs. After a week, TNT was added to the flasks, and the seedlings were cultured for another week before harvest. Dry weights were measured before and a week after TNT addition to the cultures to establish the gain of plant biomass.

When 100 seeds of ET231 mutant line were challenged with 15 mg/L TNT, the dry weight of the biomass in the presence of TNT was 68% of that the control, whereas the biomass of wild-type with TNT was 45% of that of the control (Figure 29).

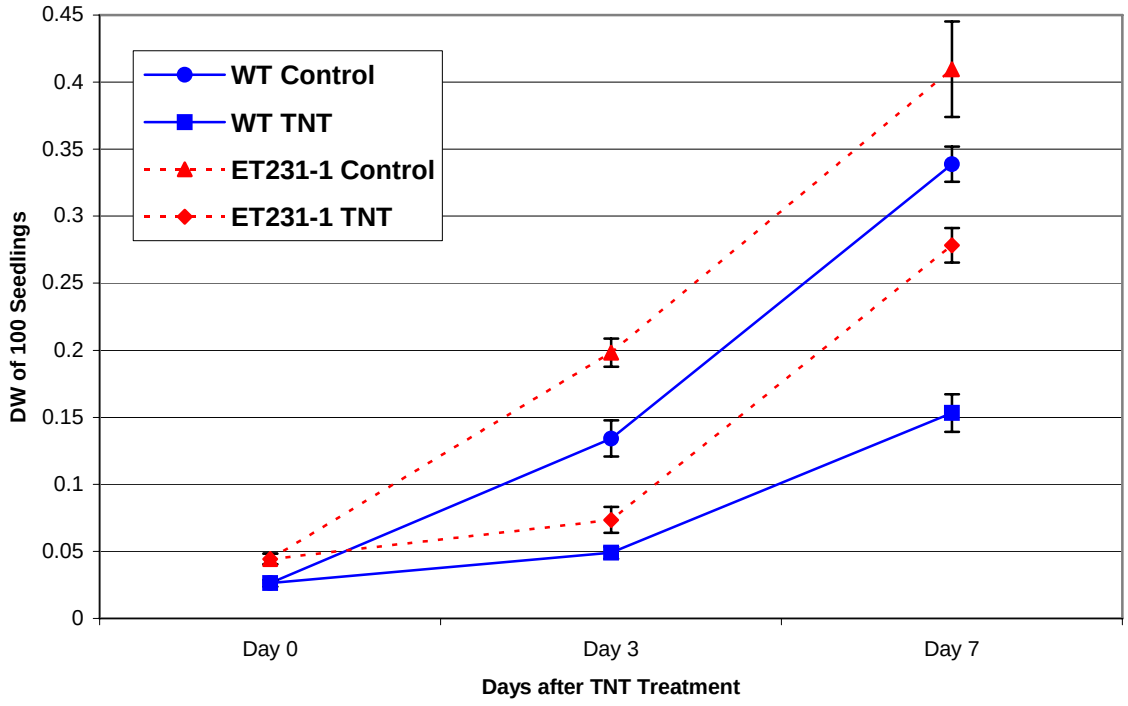


Figure 29. Effect of TNT on growth of an *Arabidopsis* mutant, ET231 and wild-type. TNT was added to a week old culture of 100 seeds to 15 mg/L concentration and the seedlings were grown for another week before harvest. The data presented are means and standard errors of three measurements.

In a separate experiment, 50 seeds of wild-type and another mutant ET32 were used in each flask. While the biomass of wild-type in the presence of 2.5 mg/L TNT was reduced by 17% compared to that of the control, the same amount of TNT had no inhibitory effect on the growth of ET32 mutant line (Table 7).

Table 7. Effect of TNT on growth of an *Arabidopsis* mutant, ET32 and wild-type plants.

TNT, mg/L	**Wild-type**			**ET32-3**		
	DW before TNT, g	DW after TNT, g	Weight gain, g	DW before TNT, g	DW after TNT, g	Weight gain, g
0	0.0203 ± 0.0014	0.381367 ± 0.017514	0.361067 (100%)	0.0172 ± 0.0003	0.352	0.3348 (100%)
2.5		0.3214 ± 0.009143	0.3011 (83.4%)		0.3535 ± 0.0158	0.3363 (100.4%)

To further delineate the effect of TNT on physiological parameters such as biomass a follow-up experiment was performed wherein fifty seeds of each four mutants (ET32, ET40, ET226, ET231) were transferred to flasks containing MS liquid media. 1 week old seedlings were challenged to 5 mg TNT/L. During 8 day exposure, total weights of flasks were measured gravimetrically. At the end of the experiment, the plant materials were blotted and dried for 3 days at 65°C. As seen in Figure 30, there was no significant differences between the dry weights at this initial TNT level (5 mg/L).

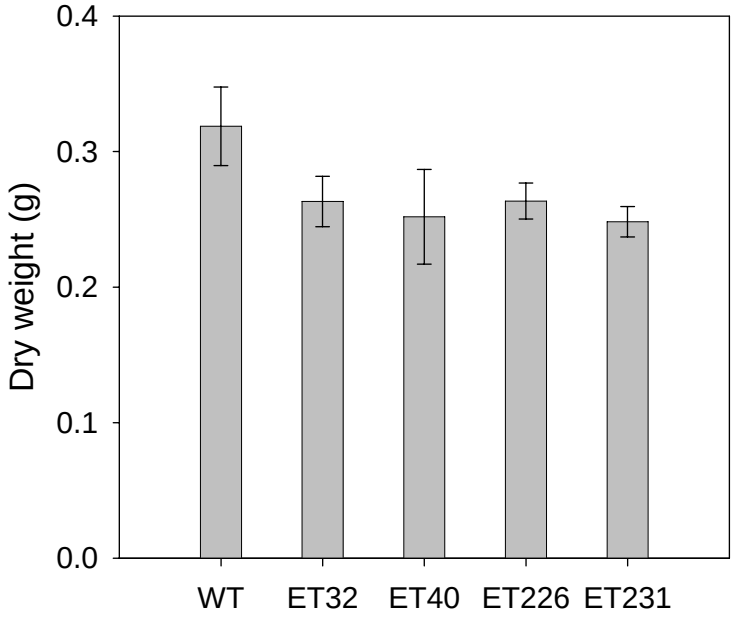


Figure 30. Dry biomass weights of wild type and mutants after 8 day exposure to 5 mg/L of TNT in liquid media. Error bar represent standard deviations.

3.2.2 *Arabidopsis* Root Experiment

In order to determine the effect of TNT on root growth in *Arabidopsis*, TNT was added to a week old culture of 50 seeds at initial concentrations up to 0.8 mg/L concentration and the seedlings were grown for another week before harvesting. Figures 31 and 32 show the length of the seedlings for the various TNT concentrations and controls. As seen TNT, even at low concentrations of 0.8 mg/L, stunts root growth considerably as compared to controls. However, no differences were observed between the wild-type and mutant root lengths.

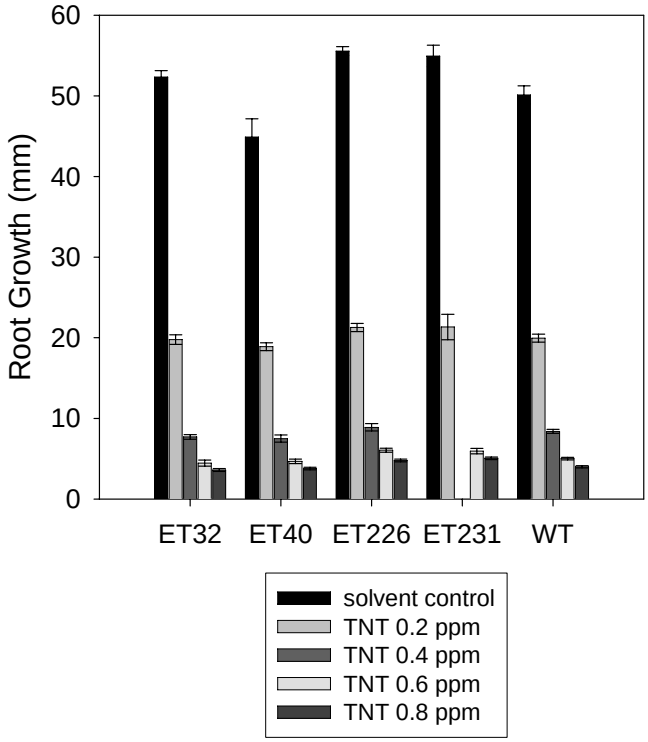


Figure 31. Root growth of the wild type seedlings exposed to different TNT concentrations. Error bars represent standard errors.

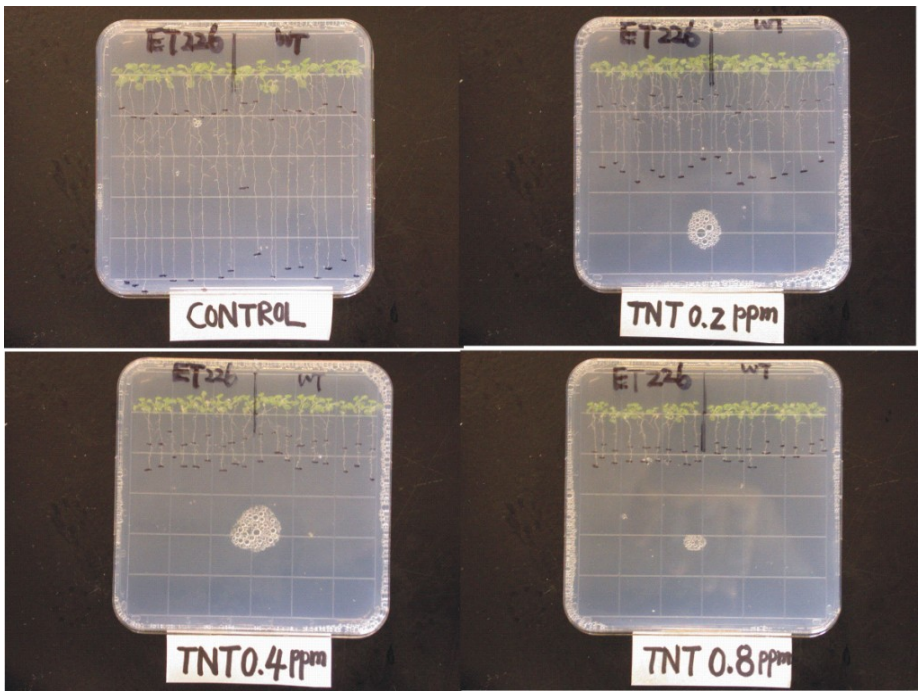


Figure 32. Photos of ET226 mutant seedlings 7 days after exposure to TNT.

3.2.3 Metabolic Analyses of Mutants

In order to determine the TNT uptake and metabolite formation rate, a number of TNT transformation studies were performed on the mutants at varying initial TNT concentrations, with seedlings at different levels of maturity. The mutants were divided into two sample sets for experimental ease. In all these experiments two-week old seedlings were used except in one set of studies where 20-day old stationary roots were used. During the course of all experiments, extracellular media samples were taken at periodic intervals and analyzed for metabolite production. In addition, at the end of the experiment, plant samples were sacrificed and analyzed for intracellular metabolite production. Table 8 lists the various experiments performed, with the initial concentration of TNT used, and the mutants tested

Initial concentrations of 6 mg/L (0.03 mM) and 75 mg/L (0.33 mM) of TNT were added to the *Arabidopsis* mutants ET-30, ET-40, ET-148 and ET-231, and TNT uptake rates and metabolite formation rates monitored. There was no significant difference in TNT removal rates between the wild-type and mutants at these concentrations. In the case of the 6 mg/L system, all the TNT disappears within 4 to 8 hours after amendment, while in the 75 mg/L system, 8 to 12 hours are required for complete TNT removal. Initial concentrations of 100 mg/L (0.44 mM) of TNT were added to two-week old wild-type seedlings and the mutants ET-25, ET-55, ET-183, ET-212, ET-218 and ET-226. Figure 33 shows the extracellular TNT concentrations for the wild-types and the mutants. There does not appear to be a difference in the rate of TNT removal between wild-type and the mutants. Hence, even at a high concentration of 100 mg/L, the mutants do not appear to possess any specific advantages in TNT removal. All the seedlings, however, showed severe signs of stress due to the high levels of TNT; browning, collapsing of the biomass and loss of form were observed in all the mutants and the wild-type seedlings. Despite the severe phytotoxic effects, the seedlings removed all the TNT within 25 hours.

Previously tested mutants ET-30, ET-40, ET-148, ET-231 and wild-type seedlings were again challenged with 120 mg/L (0.53 mM) of TNT in a separate experiment. Figure 33 shows the extracellular TNT concentrations in the wild-type and mutant seedlings; as observed from this figure, at this concentration, there is a difference in the uptake characteristics of the wild-type and mutants. While the wild-type seedlings take 120 hours to remove around 95% of the TNT, it takes the ET-148 mutant only 50 hours to do so. The mutant ET-40 takes around 70 hours to eliminate all TNT from the media, while the mutant ET-231 takes just less than 100 hours for the same. The mutant ET-30 performs marginally better than the wild-type with complete TNT removal within 120 hours. In order to probe the response of wild-type and mutant seedlings to very high levels of TNT, 140 mg/L (0.62 mM) of TNT was added to the mutants ET-25, ET-55, ET-183, ET-212, ET-218, ET-226 and wild-type seedlings. The mutants were observed to outperform the wild-type in TNT removal; the mutants ET-183, ET-218 and ET-226 remove all the TNT from the media in about 12 hours from this high an initial concentration, while mutants ET-25 took 25 hours for the same. In contrast, it took the wild-type seedlings 50 hours to remove 80% of the TNT. The health of the seedlings, both wild-type and mutants, were acutely affected by the high levels of the TNT. The seedlings browned, the leaves and shoots drooped, while the roots also showed brownish coloration. The wild-type seedling appeared to be dead by the end of the experiment, while the mutants were in comparatively

better shape. The photos depicted in Figure 35 shows the comparison in biomass health between the wild-type and the various mutants. The wild-type biomass is completely submerged in the media, while the mutants appear to be standing. Hence, the mutants, although in bad health, appear to be more resistant to the TNT than the wild-type. A separate set of axenic two-week old mutants were exposed to an initial concentration of 170 mg/L (0.75 mM) of TNT. The mutants exposed to the concentration were ET-30, ET- 40, ET-148 and ET-231 along with wild-type seedlings. The mutants ET-40 and ET-148 appear to perform better than the mutants ET-30 and ET-231 which in turn perform better than the wild-types. These four mutants appeared to remove 40 to 50% of the TNT from the media in 120 hours, in contrast to the wild-type seedlings which managed to only eliminate 20% of the TNT after 120 hours.

The difference in TNT removal capacity between the wild-type and the mutants at only very high TNT concentrations coupled with the fact that the mutant plants appeared healthier than the wild-type plants at these levels is an interesting observation. This seems to suggest that the mutants do not possess any specific advantages in transforming TNT; for example, they do not possess upregulated genes such as nitroreductases that are directly involved in TNT reduction. Instead, the mutants appear to be more resistant to the phytotoxic effects of TNT, which explains their better health. Since they are less affected by TNT than the wild-types, this manifests in a better rate of TNT transformation.

At low and high concentrations, the wild-type seedlings were not significantly affected by the toxic effects of TNT; hence mutants and wild-types removed TNT with equal efficiency. At very high concentrations, while the wild-type seedling appeared dead, the mutant seedlings appeared to be in slightly better shape (Figure 35); hence the mutants transformed TNT more efficiently than the wild-types. In addition, the genetic studies did not reveal any specific enzymes such as nitroreductases or cytochrome P450s which could be involved in TNT transformation. Genes regulating general transcription factors and metabolism controllers were seen to be affected in the mutants. Only, the mutant ET-218 appears to have mutated genes involved in direct TNT transformation. This situation, wherein the greater resistance to TNT by the mutants is not due to a specific over-expression of an enzyme directly involved in TNT transformation, is more difficult to analyze. Drawing out the precise nature of the mutation, and the phenotypical difference between the mutant and the wild-types upon exposure to TNT will require significantly more studies, beginning with plant physiology studies, and probably metabolomics of the system.

Complete details on the metabolic analyses of the ET-mutants are given in Subramanian (2004).

Table 8: Experiment and Result Summary: Summary of experiments and the results obtained is presented. The mutants ET-148 and ET-40 appear to be the best in two experiments, while the mutants ET-218 and ET-226 appear to perform the best in another study.

Initial TNT, mg/L	Growth Phase	*Arabidopsis* Mutants Tested	Fresh Weight, g	Results
6 mg/L	Exponential	ET-30, ET- 40, ET- 148, ET- 231	12 ± 2g	Equal Removal Rates
75 mg/L	Exponential	ET-30, ET-40, ET-148, ET-231	12 ± 2g	Equal Removal Rates
100 mg/L	Exponential	ET-25, ET-55, ET-183, ET-212, ET-218, ET-226	12 ± 2g	Equal Removal Rates
120 mg/L	Exponential	ET-30, ET-40, ET-148, ET-231	10 ± 1g	ET-148>ET-40>ET-231>ET-30>WT
140 mg/L	Exponential	ET-25, ET-55, ET-183, ET-212, ET-218, ET-226	12 ± 2g	ET-218>ET-226=ET-183>ET-212>ET-25=ET-55>WT
140 mg/L	Stationary	ET-25, ET-30, ET-32, ET-40, ET-55, ET-148	15 ± 2g	Equal Removal Rates
170 mg/L	Exponential	ET-30, ET- 40, ET-148, ET-231	13 ± 2g	ET-40>ET-148>ET-231=ET-30>WT

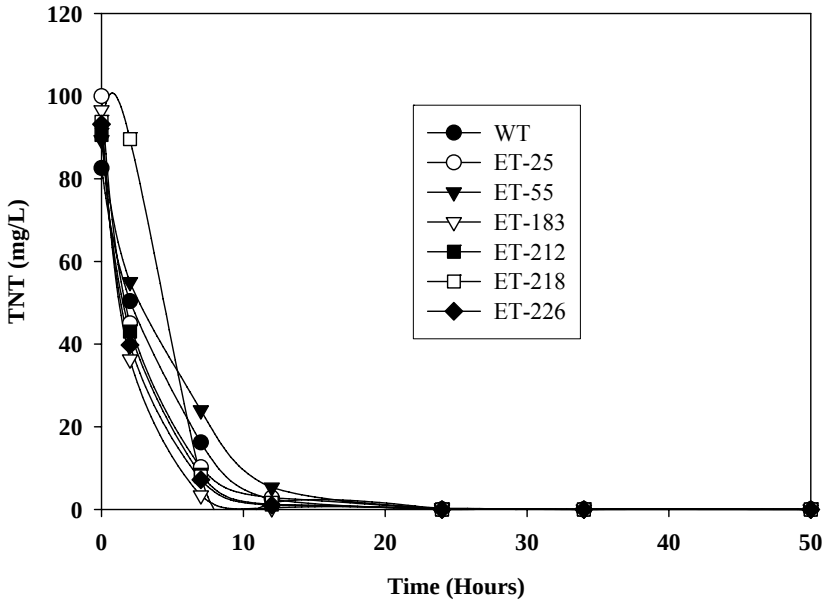


Figure 33. Mutant Study at 100 mg/L: Extracellular TNT concentrations in wild-type and mutant *Arabidopsis* seedlings, exposed to approximately 100 mg/L (0.44 mM) of TNT. No significant differences in TNT removal capacities is seen between the wild-type and mutant seedlings.

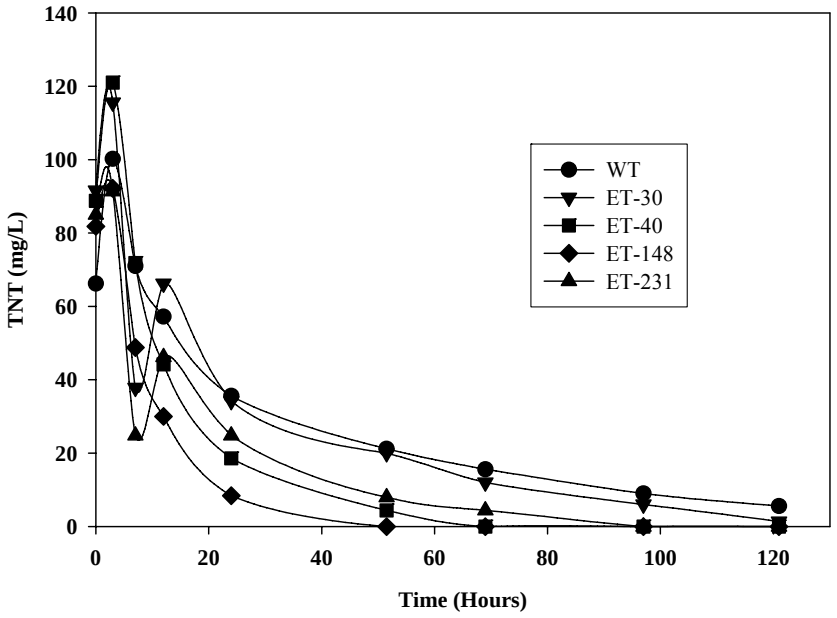


Figure 34. 120 mg/L Mutant Study: Extracellular TNT concentrations in wild-type and mutant *Arabidopsis* seedlings, exposed to approximately 120 mg/L (0.53 mM) of TNT. At this concentration, differences between the wild-type and mutants in the uptake of TNT are seen. While the mutant ET-148 removes all the TNT from the medium within 50 hours, it takes the wild-type 120 hours to remove 95% of the TNT. All the mutants perform better than the wild-type in TNT transformation.

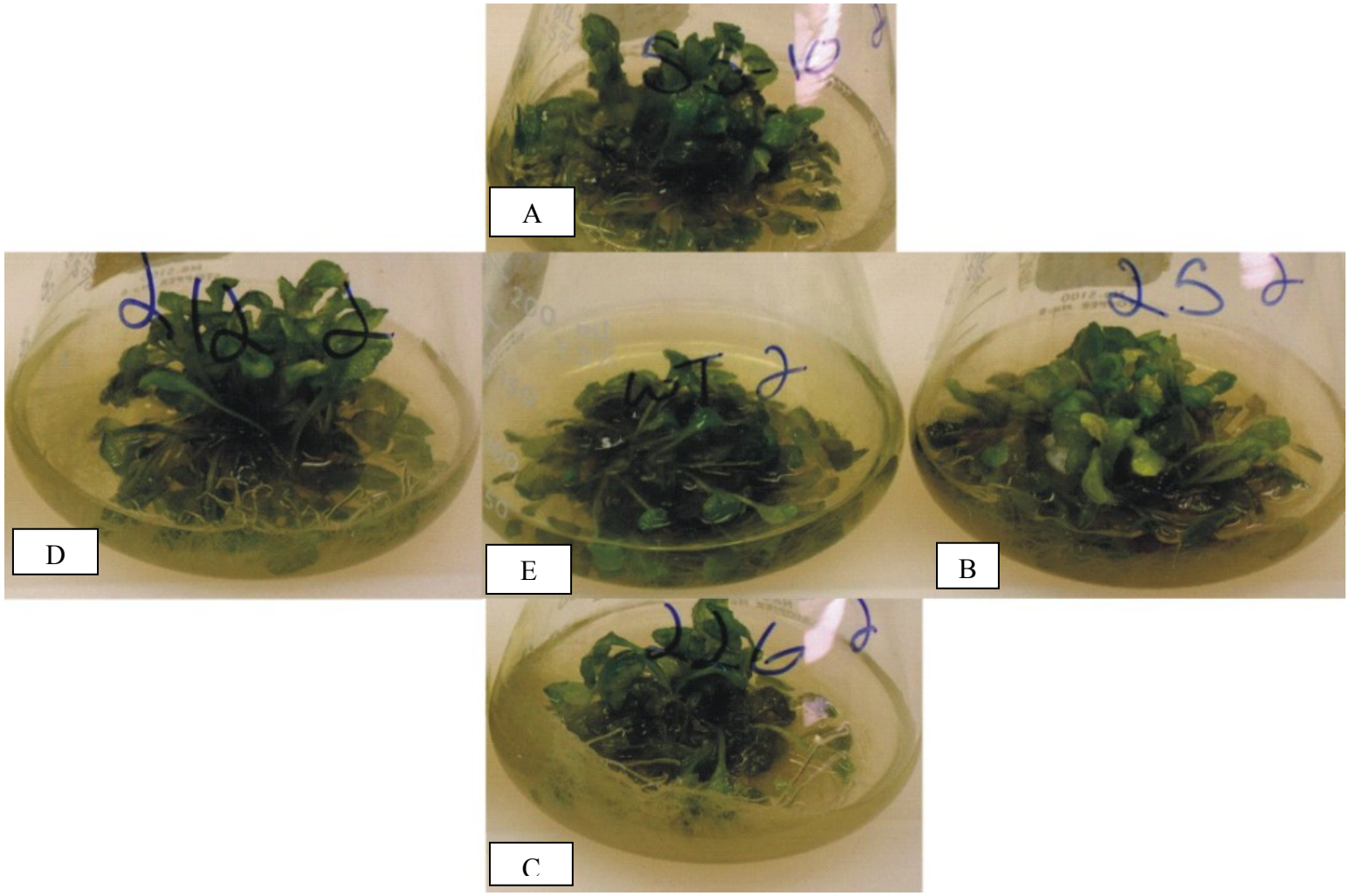


Figure 35. Mutant Health Comparison: Health of mutant seedlings ET-55 (A), ET-25 (B), ET-226 (C), ET-212 (D) and wild-type (E) 106 hours after the addition of 160 mg/L of TNT. The wild-type seedlings appear completely dead and submerged, while the mutant seedling show better health. The mutants ET-55 and ET-212 appear to be in best health.

3.2.4 Effect of RDX on TNT-resistant Mutants: Root growth assays

To determine if the TNT-resistant mutants possessed resistance to RDX too, experiments were performed to measure the root lengths of seedlings exposed to varying concentrations of RDX. The mutants ET25, ET30, ET32, ET40, ET148, ET169, ET212, ET218 and ET226 were exposed to 200 mg/RDX. There was no statistical difference between the root lengths of the seedlings 11 days after exposure to RDX. (Figure 36).

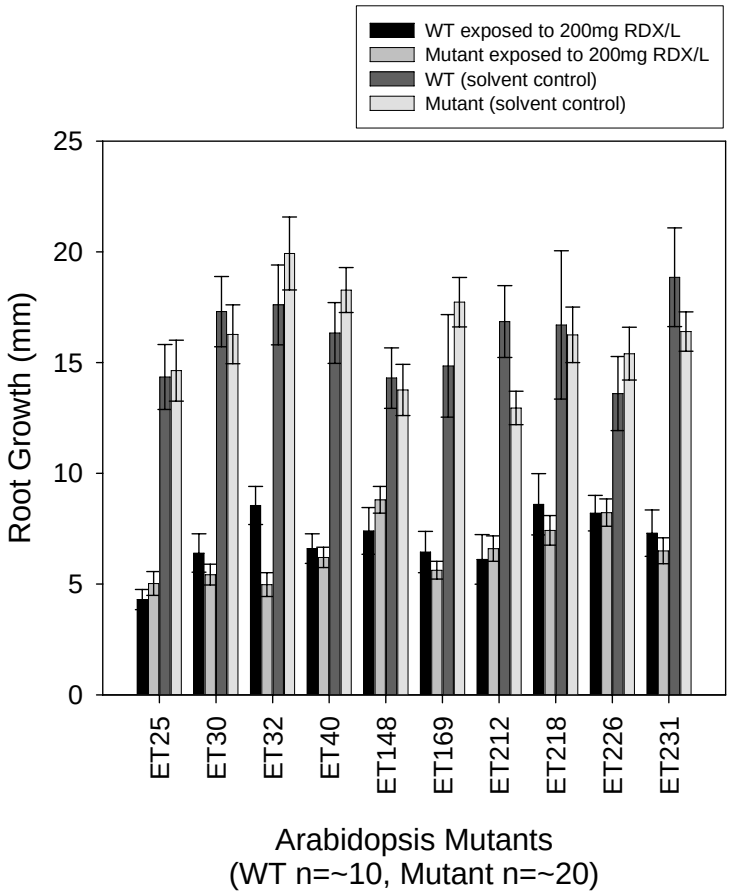


Figure 36. Root growth of *Arabidopsis* mutants in the presence of 200 mg/L of RDX. Error bars represent standard errors.

4. DNT Studies in wild-type *Arabidopsis*, in TNT and GST mutants

4.1 Toxicity of DNTs to wild-type *Arabidopsis*

For a phytotoxicity study, a biomass assay was used. Wild-type seedlings were grown in plates for 4 days. The twenty five seedlings were transferred to a flask containing sterile liquid media in order to assure the same germination. One day was allowed for the adjustment to liquid media, and then, the medium solutions were treated with different amount of 2,4-dinitrotoluene and 2,6-dinitrotoluene. The concentrations were 10, 25, 50, and 100 mg/L. The dry biomass was measured after 2 weeks.

As shown in Figure 37 and 38, 2,4-DNT is more toxic to *Arabidopsis* than 2,6-DNT. The biomass and 2,6-DNT corresponded to 43.2 % for the plants exposed to 10 mg/L of 2,4-DNT and 84.4 % for the plants exposed to 10 mg/L of 2,6-DNT, based on the biomass of the controls. The plants did not grow and blanched at over 25 mg/L of 2,4-DNT and 100 mg/L of 2,6-DNT.

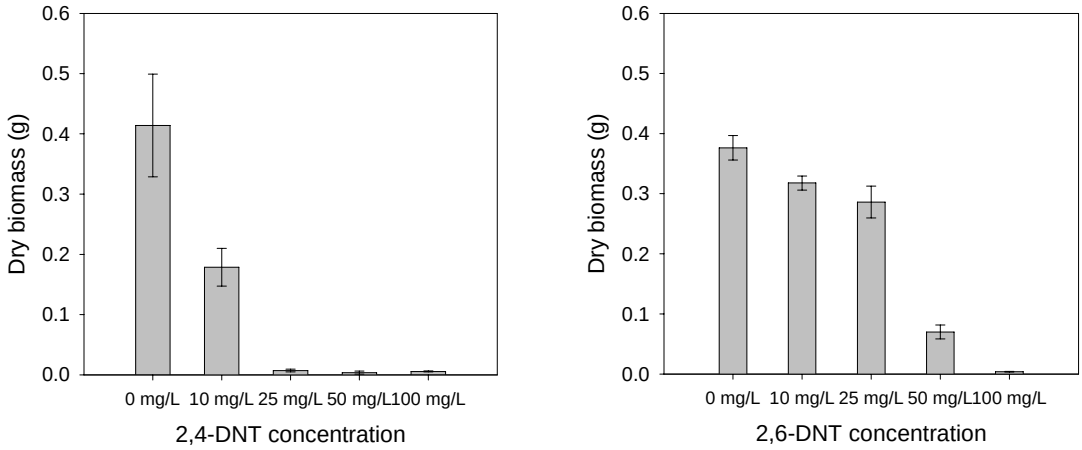


Figure 37. Biomass after 14 day exposure to different concentrations of 2,4-DNT and 2,6-DNT. Error bars represent the standard deviation (n=4).

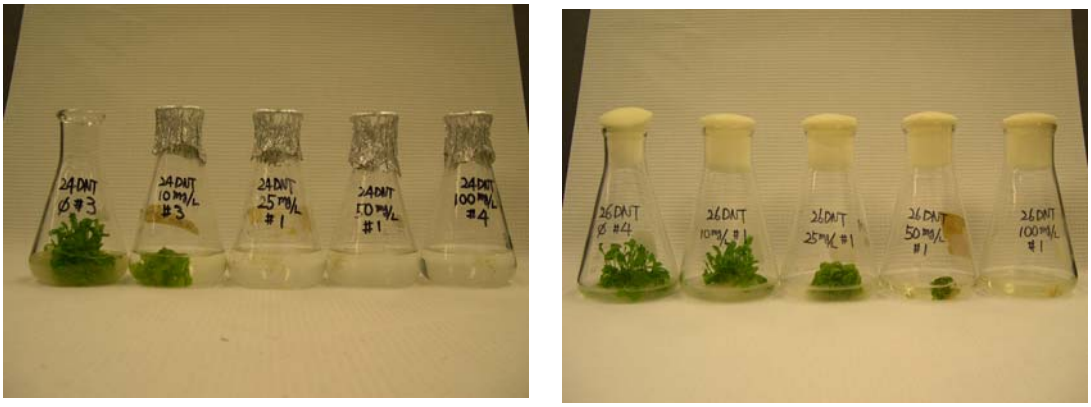


Figure 38. Photos of wild-type *Arabidopsis* after 2 week exposure to different concentration of 2,4-DNT (left) and 2,6-DNT (right).

As an alternative toxicity test, the root growth assay was conducted. Four-day-old seedlings grown in plates were transferred to different plates amended with various amounts of 2,4-DNT and 2,6-DNT. Twelve seedlings were placed per plate and duplicate plates were prepared for each treatment. The end of the root tips was marked initially on the backside of the plats. After 7 days, the length of the root growth was measured. Like the biomass assay, 2,4-DNT is more toxic to the plants than 2,6-DNT from the results of the root growth assay. In addition, the root growth assay is more sensitive than biomass assay suggesting congregation of seedlings in liquid cultures might give more resistance to the contaminants. The root growth was stunted at 1 ppm for 2,4-DNT and at 20 ppm for 2,6-DNT (Figure 39).

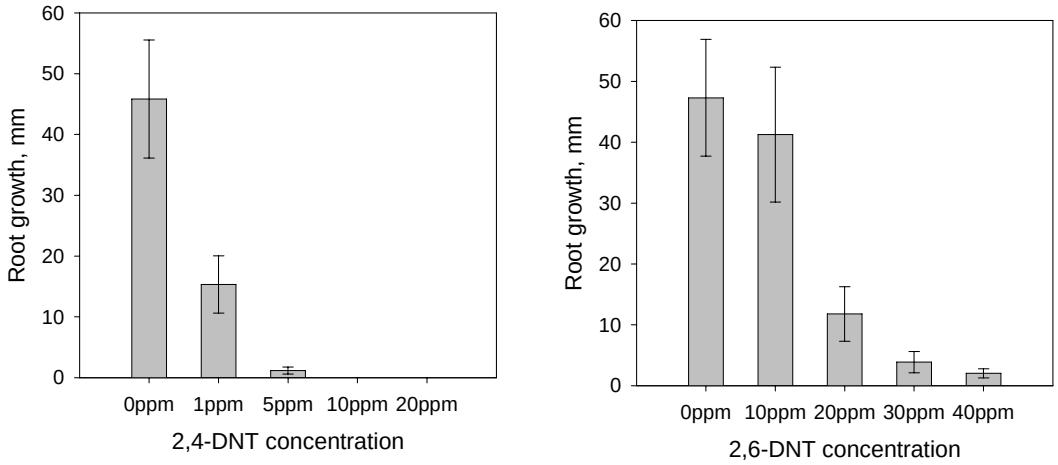


Figure 39. Root growth after 7 day exposure to DNTs in plates. Twelve seedlings were placed per plate and duplicate plates were prepared for each different concentration. Error bars represent standard deviations (n=24) except 5 ppm of 2,4-DNT (n=12).

4.2. Mass Balance Studies in wild-type *Arabidopsis*

Seedings were transferred from solid media, 250 ml wide-mouth Erlenmeyer flasks containing 50 mL MS liquid media were grown for additional 5 days, and then radiolabeled [U-14C]2,4-DNT and [U-14C]2,6-DNT stock solutions were spiked to the liquid media to make radioactivity per flask 2-3 μCi after measuring liquid volumes with 50 mL sterile pipettes. To measure mineralization of radiolabeled dinitrotoluenes by the plants, some flasks were sealed with No. 8 rubber stoppers modified with 6 mL serum vials containing 1 mL of 1N NaOH. The other flasks were plugged with foams to prevent microbial and fungal contaminations. Glass controls, glass control in dark, and autoclaved-plant controls were used also. The plant tissues harvested at different times were chopped with a spatula after freeze-drying for 1 day. About 0.01 g DW of the dry plants were used for biooxidation and the rest of them were used for extraction with methanol.

The radioactivity in media gradually decreased (Figure 40). Only 94 % of the initial radioactivity from [U-14C]2,4-DNT and 93 % from initial radioactivity from [U-14C]2,6-DNT was removed from the solution after 10 days, respectively. The mineralization of the dinitrotoluenes by plants was insignificant compared to the controls (less than 1%) as shown in Figure 41.

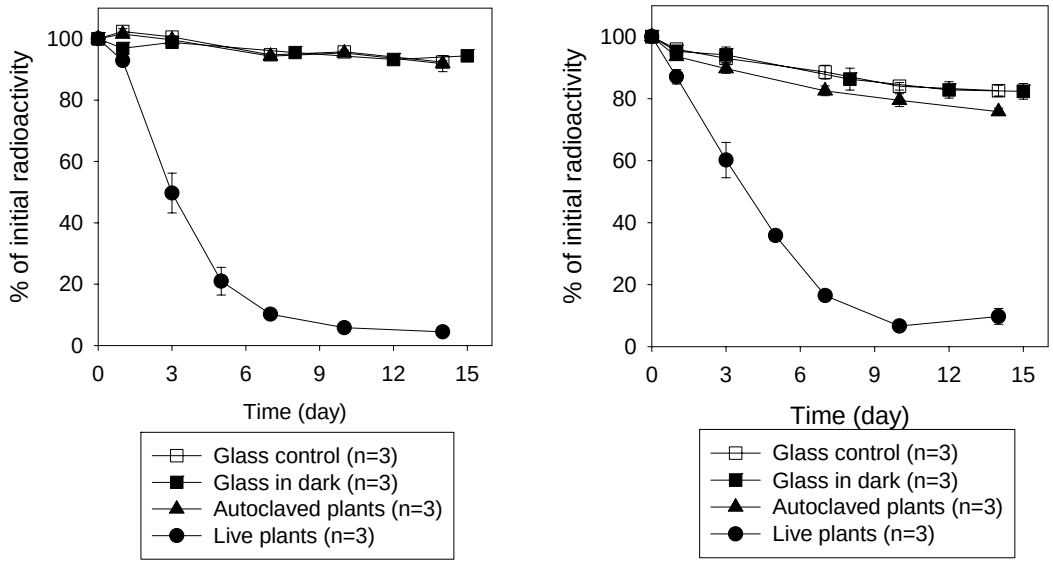


Figure 40. Uptake of ^{14C}-24DNT (left) and ^{14}C-26DNT (right) from liquid media. Plants take up dinitrotoluenes actively.

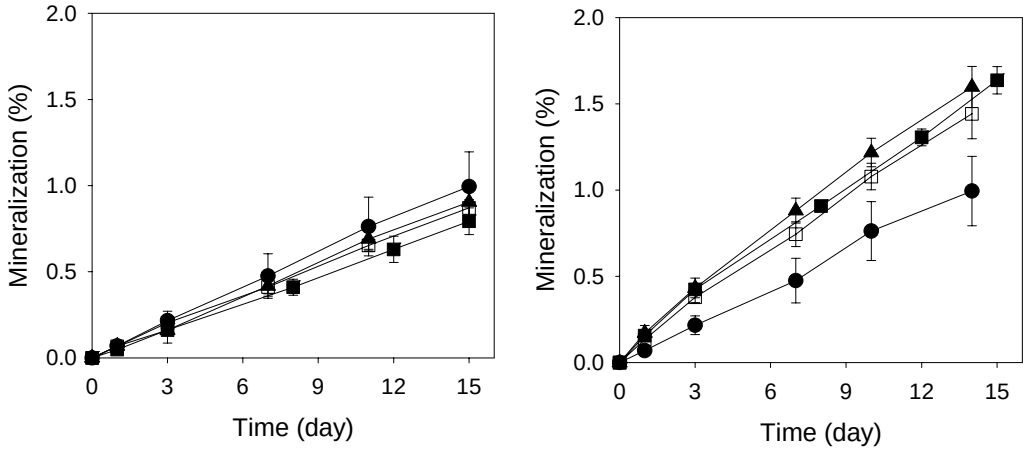


Figure 41. Cumulative mineralization of ^{14}C-24DNT (left) and ^{14}C-26DNT (right). The mineralization of dinitrotoluenes by plants were insignificant compared to the controls.

Mass balances by biooxidation and LSC analysis were shown in Tables 9 and 10. The mass balances were over 86 % for 2,4-DNT (Table 7), and 84 % for 2,6-DNT except for 7 day-exposure (Table 10).

Table 9. Mass balance of ^{14}C-2,4-DNT from biooxidation and LSC analysis.

2,4-DNT	Intracellular	Extracellular	Recovery
Glass control (14 days, n=3)	-	89.8 ± 0.9	89.8 ± 0.9
Autoclaved plants (14 days, n=3)	4.49 ± 0.55	89.1 ± 3.1	93.5 ± 2.6
3 days (n=3)	35.9 ± 9.2	51.9 ± 6.6	87.7 ± 3.6
5 days (n=3)	73.6 ± 5.4	21.0 ± 4.5	94.6 ± 1.8
7 days (n=3)	76.1 ± 8.6	10.2 ± 0.9	86.3 ± 8.9
10 days (n=4)	89.9 ± 1.8	5.81 ± 0.83	95.7 ± 1.8
14 days (n=4)	92.5 ± 2.4	4.46 ± 0.49	97.0 ± 2.4

Table 10. Mass balance of ^{14}C-2,6-DNT from biooxidation and LSC analysis.

2,6-DNT	Intracellular	Extracellular	Recovery
Glass control (14days, n=3)	-	83.6 ± 1.7	83.6 ± 1.6
Autoclaved plants (14days, n=3)	4.47 ± 0.44	79.6 ± 1.3	85.7 ± 0.8
3 days (n=3)	28.9 ± 3.6	60.2 ± 5.7	89.1 ± 2.3
5 days (n=3)	53.4 ± 2.1	35.8 ± 1.3	89.3 ± 1.5
7 days (n=3)	60.7 ± 21.2	16.5 ± 1.8	77.2 ±20.0
10 days (n=4)	82.3 ± 0.9	6.65 ± 1.28	88.9 ± 0.6
14 days (n=4)	75.1 ± 10.2	9.75 ± 2.56	84.8 ± 8.5

The dry samples were sonicated for 1 day after adding 20 mL of methanol. After filtering by using glass filters, aliquots of filtrates were analyzed with LSC for extractable portions. The residues in filters were air-dried and combusted by a biooxidizer for unextractable (bound) portions. Bound (unextractable) portions increased from 49 % to 72 % for 2,4-DNT and from 34 % to 63 % (Figure 42).

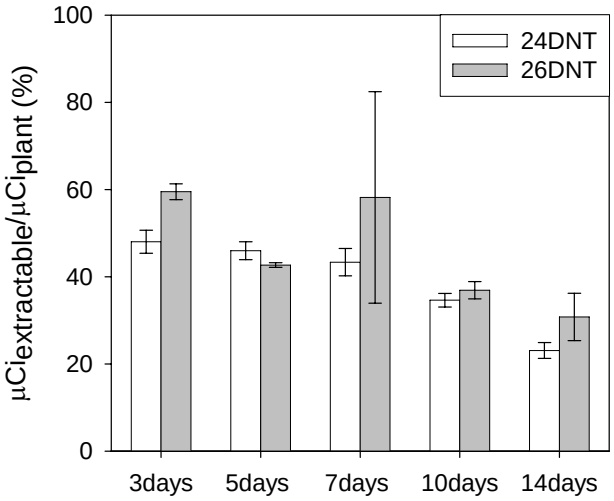


Figure 42. Percentage of extractable radioactivity from plant tissues based on uptaken radioactivity.

From radiochromatograms, several unknown peaks having short retention times and aminonitrotoluenes were detected in radiochromatograms from extracts of plants exposed to dinitrotoluenes. (Figures 43 and 44). However, diaminonitro-toluenes (24DAT and 26DAT) were not detected.

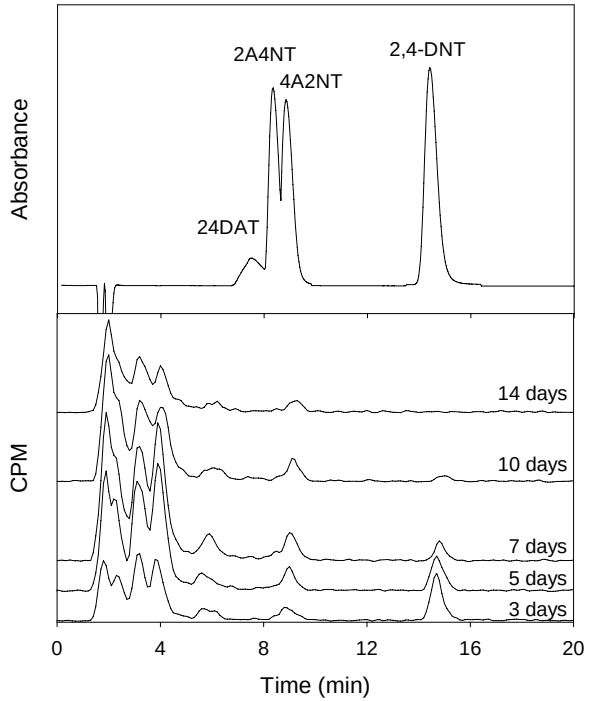


Figure 43. Radiochromatograms (bottom) of extracts from plant tissue exposed to 2,4-DNT at different times and chromatograms (top) of standards by a UV-detector. The retention times: 2,4-diaminotoluene(24DAT): 7.51 min; 2-amino-4-nitrotoluene (2A4NT): 8.35 min; 4-amino-2-nitrotoluene (4A2NT): 8.85 min; 2,4-dinitrotoluene (24DNT): 14.41 min.

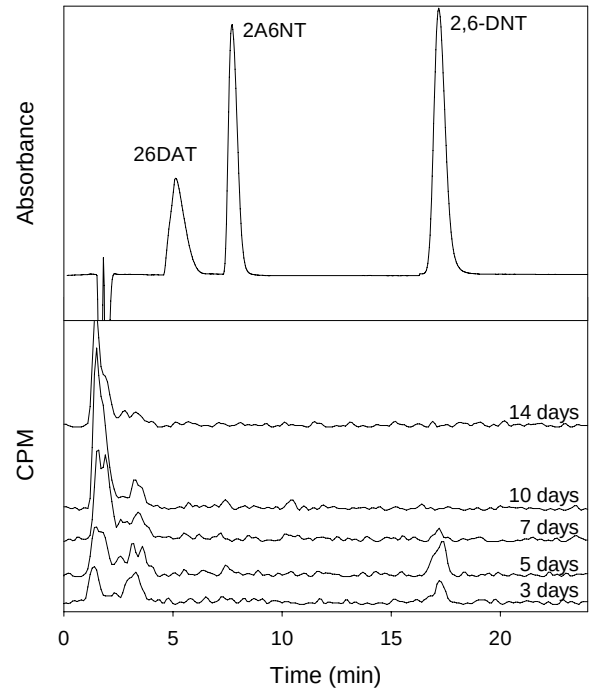


Figure 44. Radiochromatograms (bottom) of extracts from plant tissue exposed to 2,6-DNT at different times and chromatograms (top) of standards by a UV-detector. The retention times: 2,6-diaminotoluene(26DAT): 5.11 min; 2-amino-6-nitrotoluene (2A6NT): 7.71 min; 2,6-dinitrotoluene (2,6-DNT): 17.18 min.

4.3 Distribution of ^{14}C Radiolabel in Plant Tissues in wild-type *Arabidopsis*

To investigate the distribution of labels from [U-14C]2,4-DNT and [U-14C]2,6-DNT in different plant tissues, wild-type *Arabidopsis* was grown hydroponically as shown in Figure 45. After growing plants from seeds in half-strength hydroponic solutions for 21 days, 3 μCi of ^{14}C-labeled dinitrotoluenes were added to the nutrient solutions. The plants were harvested at different times and were divided into leaf, new stem, and root tissues. After drying for 3 days at room temperature, the tissues were combusted by a biooxidizer.

The tubes were rinsed with 15 ml methanol to recover the radioactivity absorbed to the tubes after decanting the media,. The radioactivity in media and tube extracts was analyzed with an LSC.

Figure 45. Photo of 21-day-old seedlings grown hydroponically.

Based on the radioactivity from [U-14C]2,4-DNT and [U-14C]2,6-DNT in the plants, 64% and 56% remained in the roots after 25 days, respectively (Figure 45). The overall mass balance decreased from 79% to 61% for 2,4-DNT (Table 11) and from 70% to 59% for 2,6-DNT (Table 12). Bacterial mineralization of dinitrotoluenes may cause the decrease of the overall mass balance. The plants removed 45.4% for 2,4-DNT and 38.8% after 25 days. A significant portion of the radioactivity corresponded to the tube extracts indicating dinitrotoluenes are absorbed to the conical polyethylene tubes.

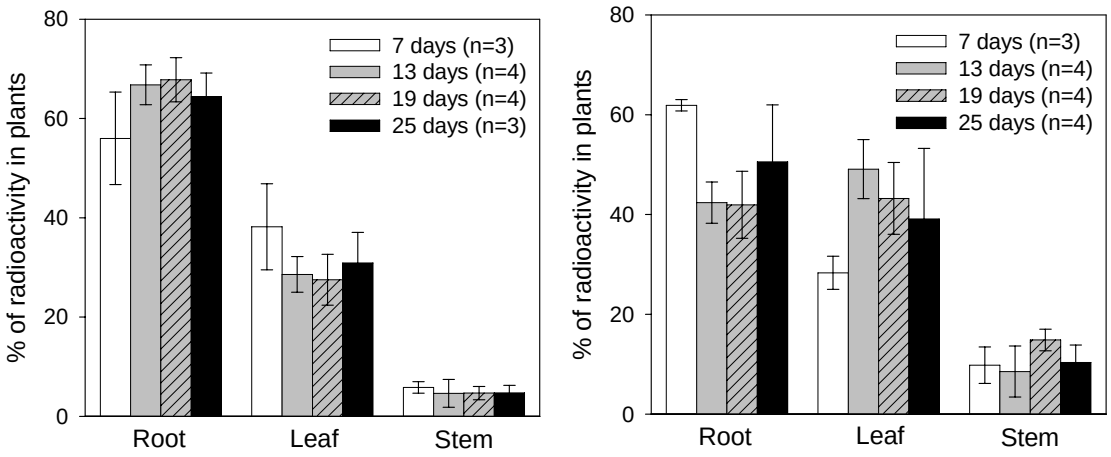


Figure 46. Distribution of ^{14}C labels in plant tissues based on the radioactivity in the whole plants exposed to [U-14C]2,4-DNT (left) and [U-14C]2,6-DNT (right).

Table 11. Percentage of the initial radioactivity (^{14}C-2,4-DNT) at different harvest times.

	7 days (n=3)	13 days (n=4)	19 days (n=4)	25 days (n=3)
Root	11.6 ± 3.48	19.2 ± 2.92	29.7 ± 5.29	29.8 ± 1.24
Leaf	7.68 ± 1.04	8.12 ± 0.33	12.1 ± 3.20	14.4 ± 3.52
Stem	1.17 ± 0.12	1.33 ± 0.84	1.99 ± 0.34	2.17 ± 0.60
Liquid medium	48.6 ± 2.20	38.3 ± 3.03	21.0 ± 3.80	11.0 ± 3.56
Methanol rinse	8.77 ± 0.69	7.24 ± 1.91	4.89 ± 1.53	3.48 ± 0.60
Recovery	78.9 ± 0.80	74.2 ± 4.69	69.6 ± 3.71	60.8 ± 5.03

Table 12. Percentage of the initial radioactivity (^{14}C-2,6-DNT) at different harvest times.

	7 days (n=3)	13 days (n=4)	19 days (n=4)	25 days (n=4)
Root	6.54 ± 0.49	5.86 ± 1.25	13.4 ± 1.97	19.4 ± 4.22
Leaf	3.00 ± 0.54	6.81 ± 1.49	14.1 ± 4.29	15.4 ± 7.04
Stem	1.02 ± 0.34	1.10 ± 0.50	4.82 ± 1.16	3.92 ± 1.09
Liquid medium	52.3 ± 5.00	45.7 ± 4.19	27.3 ± 4.11	17.0 ± 3.69
Methanol rinse	7.47 ± 0.71	6.86 ± 3.90	3.87 ± 0.76	3.04 ± 0.97
Recovery	70.4 ± 4.98	66.3 ± 7.12	63.5 ± 3.90	58.8 ± 3.97

4.4 Glutathione and Gene Expression in wild-type *Arabidopsis*

The detoxification process of xenobiotics by plants includes the conjugation with glutathione by glutathione *S*-transferases, and reactions by cytochrome P450 enzymes. It is reported that glutathione level increased and the genes of a glutathione S-transferase and a cytochrome P450 were highly induced in root tissues of *Arabidopsis* exposed to TNT. We investigated the changes of glutathione level and gene expression in response to dinitrotoluene by using a fluorescence assay and real-time PCR analysis.

Fifty seeds of wild-types were grown for 14 days before 2,4-DNT and 2,6-DNT were spiked to MS liquid media. The concentrations were 10 mg/L for 2,4-DNT and 25 mg/L for 2,6-DNT. Equal volumes of methanol were added for the control plants. The root tissues were collected after 1 day. Glutathione in the root tissues were determined by a HPLC with a fluorescence detector after derivatizing of root extracts with monobromobimane. Glutathione standards were derivatized under the same conditions for quantification.

The concentration of glutathione in the root tissues of *Arabidopsis* exposed to 2,4-DNT increased significantly (Figure 47). When the plants were exposed to 10 mg/L of 2,4-DNT for 1 day, 83.0 ± 3.7 μM (n=3) of glutathione was measured in the root tissue extracts compared to 63.2 ± 2.4 μM (n=3) for the extracts from the unexposed plants. The difference of glutathione concentrations was statistically significant by t-test. In contrast, the level of glutathione did not increase in the root tissues of the plants exposed to 25 mg/L 2,6-DNT. The difference of glutathione concentrations between the treated and untreated plants was statistically insignificant. The concentrations were 54.7 ± 8.4 μM for the untreated plants (n=3) and 57.5 ± 3.2 μM for the treated plants (n=3).

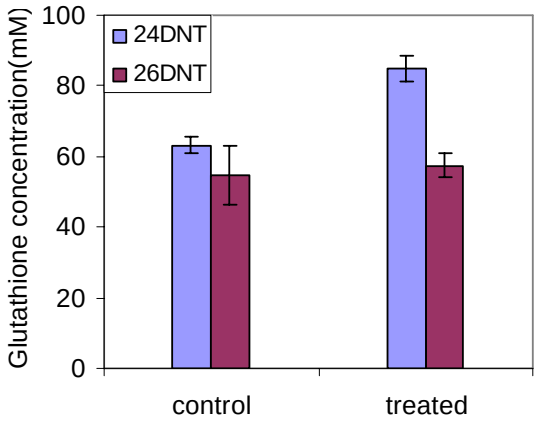


Figure 47. The levels of glutathione in the roots of the plants exposed to dinitrotoluenes compared to the control plants. The concentration of glutathione was determined by a HPLC with a fluorescence detector after derivatization with monobromobimane.

We investigated changes of expression of genes (*GSH1* and *GSH2*) involved in glutathione synthesis, and the genes (At1g17170 and At3g28740) which are highly induced by TNT exposure. After RNA extraction with Triazol (Invitrogen, Carlsbad, CA), first strand cDNA

was synthesized. Based on gene sequences at The *Arabidopsis* Information Resource (TAIR, http://www.arabidopsis.org), primer sets were designed. The sequences of primers, loci of genes, and PCR product sizes are listed in Table 13. The *Actin-8* gene was used as a control gene for both treated and untreated plants.

Table 13. Primer sets used for real-time PCR and genomic DNA contamination.

Name	Locus	Primer sets	Product size (bp)
Actin-8	At1g49240	L: TCCGGTTACAGCGTTTGGAGA R: CGCGGATTAGTGCCTCAGGT	84
Tubulin -1 chain (*TUA1*)	At1g64740	L: ATGAGGGAGATCATTAGCATTCATAT R: CTGAGGAGAAGGGTAGATGGTG	1191* 445*
GSH1	At4g23100	L: CCGTGTTCGAAGAGCTGCTG R: TTCCGGAGACTCGAATTCTTCA	106
GSH2	At5g27380	L: TGGATAACATTCACAACCATCTTGA R: GCTTTGCGGTCCTGTGGAAA	143
Putative GST	At1g17170	L: GTCGCTAAAGCCCTGCCTGA R: GAACAAAGCAACAACAGATCAACA	117
Putative cytochrome P450	At3g28740	L: CGACGATCTTGCCCTGGTTC R: GCTTTTCGCATTGTGGTTCC	146

L and R represent left primers and right primers
* PCR product sizes are 1191 bp for genomic DNA and 445 bp for cDNA.

From the results of real-time PCR analysis (Figure 47), both genes involved in glutathione synthesis, *GSH1* and *GSH2*, were induced 1.7-fold on average. The genes of a putative glutathione *S*-transferase (At1g17170) and a putative cytochrome P450 (At3g28740) were upregulated by 10-fold and 8-fold in the root tissues of 2,4-DNT-treated plants compared to the control plants.

In response to 2,6-DNT, the genes of glutathione synthesis were not induced (Figure 48). This result corresponded to that from the fluorescence assay for glutathione levels. The gene of At1g17170 and At3g28740 were induced by 6-fold and 14-fold on average. No genomic DNA contamination was confirmed by PCR product sizes from agarose gels using primers designed in the exon regions of tubulin alpha-1 chain (At1g64740). Lack of non-specific amplifications were confirmed with single peaks in the melting curves from real-time PCR analysis.

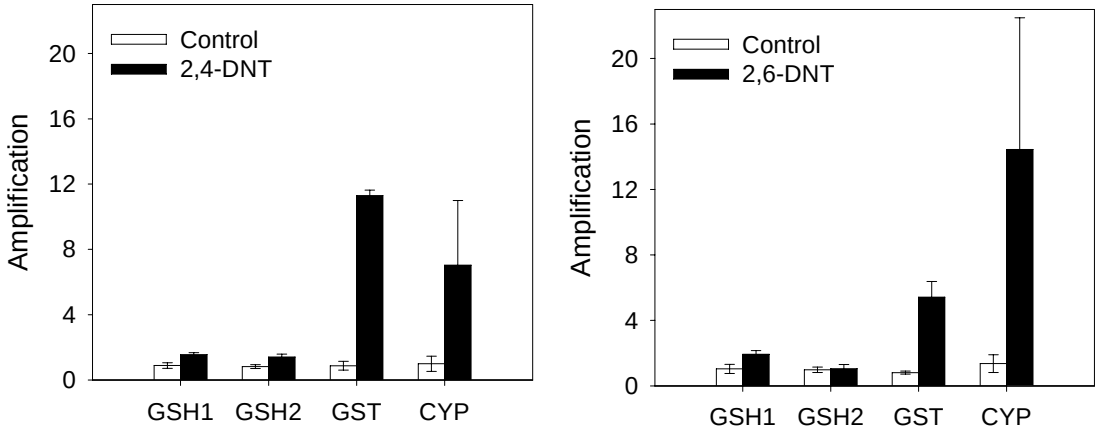


Figure 48. Gene expression of wild-type *Arabidopsis* exposed to 2,4-DNT (left) and 2,6-DNT (right) for 1 day compared to control plants (without dinitrotoluenes) from real-time PCR.

4.5 Comparison of Wild-type and ET226 Mutant

4.5.1 Uptake Study

ET226 was selected for further study since it showed fairly consistent resistance to nitroaromatic explosives. To investigate the resistance to 2,4-DNT, wild-type seedlings grown for 14 days were exposed to 25, 58, 119 mg/L of 2,4-DNT. The uptake rate by wild-type plants decreased over 58 mg/L for 2,4-DNT and 100 mg/L for 2,6-DNT as shown in Figure 49.

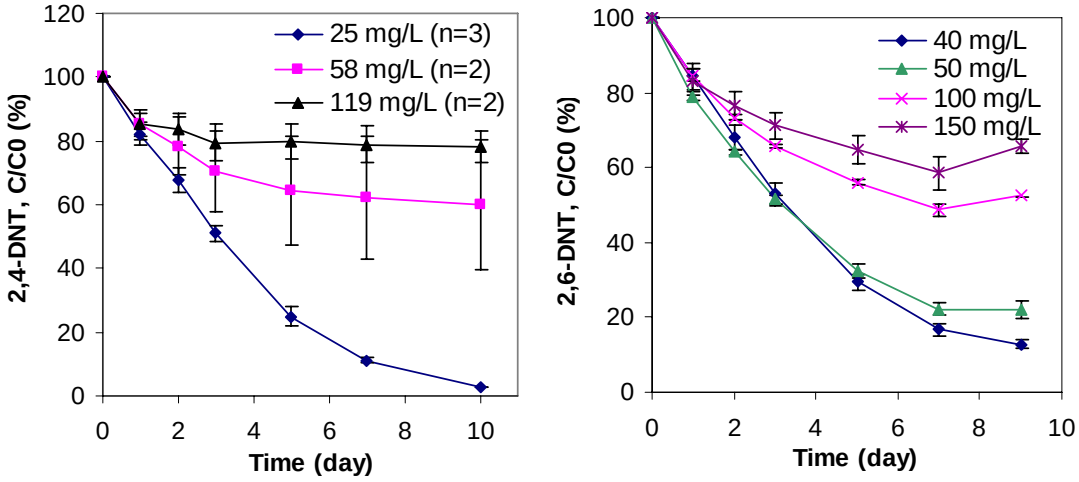


Figure 49. Percentage of the initial concentration of 2,4-DNT (left) and 2,6-DNT (right) in the media. The plant did not take up 2,4-DNT at over 58 mg/L and 2,6-DNT at over 100 mg/L.

From the above results, wild-type and ET226 mutants were exposed to the wide range of concentrations of dinitrotoluenes. When the first order rate constants (*k*) of both wild-type and the mutant exposed to 2,4-DNT (30, 40, 50, 60, and 70 mg/L) were considered (Table 14), the uptake by the wild-type was of the same or better than that by the ET226 mutants. The uptake rate by both wild-type and ET226 mutant decreased as the concentration increased.

Table 14. The first order rate constants of both wild-type and ET226 mutant exposed to different concentrations of 2,4-DNT.

2,4-DNT (mg/L)	Wild-type		ET 226 mutant	
	k (day^{-1})	r^2	k (day^{-1})	r^2
30	0.3050 ± 0.0118	0.9927 ± 0.0055	0.2065 ± 0.0294	0.9930 ± 0.0023
40	0.1817 ± 0.0011	0.9792 ± 0.0079	0.1542 ± 0.0441	0.8909 ± 0.0166
50	0.1140 ± 0.0215	0.8787 ± 0.0477	0.1224 ± 0.0148	0.8914 ± 0.0235
60	0.0781 ± 0.0062	0.6994 ± 0.0172	0.0636 ± 0.0223	0.7232 ± 0.1343
70	0.0608 ± 0.0082	0.5572 ± 0.0269	0.0547 ± 0.0045	0.5427 ± 0.0493

When wild-type and ET226 mutants were exposed to various concentrations of 2,6-DNT (50, 60, 70, and 80 mg/L), the first-order rate constant of both wild-type and ET226 mutants decreased like 2,4-DNT exposure as the concentrations increased as shown in Table 15. There was no difference of uptake rate between wild-type and ET226.

Table 15. The first order rate constants of both wild-type and ET226 mutant exposed to different concentrations of 2,6-DNT.

2,6-DNT (mg/L)	Wild-type		ET 226 mutant	
	k (day^{-1})	r^2	k (day^{-1})	r^2
50	0.1895 ± 0.0042	0.9957 ± 0.0028	0.1866 ± 0.0102	0.9983 ± 0.0012
60	0.1414 ± 0.0147	0.9958 ± 0.0011	0.1317 ± 0.0080	0.9970 ± 0.0020
70	0.1389 ± 0.0071	0.9792 ± 0.0058	0.1452 ± 0.0040	0.9872 ± 0.0085
80	0.1141 ± 0.0126	0.9670 ± 0.0164	0.1236 ± 0.0043	0.9812 ± 0.0052
90	0.1073 ± 0.0149	0.9562 ± 0.0118	0.1187 ± 0.0113	0.9626 ± 0.0134

4.5.2 Comparison of Dry Biomass Between Wild-type and ET226

After 9 day-exposure, the dry biomass of both wild-type and ET226 mutant exposed to dinitrotoluenes was measured. The difference of the biomass between wild-type and ET226 was insignificant. The biomass of wild type was greater than that of the mutant at 30 mg/L 2,4-DNT, but no difference of biomass was observed at the higher concentrations (Figure 50).

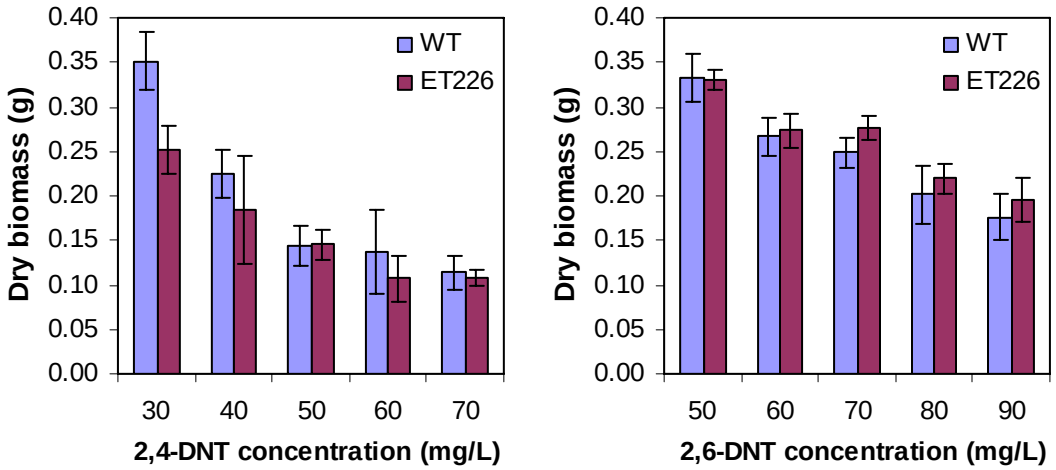


Figure 50. Dry biomass of wild-type and ET226 mutant exposed to different concentrations of 2,4-DNT (left) and 2,6-DNT (right) after 9-day exposure.

4.5.3 Root Growth Assay For Comparison Between Wild-type and ET226

Four-day-old seedlings of wild-type and ET226 were transferred to dinitrotoluene-amended plates (3, 5, 8, 10 mg/L 2,4-DNT; 20, 30, 40, 50 mg/L 2,6-DNT). As shown in Figure 51 and Figure 52, the difference of root growth between wild-type and ET226 was not clear. We attempted to measure fresh biomass of seedlings after taking them from the solid media. When seedlings exposed to 8 mg/L 2,4-DNT was compared, the fresh biomass was 0.0934 ± 0.0248 g for ET226 mutants and 0.0598 ± 0.0336 g for wild-type after 48-day exposure. At 10 mg/L of 2,4-DNT, it was 0.0581 ± 0.0360 g for the ET226 mutant and 0.0487 ± 0.0167 for the wild-type. However, the difference of the fresh biomass is not significant by t-test. In the case of 50 mg/L 2,6-DNT, the biomass was 0.0834 ± 0.0190 g for ET226 and 0.0326 ± 0.0066 g for the wild-type and the difference of the biomass was statistically significant (Table 16). For other cases, the quantitative comparison is not feasible because it was hard to separate individual seedlings from the plates.

Table 16. The difference of fresh biomass exposed to dinitrotoluenes in plates.

		ET226 (n=3)	Wild-type (n=3)	p value (one-tail)
2,4-DNT	8 mg/L	0.0934 ± 0.0248	0.0598 ± 0.0336	0.118
	10 mg/L	0.0581 ± 0.0360	0.0487 ± 0.0167	0.289
2,6-DNT	50 mg/L	0.0834 ± 0.0190	0.0326 ± 0.0066	0.024

Figure 51. The root growth of wild types (left-hand side on plates) and ET226 mutants (right-hand side on plates) exposed to different concentrations of 2,4-DNT. Photos were taken after 25 days (1st row), 31 days (2nd row), and 48 days (3rd and 4th row).

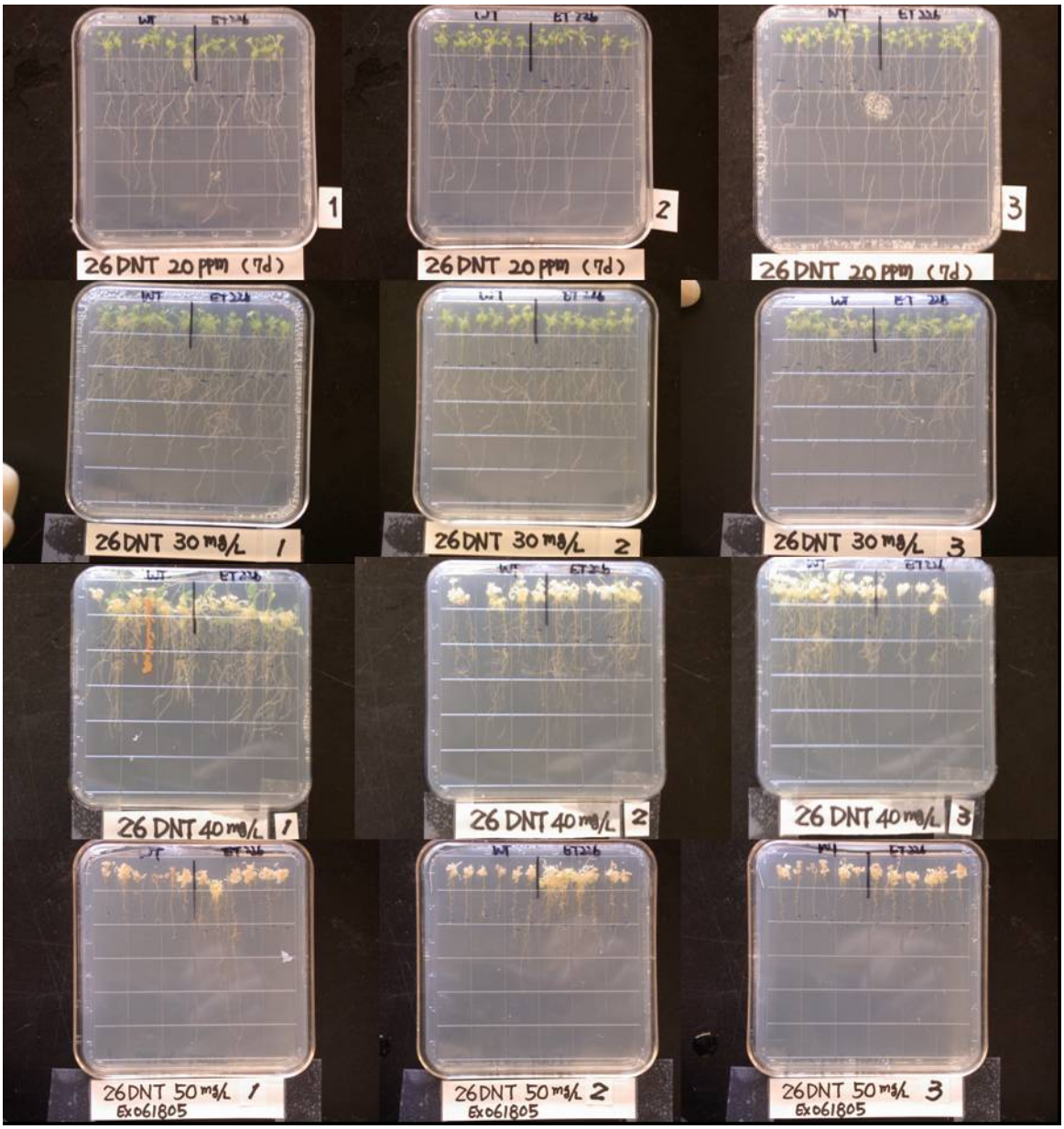


Figure 52. The root growth of wild types (left-hand side on plates) and ET226 mutants (right-hand side on plates) exposed to different concentrations of 2,6-DNT. Photos were taken after 7 days (1st row), 24 days (2nd row), 30 days (3rd row), and 45 days (4th row).

4.6. Comparison of Wild-type and Glutathione S-transferase Mutant

4.6.1 Homozygous glutathione S-transferase Mutant

We observed that the gene for a glutathione S-transferase in wild-type was highly induced in response to DNTs in the gene expression study. However, it is not clear if the gene is upregulated for degradation of dintrotoluenes or as a general response to the stress. We obtained T-DNA insertion mutant seeds for the glutathione S-transferase gene (At1g17170) from the Arabidopsis Biological Resource Center (ABRC). The seeds were grown in soil to have enough and homozygous seeds.

To check the homozygosity of the mutant plant, two-primer sets were designed and PCR was performed after DNA extraction. Homozygous mutants do not have a band in the gel electrophoresis in the usage of a primer set for mutants but not for wild-type. One out of nine plants was homozygous (Figure 53). The homozygous plant will be used for the study of the comparison of metabolite profiles between wild-type and the mutant in order to investigate the involvement of the At1g17170 gene in degradation and transformation of dinitrotoluenes.

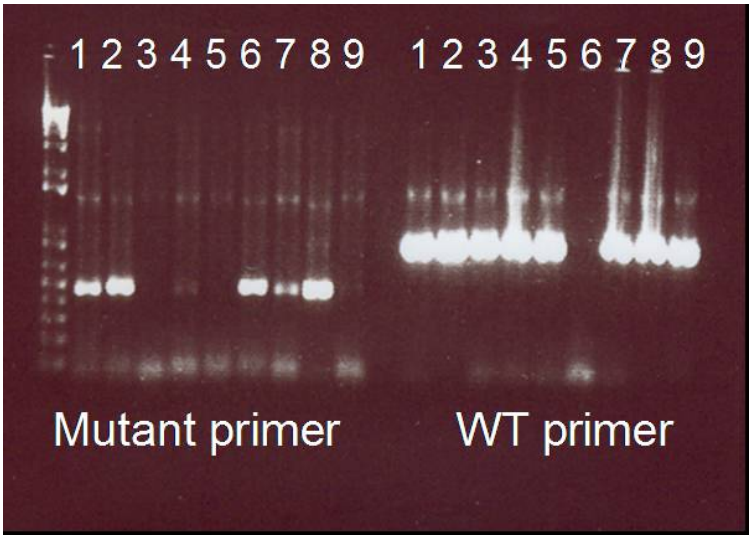


Figure 53. Gel electrophoresis using two-set primers for mutants and wild-type. The 6th lane has a band using the mutant primer set, but not using the wild-type primer set indication the plant is homozygous.

4.6.2 Uptake study at different concentrations of 2,4-DNT, 2,6 DNT, and TNT by *gst* mutant and wild-type.

The *gst* mutant and wild-type seedlings were grown on the MS solid medium plates for 4 days. 25 seedlings of each mutant and wild-type were transferred to liquid cultures and they were grown additional 5 days for 2,4-DNT uptake experiment, 11 days for the 2,6-DNT study, and 10 days for TNT uptake study. They were exposed initially to 6.6, 13.7, an 20.9 mg/L of 2,4-DNT for 14 days, 20, 40, and 80 mg l^{-1} of 2,6-DNT for 7 days, and 36.1, 63.8, and 155.1 mg/L of TNT for 6 days. The biomass was measured at the end of the experiments.

Great differences of uptake rates between the mutant and wild-type exposed to 2,4-DNT were not observed (Figure 54). Both plants removed 95 % of the initial concentration (6.6 mg/L 2,4-DNT) after 14 days, but significant decrease of uptake rate and growth was observed over 13.7 mg/L 2,4-DNT. According to biomass, the growth of plants was inhibited significantly over 13.7 mg/L 2,4-DNT (Figure 55).

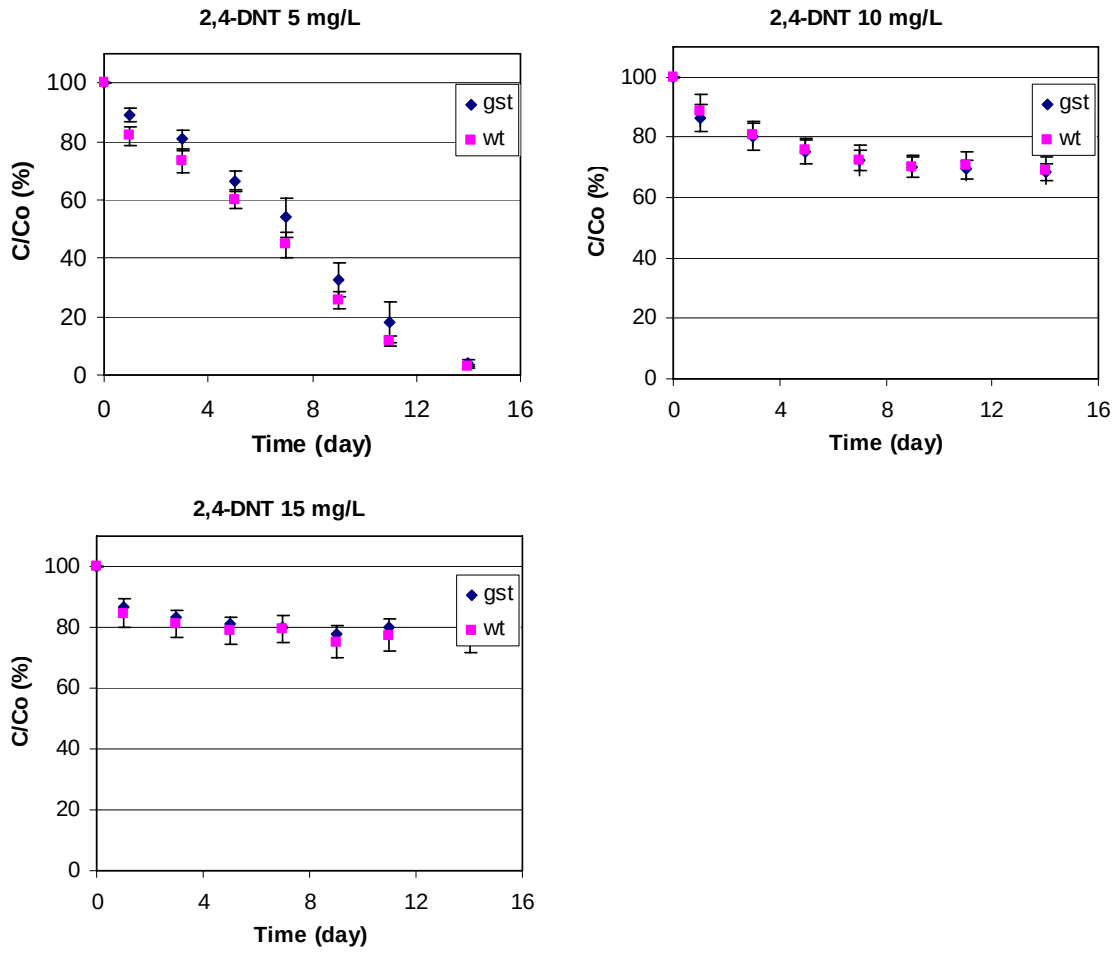


Figure 54. Uptake of the *gst* mutants and wild-types at different concentrations of 2,4-DNT for 14 days.

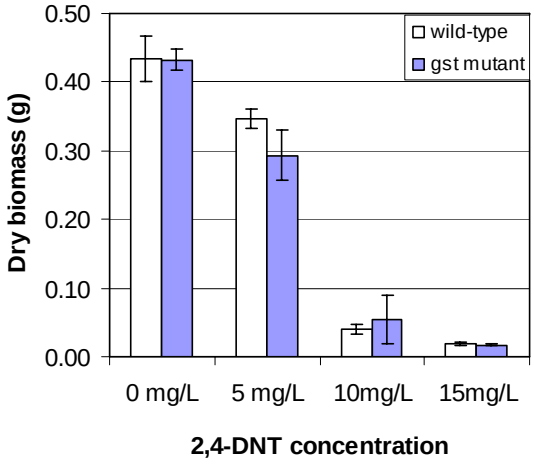


Figure 55. Dry biomass of the *gst* mutants and wild-types exposed to different concentrations of 2,4-DNT after 14 days.

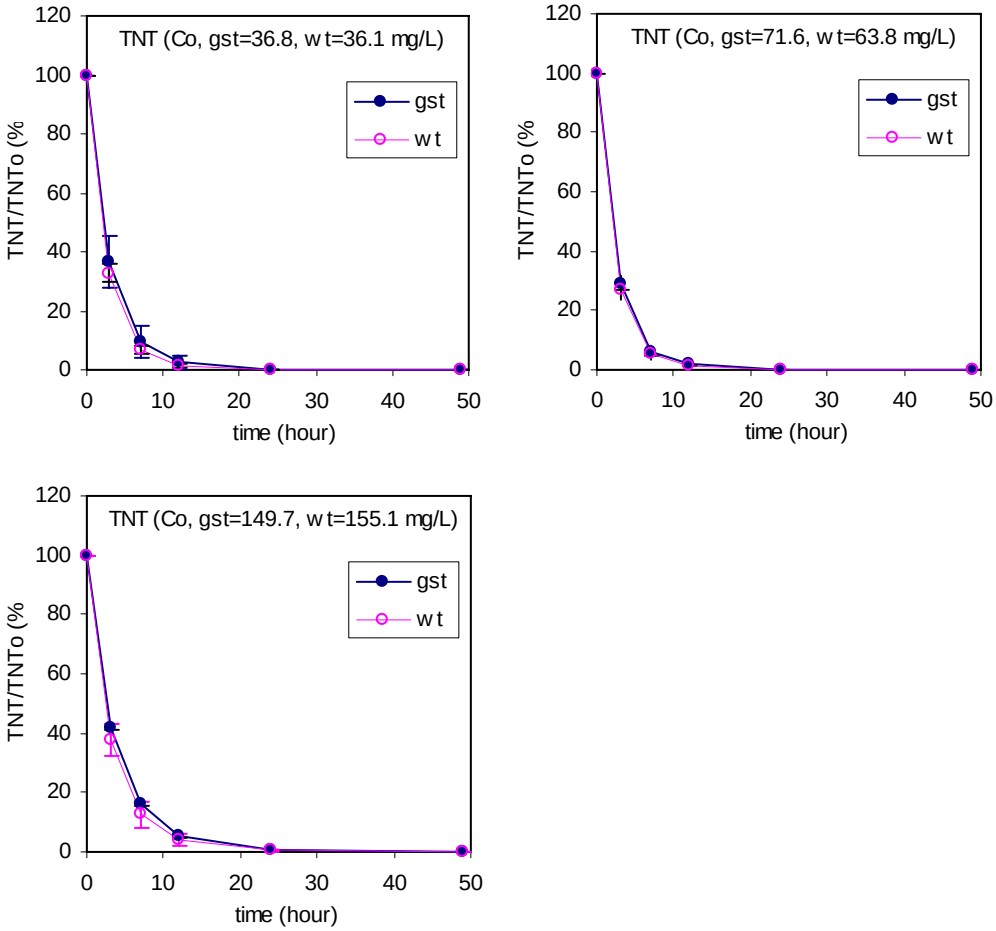


Figure 56. Uptake of the *gst* mutants and wild-types at different concentrations of TNT for 2 days.

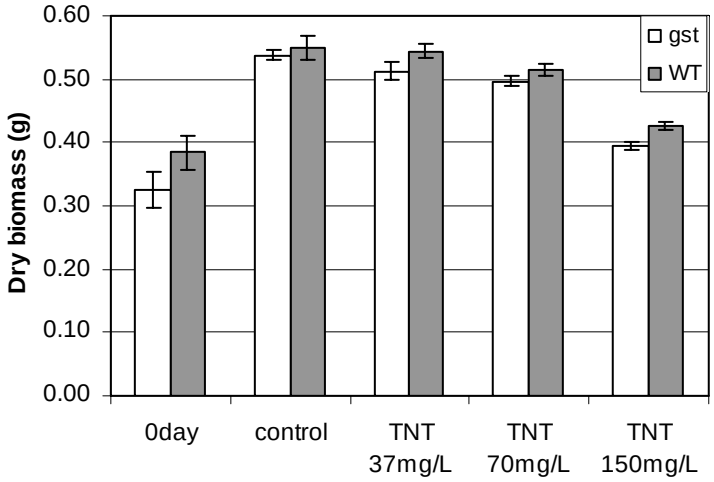


Figure 57. Dry biomass of the *gst* mutants and wild-types exposed to different concentrations of TNT after 6 days.

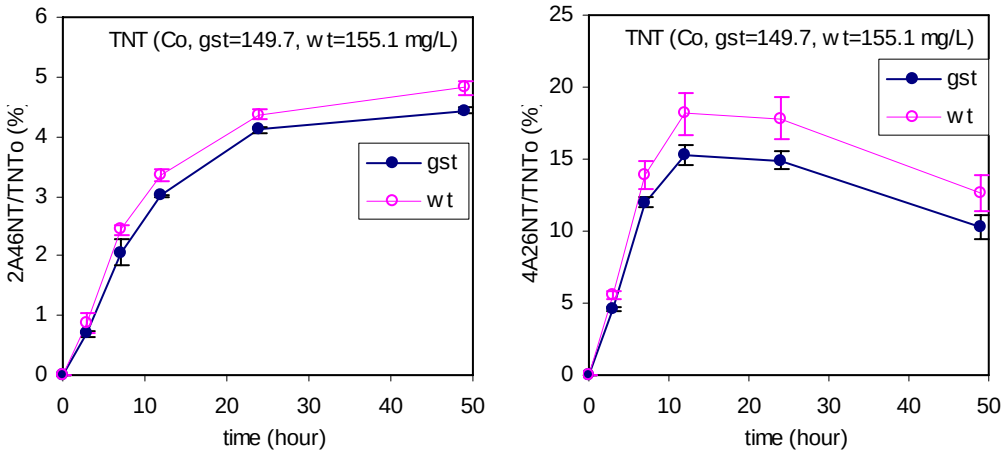


Figure 58. Molar percentage of 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene over time.

There was no significant difference in uptake rates of TNT and biomass changes between the *gst* mutant and wild-type (Figure 56 and 57). In addition, the difference in molar percentage of aminodinitrotoluenes (2A46DNT and 4A26DNT) transformed from TNT was small (less than 5 %) as shown in Figure 58.

When the uptake of 2,6-DNT and TNT by the *gst* mutant and wild-type was considered, the plants took up TNT faster than 2,6-DNT. Over 99% of the initial concentration of TNT was removed by the plants after 1 day while 30% was removed for 2,6-DNT at the same period of time (data not shown). When the uptake rates were compared, the kinetic constants of the wild-type exposed to 50 mg l^{-1} 2,6-DNT and 100 mg l^{-1} TNT were slightly greater than those of *gst* mutants (Table 17).

Table 17. Summary of pseudo-first order constants and correlation coefficient for the gst mutant and the wild-type exposed to various concentrations of 2,6-DNT and TNT for 7 days.

		gst mutant		wild-type	
		$-k$ (day^{-1})	R^2	$-k$ (day^{-1})	R^2
2,6-DNT	50 mg/L	0.2827	0.9980	0.3340	0.9958
	75 mg/L	0.2794	0.9925	0.2625	0.9992
	100 mg/L	0.2516	0.9945	0.2565	0.9833
TNT	25 mg/L	9.686	0.9984	9.002	0.9994
	50 mg/L	9.026	0.9748	9.274	0.9889
	100 mg/L	5.448	0.9840	6.490	0.9438

4.6.3. Root growth assay with *gst* mutant and wild-type exposed to 2,4-DNT

The *gst* mutant and wild-type seedlings were grown on the MS solid medium plates for 4 days. The seedlings were transferred to 2,4-DNT-amended plates (1,3, 5, 10 mg/L 2,4-DNT). The growth of root length was measured after 6 days. The toxic effect of 2,4-DNT was observed at low concentrations (1 mg/L). However, the difference of growth of root length between the *gst* mutant and wild-type was not significant (Figure 59).

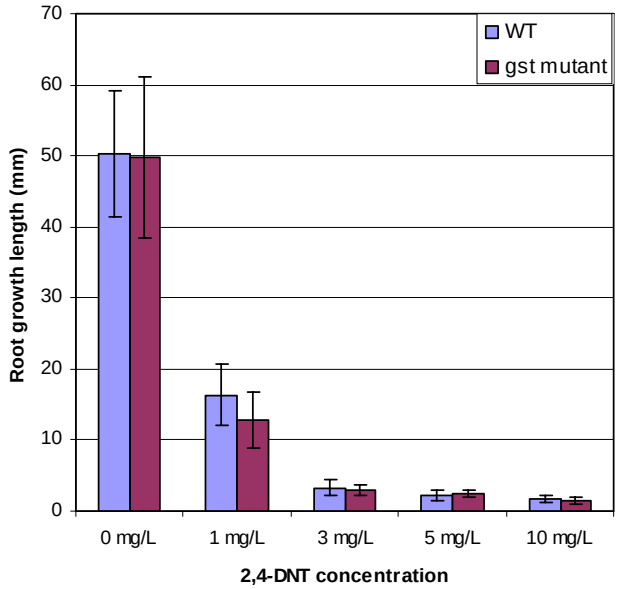


Figure 59. The root growth of the gst mutant and wild-types exposed to different concentrations of 2,4-DNT.

Similar root assays were performed for 2,6 DNT and TNT. The root elongation of both the mutant and wild-type was inhibited at lower concentrations of TNT than 2,6-DNT (Figure 60). In addition, the difference of the root elongation between the *gst* mutant and wild-type exposed to different concentrations of 2,6-DNT and TNT was statistically insignificant from t-test (two-tail, p>0.05).

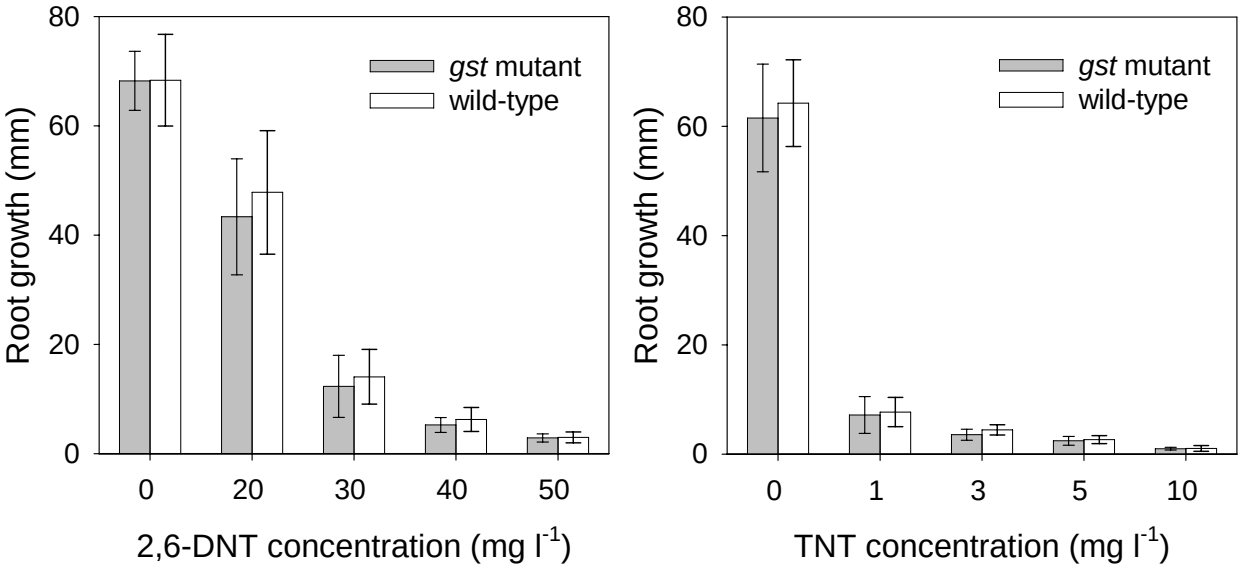


Figure 60. Comparison of the tolerance to nitroaromatic explosives between the wild-type and the *gst* mutant by the root growth assay. The difference of the root elongation between the wild-type and the *gst* mutant exposed to different concentrations of 2,6-DNT and TNT was statistically insignificant from t-test (two-tail, p>0.05). Error bar represent standard deviations (n=27).

4.6.4. Real-time PCR when *gst* mutant and wild-type were exposed to nitroaromatics

After a homozygous *gst* mutant line was isolated out of 9 seedlings from the result of gel electrophoresis following PCR reactions (Figure 54), the seeds were harvested to be used for real-time PCR, and the comparison of uptake rates and root growth between the wild-type and the *gst* mutant. To investigate the changes of glutathione *S*-transferase (GST) and cytochrome P450 (CYP) gene expression to nitroaromatic explosives, 25 seedlings of the *gst* mutant and wild-types grown on plates were transferred to liquid media. 2,4-DNT, 2,6-DNT, and TNT were added to the liquid media after the seedlings were grown for additional 10 days. The initial concentrations were 10 mg/L for TNT, 20 mg/L for 2,4-DNT, and 50 mg/L for 2,6-DNT. Equal volumes of methanol were added for the control plants. The root tissues were collected after 1 day. Expression of genes (GST and CYP) was determined by RT-PCR after RNA extraction followed by cDNA synthesis. The *gst* mutant did not have any expression of GST gene as we expected in Figure 61. In the case of the mutant, the GST gene was barely expressed when the mutant line was exposed to both controls 2,4-DNT, 2,6-DNT and TNT confirming that the

mutant line has a T-DNA insertion at At1g17170. The GST gene was upregulated when the wild-type were exposed to 2,4-DNT and 2,6-DNT except TNT. The slight downregulation of GST gene in the wild-type may be caused by toxicity of TNT. In the case of CYP, both the mutant and wild-type exposed to 2,6-DNT induced the CYP gene more compared to the controls. Induction of CYP gene in both the mutant and wild-type exposed to TNT was not observed

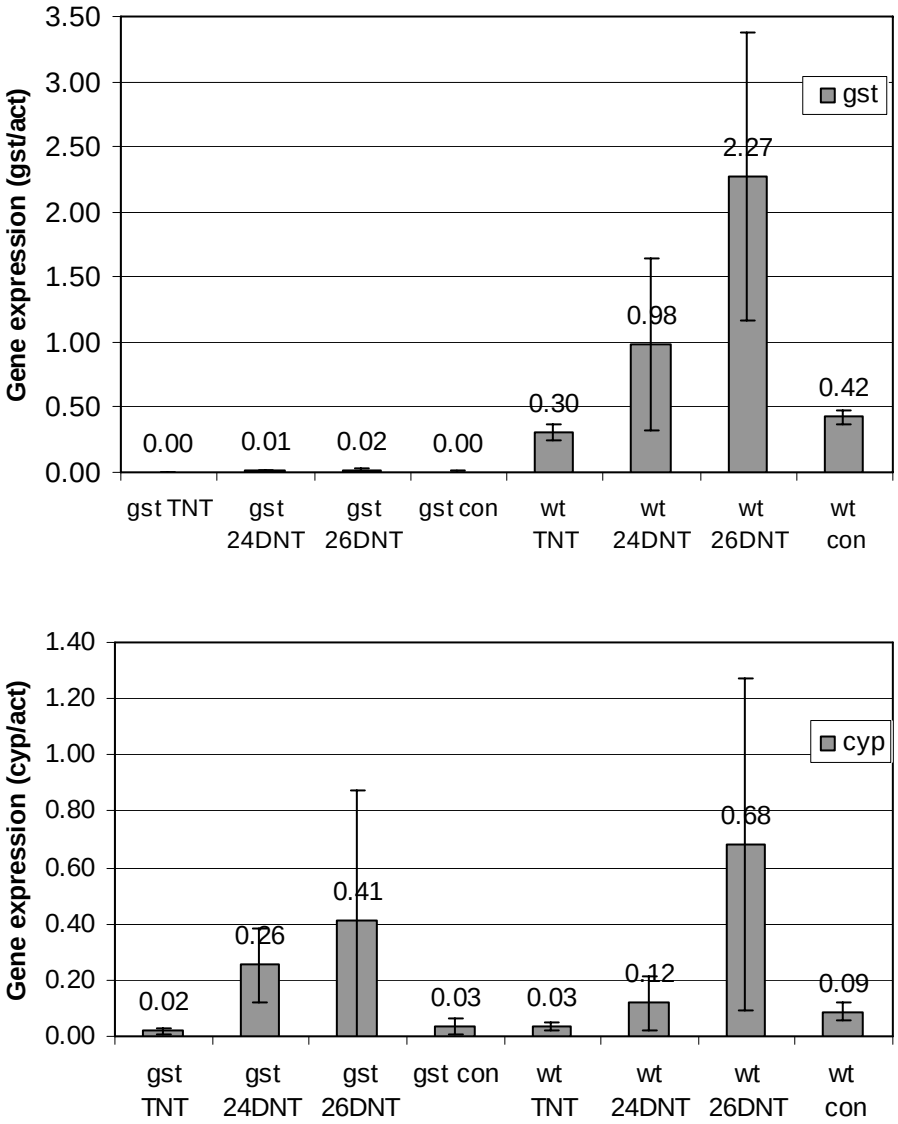


Figure 61. Expression of GST (top) and CYP (bottom) genes in the *gst* mutant and wild-type exposed to 2,4-DNT, 2,6-DNT, and TNT for 1 day compared to control plants).

Expression of GST genes in plants exposed to nitroaromatics has been reported elsewhere (Ekman, et al., 2003; Mezzari, et al., 2005). AtGSTU24 (At1g17170) is induced by 40 times when *Arabidopsis* was exposed to 0.6 mM TNT for 6 hr treatment (Mezzari, et al., 2005) and 27.5 times from the SAGE analysis with exposure of 15 mg l^{-1} TNT to *Arabidopsis* for 1 day (Ekman, et al., 2003). The GST activity of a hairy root culture of horseradish is induced at 0.1 mM TNT for 27 hr exposure by using an enzymatic assay (Nepovim, et al., 2004). In our study, the gene did not affect the uptake rate and the tolerance of *Arabidopsis* to the explosives, 2,4 DNT, 2,6-DNT and TNT even though the GST gene (At1g17170) was overexpressed when the wild-type was exposed to 2,6-DNT. Genes that are induced in response to xenobiotics from the quantitative gene expression study, such as SAGE analysis and real-time PCR does not necessarily warrant the involvement of these genes in the detoxification pathway or in their involvement in tolerance to the xenobiotic by plants.

Additional details may be found in Yoon et al (2006) and Yoon et al (in press) in Appendixes.

(V). CONCLUSIONS

Our genetic and biochemical studies in Arabidopsis of RDX, TNT, and DNTs are providing the scientific and engineering communities with a knowledge base needed to better understand plant detoxification of these compounds, thus enabling phytoremediation and natural attenuation processes. Arabidopsis plants appear able to withstand higher levels of RDX than TNT and the DNTs as evidenced by screening assays, biomass and root assays, and fate and transformation studies in liquid culture. Mineralization of RDX by plant tissue is certainly plausible, given tissue culture studies by J.L. Schnoor's laboratory and preliminary results in Arabidopsis shown in our work. Native species with fast transpiration and growth rates potentially may be selected to process RDX without toxicity to the plant. In contrast, TNT and the DNTs are much more phytotoxic, suggesting that transgenic approaches will be necessary for phytoremediation of these compounds at significant levels, a subject of the collaboration between N. Bruce and S. Strand. Furthermore, the DNTs are not as readily transformed as TNT. Since the similar green-liver pathways appear to be followed, strategies successful for TNT should be tested on these compounds, but they may require increased enzyme activity for detoxification.

This project developed screening strategies and generated mutants resistant to TNT. These mutants are resistant to TNT but do not differ in the profile of transformation products observed. In other words, likely a change in plant metabolism not directly associated with the transformation pathways of TNT occurred – the change is one that reduces phytotoxicity but not the transformation of the parent compound. This type of change has traditionally been very difficult for molecular biologists to study, since in this case, the metabolism of the whole plant needs to be analyzed to determine the change. The SALK mutant studies implied that resistance phenotype of the activation-tagged mutant lines were not caused by interruption of a gene, but possibly by an enhancement of a gene up-regulated by the 4X 35S enhancer element in the T-DNA. The cloning of the enhancer trap mutants provides a resource for the research community to probe genes upstream or downstream of the insertions sites that may be important in phytotoxicity.

Furthermore, testing of gene targets identified from gene expression studies may also provide clues to the toxicity response of plants. As shown in the *gst* mutant studies, genes that are induced in response to xenobiotics from a quantitative gene expression study does not necessarily warrant the in vivo involvement of these genes in the detoxification pathway or in their involvement in tolerance to the xenobiotic by plants. However, several other candidate genes remain to be tested, and can be accomplished with the current availability of Arabidopsis mutants.

(VII). APPENDIX 1

1 Peer Reviewed Publications

Subramanian M., Oliver, D. J., Shanks, J. V., "TNT Phytotransformation Pathway Characteristics in *Arabidopsis*: Role of Aromatic Hydroxylamines", Biotechnology Progress, **22**, 208-216, 2006 **[appended].**

Yoon, J. M., Oliver, D. J., Shanks, J. V., "Phytotransformation of 2,4-Dinitrotoluene in Arabidopsis thaliana: Toxicity, Fate, and Gene Expression Studies in vitro", Biotechnology Progress, **22**, 1524 -1531, 2006 **[appended].**

Yoon, J. M., Oliver, D. J., Shanks, J. V., "Phytotransformation and Phytoremediation of 2,6-dinitrotoluene Using a Model Plant, *Arabidopsis thaliana*", Chemosphere, accepted, 2007. **[appended].**

Hannink N., Subramanian, M., Rosser, S. J., Basran, A., Murray, J., Shanks, J. V., Bruce, N. C., "Enhanced transformation of TNT by tobacco plants expressing a bacterial nitroreductase", International Journal of Phytoremediation, accepted, 2007. **[appended].**

2 Technical Reports

None to date

3 Conference/Symposium Proceedings Papers

Shanks, J.V., Oliver, D.J., Moon H., Rollo, S., Subramanian, M., "Genetic and Biochemical Basis for the Transformation of Energetic Materials (RDX, TNT, DNTs) by Plants," Annual Meeting of the American Institute of Chemical Engineers, November 2003, San Francisco, CA **[appended].**

Moon, H., Subramanian, M., Rollo, S., Oliver, D.J. , Shanks, J.V., Hangsik Moon, "Use of Arabidopsis as a Model System for Genetic and Biochemical Studies of TNT Transformation in Plants," Battelle Conference on Sustainable Range Management, January 2004, New Orleans, LA **[appended].**

Rollo, S., Moon, H., Subramanian, M., Oliver, D.J., Shanks, J.V., "Toxicity of RDX on Germination of *Arabidopsis thaliana*," Proceedings of the Annual Biochemical Engineering Symposium, April 2004a, Lincoln, NE **[appended].**

Rollo, S., Moon, H., Subramanian, M., Oliver, D.J., Shanks, J.V., "Phytoremediation of RDX: Using *Arabidopsis thaliana* to Determine the Genetics and Biochemistry of the Transformation Pathway," Proceedings of the American Institute of Chemical Engineers Annual Meeting, Austin, TX (2004b) **[appended].**

4 Published Technical Abstracts

Subramanian M. and Shanks, J. V. "Phytotransformation of TNT through Hydroxylamine Intermediates - Pathway Confirmation", Partners in Environmental Technology Technical Symposium and Workshop, SERDP, Washington, D.C., December 2002.

Subramanian M, and Shanks J.V. "Phytoremediation of TNT through Hydroxylamine Intermediates – Pathway Confirmation", Poster Presentation, ACS, April 2003, New Orleans

Rollo, S., Moon, H., Subramanian, M., Oliver, D.J., Shanks, J.V., "Phytoremediation of RDX: Screening Studies on *Arabidopsis thaliana"* Annual Meeting of the American Institute of Chemical Engineers, November 2003, San Francisco, CA.

Subramanian M., Moon, H., Rollo, S., Oliver, D., and Shanks J.V. "Genetic and Biochemical Basis for Plant Transformation of Energetic Materials" Poster Presentation, Partners in Environmental Technology Technical Symposium & Workshop (organized by SERDP), December 2003, Washington D.C.

Subramanian M., Moon, H., Rollo, S., Oliver, D., and Shanks J.V. "Phytotransformation pathways of the energetic material TNT" American Association of Ecological Engineers, June 2004, Fayetteville, AR

Rollo, S., Moon, H., Subramanian, M., Oliver, D.J., Shanks, J.V., "The Genetic and Biochemical Phytoremediation Pathway of RDX in *Arabidopsis thaliana*," American Chemical Society Great Lakes Regional Meeting, October 2004, Peoria, IL.

Rollo, S., Moon, H., Subramanian, M., Oliver, D.J., Shanks, J.V., "Determining the Genetics and Biochemistry of the Phytoremediation Transformation Pathways of RDX and TNT," Food, Pharmaceutical and Bioengineering Poster Session, Annual Meeting of the American Institute of Chemical Engineers, November 2004 Austin, TX .

Yoon, J. M., Rollo, S., Oliver, D. J., Shanks, J. V., "Phytoremediation of Energetic Materials (DNTs and RDX) Using *Arabidopsis thaliana*", Annual Meeting of the American Institute of Chemical Engineers, October 2005, Cincinnati, OH.

Yoon J. M., Oliver, D. J., Shanks, J. V., "Phytotoxicity and Uptake of Dinitrotoluenes Using *Arabidopsis thaliana*", Partners in Environmental Technology Technical Symposium and Workshop, SERDP, November 2005, Washington, D.C.

5 Published Text Books or Book Chapters

Yoon, J. M., Oliver, D. J., Shanks, J. V., "Plant Transformation Pathways of Energetic Materials (RDX, TNT, DNTs)", pp. 103-116 in, *Agricultural Biotechnology: Beyond Food and Energy to Health and the Environment*, National Agricultural Biotechnology Council Report 17, A. Eaglesham, R. Bessin, R. Trigiano, R. W. T. Hardy, Eds. National Agricultural Biotechnology Council, Ithaca, New York, 2005. **[appended].**

6 Thesis

Subramanian M., "TNT Phytotransformation in Native and Genetically Modified Species: Significance of Aromatic Hydroxylamines in the Metabolic Pathway" PhD Thesis, 2004, Iowa State University **[appended].**

Rollo, S., "Phytotransformation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX): A study of the biochemical pathway in *Arabidopsis thaliana*" Master Thesis, 2005, Iowa State University **[appended].**

(VIII). REFERENCES

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Xiang, C., Han, P., Lutziger, I., Wang, K. and Oliver, D.J. (1999). A Mini Binary Vector Series for Plant Transformation. *Plant Molecular Biology* **40**: 711-717

(IX). APPENDIX 2

Subramanian M., Oliver, D. J., Shanks, J. V., "TNT Phytotransformation Pathway Characteristics in *Arabidopsis*: Role of Aromatic Hydroxylamines, Biotechnology Progress, **22**, 208-216, 2006.

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TNT Phytotransformation Pathway Characteristics in *Arabidopsis*: Role of Aromatic Hydroxylamines

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ABSTRACT

Basic knowledge of the plant transformation pathways of TNT will aid phytoremediation design and assessment. While TNT transformation by plant metabolism has been demonstrated in previous studies, the presence and role of hydroxylamines in the transformation pathway has not been sufficiently understood. Hydroxylamines are unequivocally shown to be formed by plant transformation of TNT by two axenic plant systems (*Arabidopsis thaliana* and *Catharanthus roseus*). In addition, confirmation was obtained for conversion of these hydroxylamines to previously identified conjugates. Further characteristics of TNT transformation in *Arabidopsis*, an increasingly popular model system for genetic and biochemical studies of TNT transformation, were elucidated by [U-^{14}C] TNT mass balance studies and metabolite feeding studies. These studies showed the rapid conversion of TNT to unextractable-bound compounds by *Arabidopsis* seedlings in agreement with the

green-liver model. *Arabidopsis* seedlings formed and transformed 4-substituted metabolites more efficiently than the 2-substituted metabolites. A qualitative kinetic rate analysis of the pathway was performed to propose rate limiting steps in the pathway and theoretical schemes for improved rates are suggested.

KEYWORDS: TNT, Trinitrotoluene, Phytotransformation, Hydroxylamines, Conjugates, *Arabidopsis thaliana*, *Catharanthus roseus*, Green liver model

INTRODUCTION

2,4,6-trinitrotoluene (TNT) and other nitroaromatics have been detected in varying concentrations at munitions production facilities and military training and testing sites world-wide (1-4). Numerous studies demonstrate the toxicity of these nitroaromatics to the local ecosystem (5-8). One potential method for cleanup is the utilization of plants which are capable of taking up and immobilizing a host of pollutants with minimal energy and nutrient inputs. Phytoremediation, the application of plants for remediation, is a potential low cost, ecologically harmonious technology that can be applied at these sites (9). However, phytoremediation is limited by the slow rate of pollutant removal and the toxicity of the pollutants to the plants. In addition, regulatory acceptance of phytoremediation would require complete knowledge of end-point fate of the pollutant. Hence, commercialization of phytoremediation as a cleanup technology would require improvements in rates of removal, determination of the final fate of the pollutant and enhancing plant resistance to the toxic effects of the pollutants.

Metabolic engineering tools are increasingly being used to achieve many of these goals. For example, transgenic plants expressing bacterial or fungal genes involved in TNT metabolism have been constructed and these have shown increased rates of TNT removal (10-12). Of late, *Arabidopsis*, which has a completely sequenced genome, is being recognized as a tool to discover genes in xenobiotic transformation and resistance (13). A recent paper by Mentewab *et al.* (2005) utilized cDNA microarray studies and RT-PCR to determine genes upregulated when low levels of TNT are added to *Arabidopsis*, while Mezzari *et. al*. (2005) studied conjugation in *Arabidopsis* cells exposed to TNT and used RT-PCR to study the regulation of glutathione S-transferases and nitroreductase genes in *Arabidopsis* transcripts exposed to TNT (14, 15). Metabolic pathway studies based on mass balances and radiolabel analysis, similar to the ones conducted in the terrestrial species *Catharanthus* and aquatic species *Myriophyllum* (16-18), have shed light on the metabolism of the TNT, the metabolites formed and their final fate and kinetics of their transformation. Some of the transformation steps and the identification of the genes and enzymes involved in the TNT transformation pathway are unknown. Expansion of the biochemical knowledge with genetic studies in *Arabidopsis* was attempted by Ekman *et al.* (2003) who performed SAGE transcription analyses in *Arabidopsis* amended with TNT and then utilized the previous metabolite and pathway studies in *Catharanthus* and *Myriophyllum* to speculate on the genes involved (19). However, differences exist in the transformation pathways in *Catharanthus* and *Myriophyllum*, and the transformation pathways have not been

identified in *Arabidopsis*. Thus, metabolic pathway studies for TNT transformation in *Arabidopsis* are necessary for it to be used as a genetic tool.

The metabolic pathway for TNT transformation in terrestrial plants is shown in Figure 1 (20, 21). The confirmed portions of the pathway are shown using bold arrows, whereas the speculated branches are shown using dotted arrows. The metabolites that are hypothesized to be present are enclosed in a dotted square. As delineated in this pathway, plants follows a "green-liver" scheme of TNT transformation, starting with the reduction of TNT to its aminodinitrotoluenes, 2-amino-4,6-dinitrotoluene (2ADNT) and 4-amino-2,6-dinitrotoluene (4ADNT). This is postulated to be accomplished via the partially reduced intermediates, the hydroxylamines, 2-hydroxylamine-4,6-dinitrotoluene (2HADNT) and 4-hydroxylamine-2,6-dinitrotoluene (4HADNT). These hydroxylamines are also thought to be capable of direct conjugation, oxidation or bimolecular nucleation (21-23). The aminodinitrotoluenes thus formed are subjected to further conjugative reactions, wherein a sugar or another plant biomolecule is added to the amino group. All the conjugates are thought to subsequently polymerize and form unextractable compounds bound to plant macromolecules (20, 21).

Aminodinitrotoluenes have ubiquitously been identified as the first stable metabolites formed during TNT transformation by plants (20, 21). Since these aminodinitrotoluenes are less toxic than TNT (24-27), phytoremediation is assumed to lower the toxicity of the system. Recent research suggests that hydroxylamines are the first metabolites during TNT phytotransformation. Both Wang *et al.* (2003) and Pavlostathis *et al.* (1998) showed the formation of hydroxylamines during TNT transformation by aquatic plants; however complete axenic conditions were not achievable (28, 29). Since many mainly anaerobic bacterial systems have shown the formation of hydroxylamines (30), the possibility that bacterial enzymes were responsible for the presence of hydroxylamines in these plant-based studies cannot be ruled out. A recent paper on TNT transformation by axenic tobacco cell cultures failed to identify hydroxylamines but did show the formation of hydroxylamine-derived conjugates (23). Hence, the unique role of plants in the formation of hydroxylamines was not fully ascertained in those studies.

This paper focuses on metabolic pathway studies for TNT phytotransformation in *Arabidopsis*. The presence of two key metabolites- the hydroxylamines- are shown in two axenic systems, *Arabidopsis* and *Catharanthus*, and their role in the transformation pathway elucidated. The TNT transformation pathway in *Arabidopsis* is contrasted with those reported for *C. roseus*, tobacco and *M. aquaticum*, and the results are used to suggest improvements in TNT removal rates.

MATERIALS AND METHODS

CHEMICALS. Solid TNT for feeding experiments was purchased from ChemServices (West Chester, PA), while liquid HPLC standards of TNT, 2ADNT, 4ADNT, 2HADNT, 4HADNT and 2,2',6,6'-tetranitro-4,4'-azoxytoluene (4,4'-azoxy-*bis*-TNT) were purchased from AccuStandards (New Haven, CT). [ring-U ^{14}C] 2,4,6-trinitrotoluene of 5 mCi activity in solid form was purchased from Perkin-Elmer Life Sciences (Boston, MA). The 2HADNT, 4HADNT, 2ADNT, 4ADNT and 4,4'-azoxy-

bis-TNT standards were used in the low concentration metabolite feeding studies, since the solid form of these compounds were not always available. Previously purified standards of TNT-5.5, TNT-6.7, 2A-5.6 and 4A-6.6 (which correspond to TNT-1, TNT-2, 2A-1 and 4A-1, respectively) were used in the identification of conjugates (31, 17). All solvents, including 2-propanol, methanol, ethanol, ethyl ether and acetonitrile were purchased from Fisher Scientific. The scintillation cocktails Ultima Flo* M, Ultima Gold MV, Insta Fluor, PermaFluor and CarboSorb were purchased from Packard Instruments (Boston, MA).

PLANTS. *Arabidopsis thaliana* was grown axenically from seeds. 50 seeds of the plant were surface sterilized with 20% bleach for 15 minutes and subsequently rinsed three times with sterile water. They were next transferred to 50 ml of *Arabidopsis* growth media (4.2 mg of MS media salts, 20 g Sucrose, 1 ml Gamborg B-5 vitamins, MES buffer, Potassium phosphate in 1 L of nanopurified water at a pH of 5.7) in a 250 ml Erlenmeyer flask and shaken at 100 rpm under light. Seven or 14-day-old exponential phase seedlings were used in the TNT and metabolite feeding experiments. These seedlings consisted of both roots and leaves.

Hairy roots of *Catharanthus roseus*, which were grown as described in Hughes *et al*. (1997), propagate solely as roots. *C. roseus* roots were grown in 50 ml of half strength Gamborg B5 media in 250 ml flasks, and shaken at 100 rpm in the dark (18). Seven or 14-day-old, exponential phase roots were used in the TNT and metabolite feeding experiments.

ANALYTICAL METHODS. Reverse-phase HPLC was used for the separation and identification of metabolites from the phytotransformation studies. A Waters system with a 717 autosampler equipped with a PDA detector was used for these studies. A C8 NovaPak column with 82% water and 18% 2-propanol was used for quantification of TNT, 2ADNT, 4ADNT and the previously identified conjugates of TNT-1, TNT-2, 2A-1 and 4A-1 (17). A C18 NovaPak column with a gradient mobile phase of 60% water and 40% acetonitrile ramped to 40% water and 60% acetonitrile was used for the identification of 2HADNT, 4HADNT, 4,4',6,6'-tetranitro-2,2'-azoxytoluene (2,2'-azoxy-*bis*-TNT) and 4,4'-azoxy-*bis*-TNT (28). Ion-suppressed HPLC separation with a mobile phase of 82% 50 mM H_3PO_4 and 18% 2-propanol on a C8 NovaPak column was performed to detect any oxidative metabolites (16). Secondary confirmation for hydroxylamine presence was accomplished using Electron Impact Mass Spectroscopy. The sample was collected following HPLC separation, twice extracted into ethyl ether, and concentrated by evaporation. Electron Impact ionization experiments were performed on a Finnigan TSQ700 triple quadrupole mass spectrometer Finnigan MAT, (San Jose, CA) fitted with a Finnigan EI/CI ion source. Samples were introduced to the mass spectrometer using the solids probe. The probe was heated gradually from 100 to 400 degrees. The instrument was used as a single quadrupole and scanned from 50 to 1000 daltons.

In the radiolabeled TNT feed experiment, quantification of radioactivity under the HPLC peaks was accomplished by having a Packard 505 Flow Scintillation Counter in serial attachment to the PDA detector. A ratio of 1:1.5 of mobile phase to Ultima Flo* M scintillation cocktail was used. Quantification of the radioactivity of extracellular, intracellular extractable and intracellular bound metabolites fractions were done in a Packard 2900 TR Scintillation counter. A 1:5 ratio of sample to Ultima Gold MV cocktail was used for aqueous samples, while a ratio of 1:5 of sample to

Insta Fluor was used for organic samples. Residual radioactivity in the plant biomass was measured by combusting a portion of the biomass in an OX700 Harvey Biological Oxidizer. Oxygen was used to complete combustion, while nitrogen was used to flush out residual radioactivity. 15ml CarboSorb was used to collect the radioactive carbon dioxide from the combustion chamber, which was combined with PermaFluor E^+ in a ratio of 1:5 and analyzed with the scintillation counter.

EXTRACTION OF INTRACELLULAR METABOLITES (INTRACELLULAR-EXTRACTABLES). Extraction of intracellular metabolites for identification and quantification was accomplished by freeze-drying the biomass for 48 to 72 hours and subsequently sonicating the dried biomass twice in methanol for 48 hours at 15°C. The methanol was collected by centrifugation, decanted, evaporated down to 5 ml, and subsequently analyzed via HPLC or LSC. In the radiolabeled TNT mass balance experiments, the residual radioactivity in the biomass was analyzed in a bio-oxidizer.

TNT PATHWAY ANALYSIS STUDIES. 100 mg/L (0.44 mM) TNT was added to seven-day old *Arabidopsis* seedlings in their early exponential phase (in triplicate), and extracellular samples were periodically analyzed for the presence of hydroxylamines and other metabolites. A control with heat-killed biomass and a biomass free control were also amended with similar TNT concentrations. The biomass-killed control was used to isolate the effect of absorption of TNT by the biomass, while the biomass-free control was used to determine the photodegradative and evaporative effects on TNT concentrations. A solely evaporative control was maintained by keeping biomass-free TNT amended medium at 100 rpm in the dark. All reported concentrations are corrected for evaporation losses. Plants were sacrificed at 12 hours and at the end of the experiments (120 hours) and analyzed for their intracellular-extractable metabolites. In a separate experiment, 14-day-old *C. roseus* roots, in their mid- or late- exponential phase, were amended with TNT concentrations in the range of 75 mg/L (0.33 mM) to 110 mg/L (0.48 mM). Media (extracellular) and intracellular-extractable samples were taken periodically and analyzed in an HPLC with a C8 column.

METABOLITE FEEDING STUDIES. In *Arabidopsis*, 2HADNT, 4HADNT, 4,4'-azoxy-*bis*-TNT, 2ADNT and 4ADNT were added individually to different seven-day old axenic *Arabidopsis* seedlings, in duplicate. 25 seeds per flask were used in these studies, and a volume of 25 ml of medium was used per flask. Media samples were taken periodically to determine metabolite concentrations. Wet weights were measured at the end of the experiment (44 hours). 2HADNT, 4HADNT, 2ADNT and 4ADNT were added to seven-day old *C. roseus* roots in duplicate and the medium analyzed for metabolite levels.

TNT MASS BALANCE STUDY IN *ARABIDOPSIS*. 14-day-old *Arabidopsis* seedlings were fed [ring-U ^{14}C] TNT at an initial concentration of 15 mg/L (0.07 mM) and initial radioactivity levels of 5 μCi per flask. Media samples were taken periodically and analyzed for radioactivity and metabolite formation, and seedlings were sacrificed at regular time intervals to determine the amount of radioactivity lodged inside the plants. TNT and metabolite uptake rates were determined assuming pseudo-first order kinetics as shown in (31).

RESULTS

HYDROXYLAMINES DETECTED IN *ARABIDOPSIS* AND *C. ROSEUS*. When high levels of TNT were added to *Arabidopsis and C. roseus* hydroxylamines (2HADNT and 4HADNT) were detected for short periods of time in both systems. Primary confirmation for hydroxylamine presence was obtained by matching their retention time and absorbance spectra with standards (Figure 2). Secondary confirmation for hydroxylamine presence was obtained through Electron Impact Mass Spectroscopy. Mass fragments from the sample were compared with standards from literature (32). The hydroxylamines showed major mass fragments of 212, 197, 165 and 155 (Figure 3). Since hydroxylamines are highly unstable in aqueous media, their detection was found to be a very strong function of sample handling and applied analytical chemistry. Samples run immediately, in a C18 column, under a strong non-polar mobile phase, were more likely to reveal the formation of the hydroxylamines. Samples stored at -20 °C were likely to result in the degradation of the hydroxylamines. Samples run through a C8 column were also likely to result in the hydroxylamine degradation during their separation in the column. Hence, the C18 column was used for hydroxylamine detection, with minimal storage time. Samples that were not run immediately were stored at -80 °C until they could be analyzed.

In *Arabidopsis*, 2HADNT and 4HADNT were observed for the first 12 to 18 hours after 100 mg/L (0.44 mM) of TNT was added to the seedlings (Figure 4). 4HADNT (0.11 mM or 25% of initial TNT) was observed in greater levels than 2HADNT (0.08 mM or 19% of initial TNT). In *C. roseus*, when 75 to 100 mg/L (0.33 to 0.44 mM) of TNT were added to the roots, 2HADNT was detected in levels up to 3 mg/L (14.1 μM), but disappeared within 20 hours of TNT addition (Figure 5). The other hydroxylamine, 4HADNT was detected at only one time point in the 80 mg/L (0.38 mM) and 100 mg/L (0.44 mM) systems, at a maximum concentration of 1.7 mg/L (7.8 μM) four hours after TNT amendment. The detection of these metabolites in these systems was possible because of the high initial levels of TNT, since previous experiments with lower levels of TNT feed had failed to reveal their formation (18, 33). In both systems, hydroxylamines, to the best of our detection scheme, appeared within the first few hours after TNT addition and disappeared in 12-18 hours. Hence, a narrow window exists for the identification of these metabolites. This is the first detection of hydroxylamines in an axenic plant transforming TNT.

CONJUGATION OF HYDROXYLAMINES. Metabolites of TNT transformation were fed to *Arabidopsis* and *C. roseus* to determine their final fates and to further elucidate the transformation pathway characteristics. When *Arabidopsis* seedlings were fed with 4ADNT the conjugate 4A-1 was detected extracellularly; when they were fed with 4HADNT the conjugates TNT-2 and 4A-1 were both detected in the extracellular fraction. Since 4HADNT is upstream of 4ADNT in the TNT transformation pathway, it follows that a portion of 4HADNT is being reduced to 4ADNT that is subsequently being conjugated to 4A-1; simultaneously a parallel branch of 4HADNT is being directly conjugated to form TNT-2. No metabolites or conjugates were observed in the intracellular-extractable fraction, probably because they were bound to the biomass.

When *C. roseus* roots were fed 2ADNT they produced only the conjugate 2A-1 in the extracellular fraction; when they were fed with 2HADNT, they formed the

conjugates 2A-1 and TNT-1, both in the extracellular fraction. Similar to the logic pursued in *Arabidopsis* for 4ADNT and 4HADNT feeding studies, these results imply that 2HADNT is being directly conjugated to form TNT-1. When 4HADNT was fed to the *C. roseus* roots no metabolites were detected in both the extracellular and the intracellular-extractable fraction.

Based on previously published feeding studies (16, 17) it was postulated that hydroxylamines can be directly conjugated (21). In those studies, TNT added to *C. roseus* roots showed the formation of the conjugates TNT-1, TNT-2, 2A-1 and 4A-1; however when 2ADNT was added to the roots, only 2A-1 was formed, and when 4ADNT was added to the roots, only 4A-1 was observed (16). Hence, it was speculated that a metabolite upstream of the aminodinitrotoluenes was responsible for the formation of TNT-1 and TNT-2. In feeding studies presented here, ADNT-fed roots showed the formation of 2A-1 and 4A-1, while HADNT-fed roots showed the formation of all four conjugates. This confirmed that hydroxylamines are those upstream metabolites capable of direct conjugation. Vila *et al.* (2005) also have proven the formation of monoglycoside and diglycoside conjugates from both 2HADNT and 4HADNT.

The significance of hydroxylamines in TNT transformation can be gauged from the numerous reactions these metabolites can undergo. Hydroxylamines can be biotically further reduced to aminodinitrotoluenes (22), abiotically polymerized to azoxy dimers (28), may be involved in the formation of oxidative metabolites (21) and can directly conjugate (23). In addition, our preliminary toxicity analyses showed that the hydroxylamines are more toxic to plants than TNT and the aminodinitrotoluenes (Table 1). The weight of the seedlings exposed to hydroxylamines is reduced by around 40%, whereas the aminodinitrotoluene and TNT-amended seedlings showed no decrease in biomass. Hence, hydroxylamines are not only potential central variables in the TNT transformation pathway, but are also important from a toxicity perspective.

TNT MASS BALANCE STUDIES. ^{14}C radiolabeled TNT was taken up and transformed efficiently by *Arabidopsis* seedlings. Table 2 shows the distribution of the carbon from [ring-U ^{14}C] TNT in the extracellular, intracellular and intracellular-bound portions of the seedlings. Initially, carbon from the TNT was completely in the extracellular portion, but is quickly taken up by the seedlings. The extracellular radioactivity fell to less than 5% by the end of the experiment (168 hours), while the intracellular fraction peaked at around 20% within 12 hours of TNT addition, and then steadily declined to around 5% by 168 hours. The amount of intracellular-bound metabolites, the portion of the radiolabel that could not be extracted by methanol sonication rose steadily to more than 74% by 168 hours. The intracellular-bound metabolites represent a final fate of the carbon from TNT, and its increasing number signifies the end of metabolism and reduced bioavailability of TNT. Previous mass balances on *C. roseus* roots revealed a total of 30 to 40% of the label as intracellular-bound metabolites after 8 days, while the bulk of the label remained as intracellular extractables (63%) (17). 10 to 16% of the label remained in the extracellular phase (17). In contrast, in *Arabidopsis*, only 5% of the label remained in the extracellular portion and 5% in the intracellular-extractables, while 74% of the label accumulated as intracellular-bound metabolites seven days after TNT addition. The greater levels of the intracellular-bound metabolites in *Arabidopsis* seem to indicate that *Arabidopsis* transforms TNT to its final fate faster than *C. roseus*.

TNT TRANSFORMATION CHARACTERISTICS IN *ARABIDOPSIS*. In addition to the hydroxylamines, the metabolites 4,4'-azoxy-*bis*-TNT, 2ADNT, 4ADNT and the previously identified conjugates of 4A-1, TNT-1, TNT-2 and 2A-1 were also detected when 100 mg/L (0.46 mM) of TNT was added to *Arabidopsis* seedlings (Figure 4b). TNT was initially reduced to the hydroxylamines, which in turn underwent further rapid metabolism. Biotic and abiotic reactions competed for hydroxylamines, resulting in the formation aminodinitrotoluenes and azoxy dimers, respectively. The aminodinitrotoluenes were observed through out the course of the experiment at levels less than 15% of the initial TNT added. All these reactions are depicted in the TNT transformation pathway (Figure 1). Greater amounts of 4-substituted conjugates (TNT-2 and 4A-1) than 2-substituted conjugates (TNT-1 and 2A-1) were observed. Over time, all the conjugates were incorporated as intracellular-bound metabolites. 4,4'-azoxy-*bis*-TNTwas observed in the system only at one time point of 4 hours. Ion suppression HPLC did not reveal the formation of oxidative metabolites; hence, either no oxidative metabolites were being produced, or they were formed in below detectable concentrations.

Further characteristics of TNT transformation in *Arabidopsis* was obtained from analysis of the hydroxylamine and aminodinitrotoluene feeding studies (data not shown). During the hydroxylamine feeding studies in *Arabidopsis*, it was observed that the rate of formation of 2ADNT was five times lower than that of 4ADNT formation. Additionally, from both the hydroxylamine and aminodinitrotoluene feeding experiments, the rate of 2ADNT removal was around three times less than that of 4ADNT removal. From these observations, it appears that *Arabidopsis* is better equipped to handle 4-substituted metabolites than their 2-substituted isomers. This characteristic of the pathway can be of importance in future metabolic engineering efforts.

DISCUSSION

With the extensive use of native and genetically modified *Arabidopsis* for phytoremediation studies, characterizing the xenobiotic metabolic pathway in this system is important, and has been accomplished for TNT transformation. Apart from *Arabidopsis* seedlings, TNT transformation has been previously studied in *C. roseus* hairy roots, tobacco cell cultures and *M. aquaticum* aquatic plants. In previous studies on *C. roseus* roots it was shown that 2 and 4-substituted metabolites were formed in approximately equal proportions (16, 17). TNT was shown to progress through the aminodinitrotoluene pathway into bound metabolites. No hydroxylamines were detected in that study. Our studies on *C. roseus* showed the formation of hydroxylamines, aminodinitrotoluenes and conjugates, but the roots formed 2-substituted metabolites in greater proportions. Both these studies did not reveal the formation of oxidative metabolites. Large amounts of oxidative metabolites (36% after 12 days of TNT addition) were detected by Bhadra *et al.* (1999) in the aquatic plant *M. aquaticum*, with 4-substituted metabolites being formed in slightly larger amounts than 2-substituted metabolites (16). The oxidative metabolites formed included 2-amino-4,6-dinitrobenzoic acid, 2,4-dinitro-6-hydroxybenzyl alcohol, 2-*N*-acetoxyamino-4,6-dinitrobenzaldehyde and 2,4-dinitro-6-hydroxytoluene.

Wang *et al.* (2003) also studied *M. aquaticum* and did not detect any oxidative metabolites but showed the formation of hydroxylamines. They offer positive UV-visible spectral comparison and NMR confirmation for the presence of

both 2HADNT and 4HADNT in non-axenic *Myriophyllum aquaticum*. When TNT was added at an initial concentration of 25 mg/L (0.11 mM) , these metabolites were shown to be present in the system from the second day to the end of the experiment (15 days), at levels less than 10% of initial TNT. They speculate, and we concur, that the reason for non-observance of hydroxylamines in previously published TNT phytotransformation studies are artifacts of the analytical chemistry used. Essentially, the frequently used EPA 8330 scheme of explosive and metabolite detection does not reliably work for hydroxylamine detection. In addition, hydroxylamines are highly unstable in aqueous, aerobic media (34, 35) and turned over extremely rapidly (21). In a recent study, Vila *et al.* (2005) also attempted separation and identification (using acid and enzymatic hydrolysis, Electrospray Ionization-MS and ^{1}H and ^{13}C NMR) of all metabolites formed during TNT phytotransformation but could not detect hydroxylamines; only conjugates derived from hydroxylamines were identified (23). A combination of both of low stability and high turnover could be responsible for the absence of hydroxylamines from previous axenic TNT phytotransformation studies.

Wang *et al.* (2003) also showed that hydroxylamines are abiotically reduced to azoxy dimers and biotically reduced to aminodinitrotoluenes and speculate that azoxy dimers and aminodinitrotoluenes are involved in the formation of conjugates, oxidative metabolites and bound metabolites. Our studies on *Arabidopsis* showed the formation of hydroxylamines, aminodinitrotoluenes, aminodinitrotoluene-derived conjugates, hydroxylamine-derived conjugates, 4,4'azoxy-*bis*-TNT and the final product of unextractable bound metabolites. No oxidative metabolites were detected in *Arabidopsis* seedlings. Lack of evidence for a major oxidative pathway for TNT metabolism in *Arabidopsis* might require a revision in the interpretation of TNT-responsive gene expression data (19). *Arabidopsis* seedlings were seen to form and transform 4-substituted metabolites at a higher rate than 2-substituted metabolites. This was also observed in tobacco cell cultures, where 68% of the metabolites had substitutions in the 4-subtituted position A transgenic species of tobacco, expressing a bacterial nitroreductase, removed TNT more rapidly from the medium via aminodinitrotoluenes when compared to controls and this correlated with improved plant health (10). Based on the above discussion, it is clear that the TNT transformation pathway is substantially similar across all plant species. However, specific characteristics of the pathway, such as ratio of 2 and 4-substituted metabolites and role of oxidative metabolites, differ between species indicating diversity in the enzymes targeting TNT. Choosing an appropriate species for TNT phytoremediation will hence depend on the ability of the plant to grow in the target area and the mechanisms it uses to remediate pollutant.

In addition to these well-characterized portions of the TNT pathway in various plant species, there exists a significant percentage of TNT that appears to proceed through unidentified metabolites. This has been shown in our ^{14}C labeled TNT feeding studies and has shown to be the case in *C. roseus* too (21) wherein a significant percentage (up to 50%) of the ^{14}C label remained unidentified. Since TNT is a strongly electronegative compound, the initial mode of attack is necessarily via reduction of the nitro groups. This seems to indicate that the hydroxylamines, which are formed by partial TNT reduction, are potentially being transformed to various unidentified metabolites, possibly polar in nature. Hence, identification of hydroxy-lamines in the TNT transformation is useful in determining alternative TNT phytotransformation pathways.

There appear to be, qualitatively, two rate-limiting steps in the TNT transformation pathway- (1) TNT to hydroxylamines and (2) aminodinitrotoluenes to conjugates. When TNT is partially reduced, the product hydroxylamines are rapidly turned over. The intrinsic rate constant for TNT reduction to hydroxylamines is of the order 10^0 (ml/gFW/hr), while the intrinsic rate constant for hydroxylamine removal is of the order of 10^1 (ml/gFW/hr). These rate constants were calculated assuming pseudo-first order TNT transformation kinetics. This indicates that the reduction of TNT to hydroxylamines is rate limiting in the initial part of the pathway. In the next section of the transformation pathway, aminodinitrotoluenes are formed from the rapid reduction of hydroxylamines and are subsequently conjugated. The rate of hydroxylamine removal is around 10 times the rate of aminodinitrotoluene removal. This is also shown graphically in Figure 5b- hydroxylamines disappear from the system within 20 hours, while the aminodinitrotoluenes persist for the length of the experiment (up to 120 hours). These observations imply that ADNT removal by conjugation is rate-limiting in this section of the pathway. Based on these two rate limiting steps of the pathway, we hypothesize that overexpression of those genes which encode enzymes that reduce TNT to hydroxylamines or aminodinitrotoluenes to conjugates are potentially the most promising in terms of speeding up TNT removal. The unreactive nature of the end point of TNT transformation- the unextractable bound metabolites- may represent lowered xenobiotic phytotoxicity. Hence, acceleration of the rate of formation of bound metabolites is of equal interest as merely accelerating TNT removal.

CONCLUSIONS

Arabidopsis and their mutants are being highly utilized in current day research in deciphering the genetics behind TNT phytoremediation. This study determined the basic pathway of TNT metabolism in *Arabidopsis* in addition to clarifying an earlier picture of TNT metabolism in *C. roseus*. The presence of hydroxylamines in the TNT transformation pathway has been proven. The varied role of these metabolites in the metabolic pathway- from reduction to conjugation- has been demonstrated. The higher toxic effect of hydroxylamines, when compared to TNT and aminodinitrotoluenes has also been shown. Finally, rate limiting steps of the pathway were qualitatively determined. Hence, the potential role of hydroxylamines in the manipulation of the TNT metabolic pathway has been demonstrated. Further investigations into understanding the reason for the preference of 4-substituted metabolites in *Arabidopsis*, determining the fate of the azoxy dimers, and identifying the other polar metabolites during TNT transformation are warranted. In addition, research in determining the enzymes and genes involved in TNT transformation can lead to the development of efficient transgenic plants or conditions to maximize remediation.

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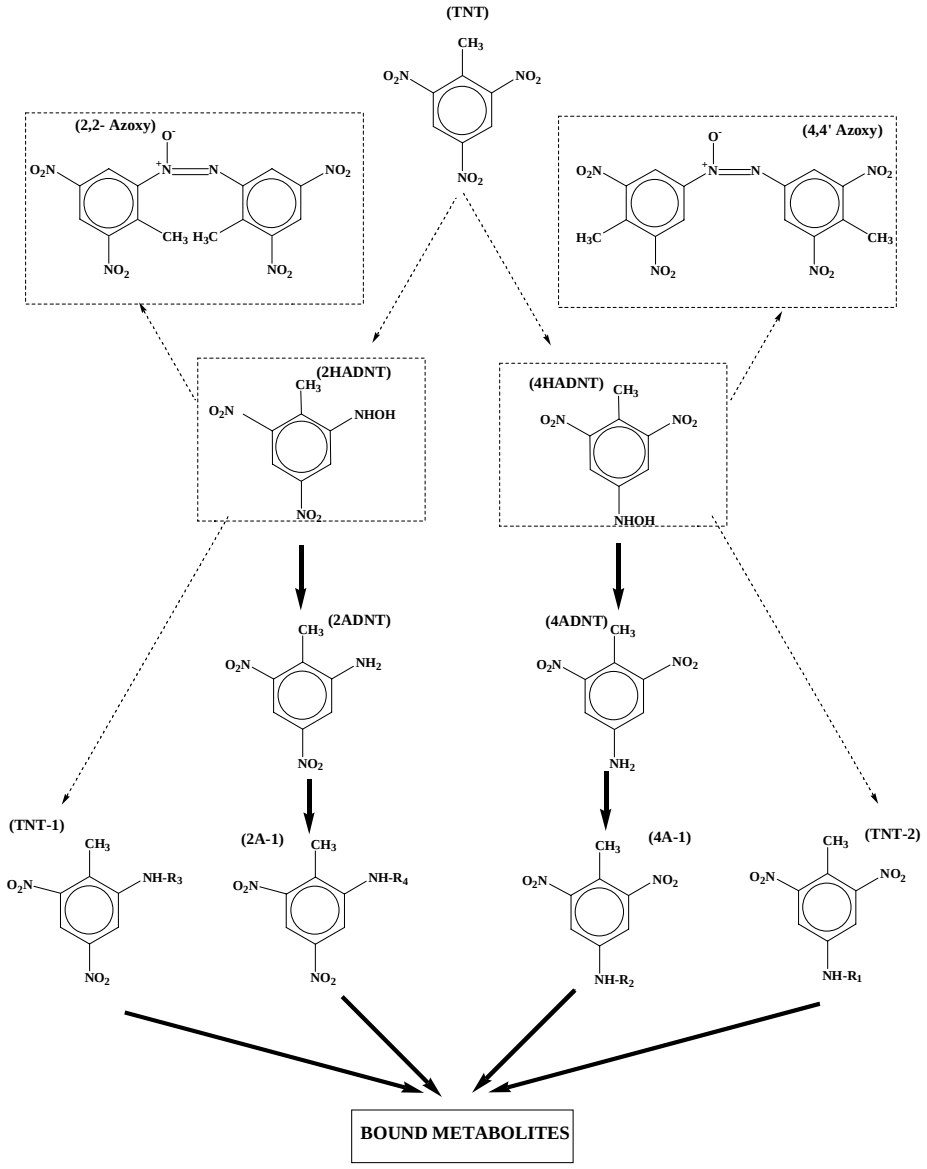

Figure 1. Proposed TNT transformation pathway in axenic plants as determined by xenobiotic feeding experiments in *C. roseus* and *Arabidopsis*. High level TNT feeding studies in *C. roseus* and *Arabidopsis* proved the presence of hydroxylamines and azoxy dimers (shown in boxes) while the dotted arrows show newly determined sections of the pathway. The bold arrows represent sections of the pathway confirmed by previously published studies (20). All these steps, in total, represent the "Green Liver" model of TNT transformation by plants.

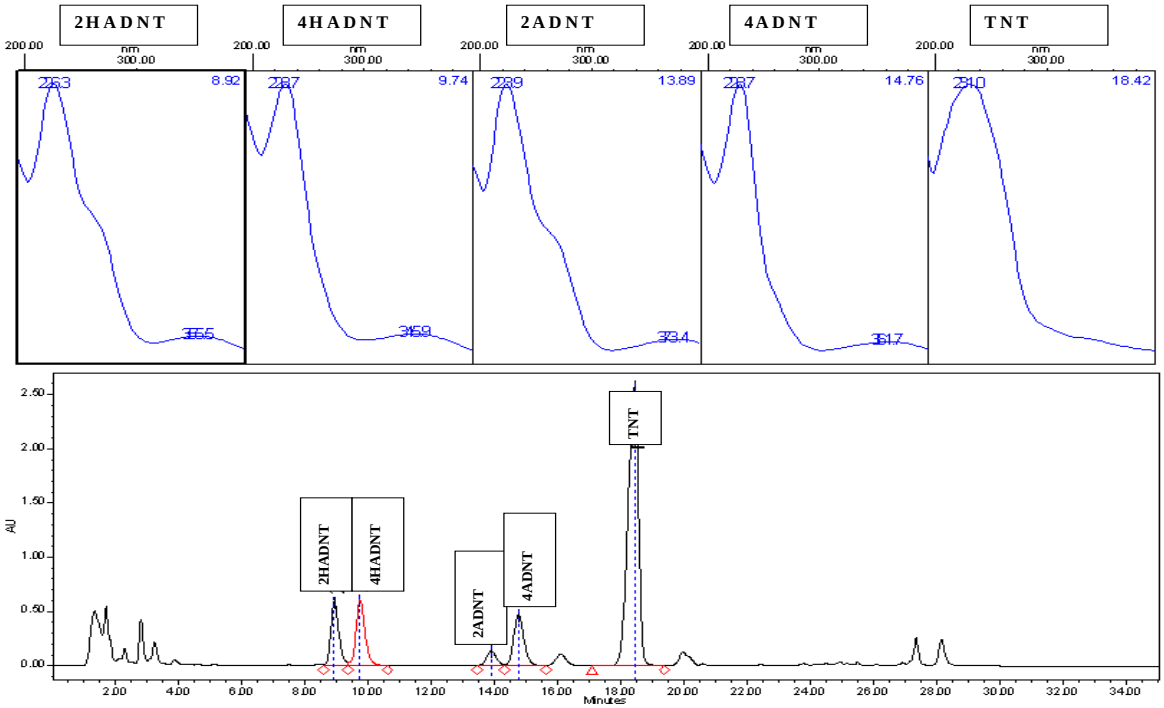

Figure 2. Chromatogram showing the elution of TNT, 2HADNT, 4HADNT, 2ADNT and 4ADNT on a Nova-Pak C18 column with 2-propanol and water in the mobile phase 6 hours after 100 mg/L (0.44 mM) TNT was added to *Arabidopsis* seedlings. The UV-visible absorption spectra for the metabolites are also shown, normalized along the y-axis for absorbance and x-axis for wavelength.

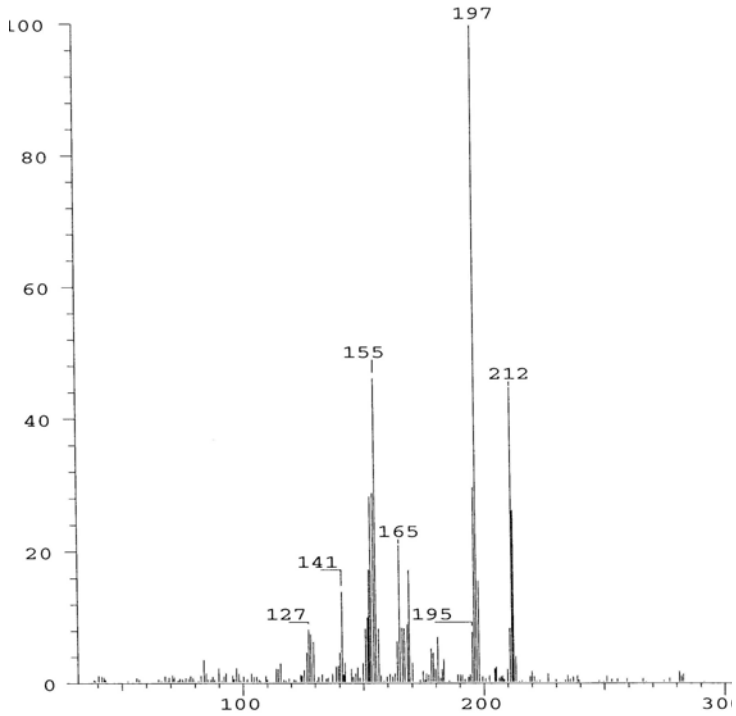

Figure 3. Electron impact spectra of hydroxylamine sample as determined by a Finnigan TSQ700 triple quadrupole mass spectrometer. The sample was extracted from *Arabidopsis* medium exposed to 100 mg/L (0.44 mM) TNT for 6 hours. The x-axis represents (mass/charge), while the y-axis represents the relative intensity. The two main peaks are observed at 212 and 197; the first peak represents the molecular weight of the HADNT, while the second peak represents its largest ion.

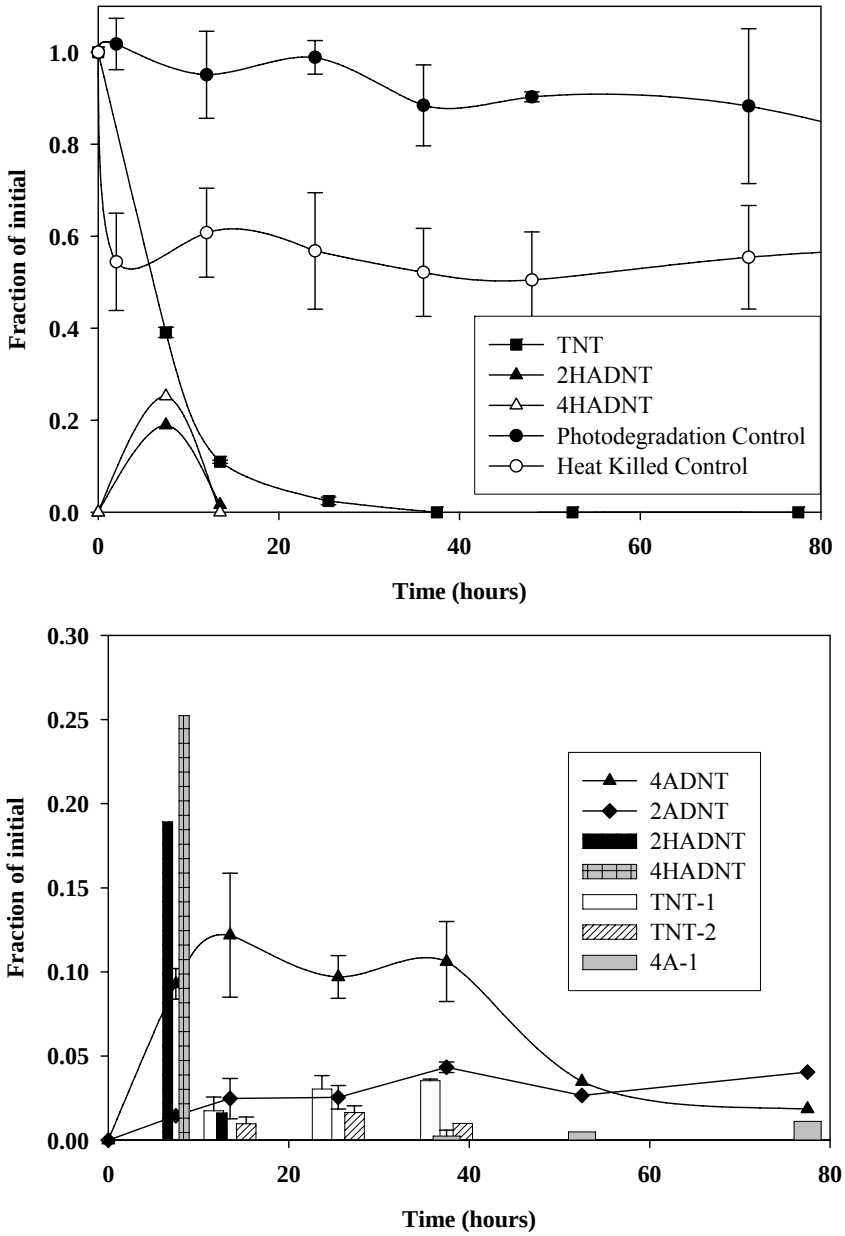

Figure 4. Extracellular levels of TNT, metabolites, conjugates and controls in 7-day-old *Arabidopsis* seedlings fed with 100 mg/L (0.44 mM) of TNT. In Figure 4a, the y-axis for the photodegradation and heat killed controls represent TNT concentrations. In Figure 4b, the hydroxylamine and conjugate concentrations are represented as bar graphs while the aminodinitrotoluenes are represented as line graphs for clarity of presentation. The metabolites TNT-1, TNT-2 and 4A-1 represent the conjugates referred to in Figure 1. The formation of hydroxylamines coincided with the removal of TNT, and they were present until 15 hours from TNT addition. Values of the controls, 2ADNT, 4ADNT, TNT-1 and TNT-2 are averages and standard deviations of duplicates, while the HADNT levels represent single measurements. The TNT concentrations are averages and standard deviations of triplicates. All concentrations have been corrected for evaporation losses.

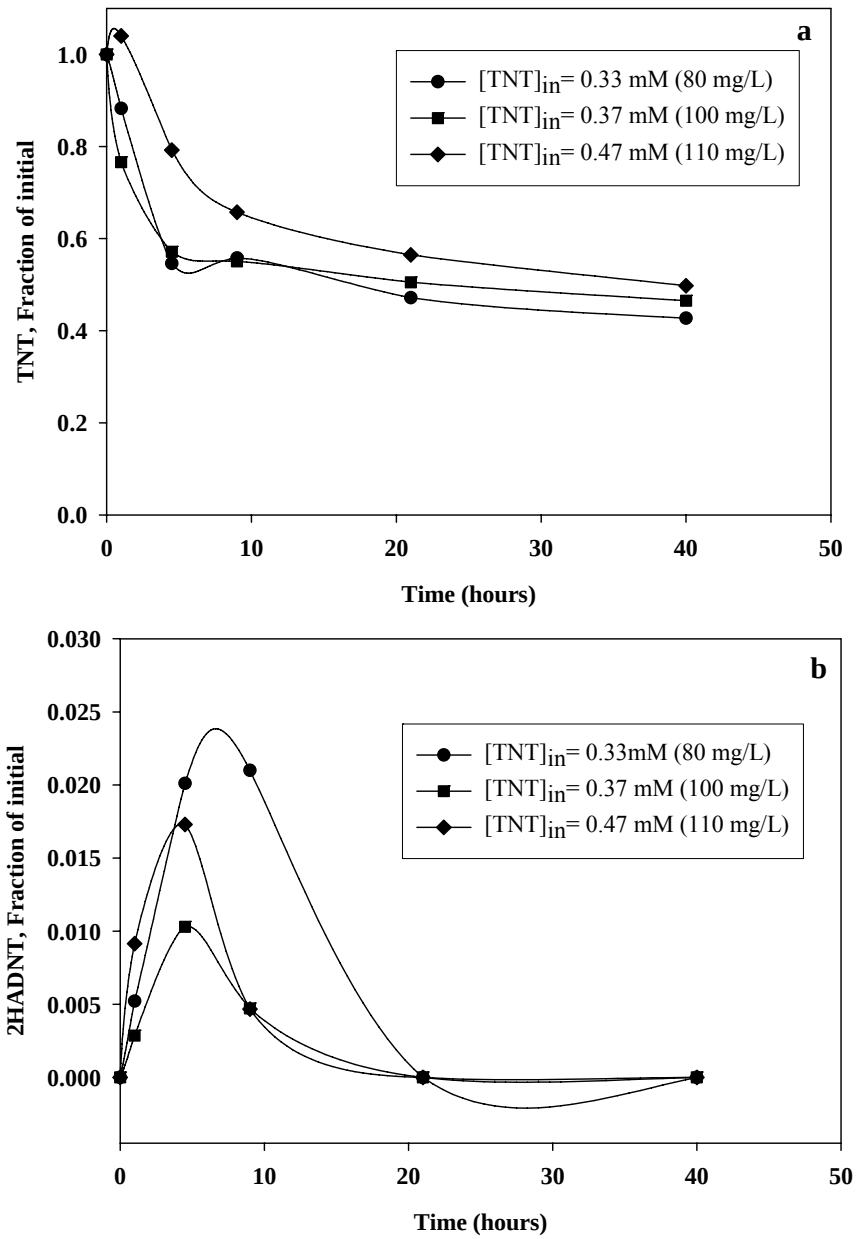

Figure 5. Transient concentration profile of extracellular TNT (5a) and 2HADNT (5b) in *C. roseus* hairy roots in high concentration TNT feeding studies. The higher levels of TNT used ensured higher concentrations of all metabolites, including hydroxylamines. This was the first instance of hydroxylamines being determined in an axenic phytoremediation study. 2HADNT was formed at low levels, but disappeared rapidly within 20 hours. The *C. roseus* seedlings were inefficient in their transformation of TNT, as evidenced by the slow rate of removal. All concentrations have been corrected for evaporation losses.

Table 1. Wet weights of *Arabidopsis* seedlings exposed to various TNT transformation metabolites. The weights were measured 55 hours after exposure of the metabolites to the one-week old seedlings. The weights are means and standard deviations of duplicates. The initial concentration of all the metabolites was 5 mg/L, except TNT which was fed at 75 mg/L, and the control which did not have any xenobiotic added to it. An ANOVA analysis of all the wet weight means gave P= 0.002, which indicates the difference in wet weights is due to the presence of the compound added..

Metabolite Added	Concentration, mM	Fresh Weight, % of control
2HADNT	0.023	70 ± 18
4HADNT	0.023	71 ± 9
2ADNT	0.012	123 ± 11
4ADNT	0.025	120 ± 4
4,4'Azo	0.025	72 ± 6
TNT	0.320	110 ± 3
Control	0	100 ± 6

Table 2. Distribution of ^{14}C label from TNT in *Arabidopsis* seedlings fed with 15 mg/L (0.07 mM) and 1 to 10 μCi of [ring-U ^{14}C] TNT. The fractions of initial label values are shown in the table. The extracellulars refer to the media levels, the intracellular-extractables refer to the extractable compounds in the biomass, while the intracellular bound metabolites refer to the label that could not be extracted from the biomass. Results are means and standard deviations of duplicates.

Time (hours)	Extracellular	Intracellular-Extractables	Intracellular-Bound metabolites	Total Recovered
0	1.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.00 ± 0.00
4	0.74 ± 0.01	0.21 ± 0.16	0.09 ± 0.11	1.01 ± 0.09
12	0.44 ± 0.05	0.21 ± 0.20	0.20 ± 0.08	0.84 ± 0.14
25	0.21 ± 0.03	0.21 ± 0.19	0.40 ± 0.07	0.80 ± 0.11
117	0.03 ± n.a.	0.06 ± n.a.	0.67 ± n.a.	0.76 ± n.a.
168	0.05 ± 0.03	0.05 ± 0.02	0.74 ± 0.02	0.84 ± 0.06

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Phytotransformation of 2,4-Dinitrotoluene in *Arabidopsis thaliana*: Toxicity, Fate, and Gene Expression Studies In Vitro

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Abstract

Basic knowledge of the plant transformation pathways and toxicity of 2,4-dinitrotoluene (2,4-DNT) will assist in the design and assessment of a phytoremediation strategy. This study presents the toxicity and fate of 2,4-DNT, and gene expression in response to 2,4-DNT exposure using the model plant *Arabidopsis thaliana*, an increasingly popular system for genetic and biochemical studies of phytotransformation of explosives. From the results of biomass and root growth assays for toxicity, 2,4-DNT was toxic to the plants at concentrations as low as 1 mg/L. In the uptake study, 95% of the initial 2,4-DNT was removed by 15-day-old seedlings from liquid media regardless of the initial 2,4-DNT concentrations while 30% was accounted for by the adsorption to the autoclaved plant materials. The mass balance was over 86% using [U-^{14}C]2,4-DNT and the mineralization by the plants was less than 1% under sterile conditions during 14 days of exposure. The percentage of the bound radioactivity increased from 49% to 72% of the radioactivity in the plants suggesting transformed products of 2,4-DNT may be incorporated into plant tissues such as lignin and cellulose. Monoaminonitrotoluene isomers and unknown peaks with short retention times were detected as transformed products of 2,4-DNT by the plants. Most (68%) of the radioactivity taken up by the plants was in the root tissues in nonsterile hydroponic cultures. Glutathione and expression of related genes (*GSH1* and *GSH2*) in plants exposed to 2,4-DNT were 1.7-fold increased compared to untreated plants. Genes of a glutathione *S*-transferase and a cytochrome P450, which were induced by 2,4,6-trinitrotoluene exposure in previous studies, were upregulated by 10 and 8 fold, respectively. The application of phytoremediation and the development of transgenic plants for 2,4-DNT may be based on TNT phytotransformation pathway characteristics because of the similar fate and gene expression in plants.

Keywords: 2,4-dinitrotoluene, Phytoremediation, *Arabidopsis thaliana*, Glutathione,

Glutathione *S*-transferase, Cytochrome P450, real-time PCR.

Introduction

2,4-dinitrotoluene (2,4-DNT) has been found as a contaminant in both soil and groundwater at munitions production facilities and military training sites, as it is used as an explosive and produced as a by-product in 2,4,6-trinitrotoluene (TNT) synthesis *(1)*. In addition, 2,4-DNT is a precursor for the synthesis of toluene diisocyanates in the polyurethane manufacturing industry *(2)*. Numerous studies indicate the toxicity of dinitrotoluene to various organisms. 2,4-DNT induced cancers and has toxic effects on reproductive organs in rats *(3,4)*. Hepatobiliary cancer and urethal tumor possibly caused by DNT exposure were found in workers at munition facilities and miners who have used dinitrotoluenes as the explosive in underground copper mining *(5,6)*. 2,4-DNT was cytotoxic, but less so than TNT, in H4IIE rat hepatoma cell cultures *(7)*. In contrast, Rocheleau *et al*. reported that 2,4-DNT was more toxic to the plants than TNT in the soil freshly amended with the explosives *(8)*.

Possible treatments of soil and groundwater contaminated with dinitrotoluenes have been proposed including alkaline hydrolysis *(1)* and photolysis with surfactants *(9)*. However, those technologies require excavation and transport of the contaminated soil and groundwater as well as high maintenance and operating costs. Phytoremediation is an environmentally-friendly, cost-effective, and in-situ treatment *(10,11)*. However, limitations such as slow removal by plants, toxicity of high levels of contaminants to the plants, and incomplete knowledge of fate after uptake restrict the application of phytoremediation on contaminated sites.

Metabolic engineering tools are being used to overcome these limitations. Engineered tobacco plants expressing a bacterial nitroreductase have shown faster removal rates and better growth than the wild-type at high concentrations of TNT *(12)*. In addition, transgenic *Arabidopsis* expressing a bacterial cytochrome P450 has been engineered for testing the potential of transgenic plants to remediate another explosive, hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) *(13)*. Metabolic pathway studies, using mass balances and radiolabel analysis, are being used to explore the metabolism, kinetics, and fate of explosives in plants for phytoremediation design. Pathway studies in aquatic systems of *Myriophyllum (14)*, and in axenic cultures of *Catharanthus roseus (15)* and *Arabidopsis thaliana (16)* have led to a proposed metabolic network topology for the "green-liver" scheme for TNT phytotransformation. Metabolic pathway studies for RDX and HMX have been less extensive than those for TNT, but reveal that in contrast to TNT, RDX may be mineralized by plants *(17)*. In the case of 2,4-DNT, most studies have been restricted to its toxicity to plants in soil systems *(8)*, and little information about the metabolism and fate of 2,4-DNT in axenic plants is available for the phytoremediation applications. Based upon its chemical structure, 2,4-DNT may follow transformation processes more similar to those for TNT rather than RDX or HMX, since it is a nitroaromatic rather than a nitroamine. However, bacteria can mineralize 2,4-DNT *(18)* and RDX *(19)*, but not TNT *(20)* , and plants can mineralize RDX *(21)* but not TNT *(22)*, so clearly metabolic studies need to performed for the dinitrotoluenes in plants.

Although some of the transformation steps are being elucidated through the radiolabel studies, identification of the genes and enzymes involved in these pathways and the detoxification response are unknown. Increasingly, *Arabidopsis* is being used as a tool for information about gene expression in response to the explosives *(23,24)*. *Arabidopsis* is commonly used as a model plant because the genome is completely sequenced and seeds of T-DNA insertion mutants for specific genes are publicly

available for the further research on the genes involved in transformation of xenobiotics. Several studies about the regulation of genes of *Arabidopsis* exposed to TNT have been investigated by using molecular techniques such as SAGE analysis and RT-RCR *(23,24)*.

The detoxification process of xenobiotics by plants includes the conjugation with glutathione by glutathione *S*-transferases and reactions catalyzed by cytochrome P450 enzymes *(25)*. Glutathione is a tripeptide composed of glutamate, cysteine, and glycine and its increase in root tissues of plants exposed to nitroaromatic explosives has been reported *(23)*. Glutathione is produced by a two-step reaction. γ-glutamylcysteine synthesis from the reaction of glutamate and cysteine, which involves the gene *GSH1*, is followed by ligation of γ-glutamylcysteine and glycine where the gene *GSH2* is involved *(26)*. The glutathione produced may be conjugated with xenobiotics for detoxification processes *(25)*. This conjugation is catalyzed by a family of enzymes, the glutathione *S*-transferases, with each member of the family reacting with a range of xenobiotics. The glutathione-conjugates are then transported into the vacuole or cell wall thereby protecting the plant from the toxic chemical *(27)*. Another family of enzymes that have been implicated in xenobiotic detoxification is the cytochrome P450s. Recently, genes such as glutathione *S*-transferases and cytochrome P450s in *Arabidopsis* were proposed by Ekman *et al*. *(24)* to be involved in degradation of TNT. The pathway of glutathione synthesis and the detoxification mechanisms by plants are shown in Figure 1.

The objectives of this study are to evaluate the phytotoxicity of 2,4-DNT under sterile conditions that exclude the adsorption to soil and bacterial degradation, to investigate the fate of 2,4-DNT and its transformation metabolites in *Arabidopsis* after uptake, and to measure the changes in the expression of specific genes in the plants exposed to 2,4-DNT. These phytotransformation characteristics for 2,4-DNT are compared to those reported for TNT *(16,22-24)* and the results are used to suggest strategies for the application of phytoremediation and the development of transgenic plants for 2,4-DNT.

Materials and Methods

Chemicals. 2,4-dinitrotoluene (2,4-DNT, purity 98%) was purchased from Chemservice (PA). ^{14}C-ring labeled 2,4-DNT (radioactivity 6.23 mCi/mmol, purity 99%) were obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). The standards of 2-amino-4-nitrotoluene (98.1%), 4-amino-2-nitrotoluene (99.8%), and 2,4-diaminotoluene (99.8%) were provided by Sigma-Aldrich (St. Louis, MO). Other chemicals and solvents were reagent or better grade.

Plant Materials. Wild-type *Arabidopsis thaliana* (Columbia) seeds were obtained from the Arabidopsis Biological Resource Center (ABRC) (Columbus, OH). Seeds were sterilized in 1.5 mL microcentrifuge tubes with 1 mL of 50% Clorox bleach solution and 0.1% Triton X100 by vortexing vigorously for 15 minutes and were washed four times with sterile deionized water. For liquid cultures, the sterile seeds were transferred to 50 mL MS liquid media (half-strength Murashige and Skoog salts *(28)*, 0.5 g of MES, 20 g of sugar, and 3 mL of a 6% KH_2PO_4 solution per liter, pH 5.8) on a shaker at 80 rpm. In the case of MS solid media, sterile seeds were mixed with a 0.1% sterile agar solution, and then were placed on solid half strength MS media (1% agar). After incubation at 4 ^{o}C for 2 days in the dark, the plates were placed vertically in a culture room. All plants were grown under continuous light at 24 ^{o}C.

Toxicity Tests. For the root growth assay, 4-day-old seedlings grown on solid MS media (pH 5.8) were transferred to 1% agar plates amended with various concentrations of 2,4-DNT. The plates were prepared in duplicate and twelve seedlings were transferred per plate. The end of root tips were marked initially on the backsides of the plates and the growth of the root length was measured after 7 days.

The biomass assay was used for additional toxicity tests. Twenty five seedlings grown on solid media for 4-5 days were transferred to 50 mL MS liquid media (pH 5.8) in order to assure the same initial biomass. A day was allowed for adjustment before different volumes of 2,4-DNT stock solution were spiked into the liquid media to make different final concentrations. The plants were harvested after 10 days and were rinsed with deionized water before being dried at 65 ^{o}C in an oven for over 3 days. The dry biomass was measured gravimetrically.

Uptake and Mass balance. For the uptake study, 2,4-DNT stock solutions in methanol were added to 15-day-old seedlings grown from 50 seeds in sterile 50 mL MS liquid media. The plants were exposed to 8, 15, and 30 mg/L 2,4-DNT for 5 days. Samples from the media were taken periodically and mixed with acetonitrile (1:1 vol) for HPLC analysis. The liquid samples were filtered with Xpertek syringe filters (0.2 μm) from P.J. Cobert Associates (St. Louis, MO) before injection.

The uptake of 2,4-DNT was monitored by using an HPLC (Waters, Milford, MA) equipped with a 996 diode-array detector, 510 pumps, and a 717 plus autosampler. Spectra were scanned between 200 and 400 nm and chromatograms extracted at 240 nm for quantification. A Nova Pak C8 column (5 μm, 3.9x150 mm) from Waters and an isocratic mobile phase of 82/18 (water/2-propanol, v/v%) was used for analyte separation.

For the mass balances, 25 four-day-old seedlings were transferred to each 250 ml wide-mouth Erlenmeyer flask containing 50 mL MS liquid media. Radiolabeled 2,4-DNT stock solutions were spiked into the liquid media after 5 days of additional growth. Glass controls and autoclaved-plant controls were prepared also. For controls for autoclaved plants, seedlings were autoclaved immediately after being transferred to liquid media. The radioactivity per flask was about 2 μCi (1.8 mg/L from HPLC analysis) and initial volumes of the liquid media were measured with 50 mL sterile pipettes. To measure the mineralization of [U-^{14}C]2,4-DNT by the plants, several flasks were sealed with No.8 rubber stoppers under which were installed 6 mL serum vials with 1 mL of 1N NaOH. The other flasks were plugged with foams to prevent microbial and fungal contaminations. The plants exposed to [U-^{14}C]2,4-DNT were harvested at 3, 5, 7, 10, and 14 days, and freeze-dried. The dry biomass was weighed and a portion (0.03-0.05 g) was combusted using an OX700 Harvey Biological Biooxidizer. A sample (500 μL) out of 15 ml CarboSorb E cocktail trapping $^{14}CO_2$ from biooxidation was mixed with 3 mL PermaFluor E^{+} cocktail before analyzing by a Packard 2900 TR Liquid Scintillation Counter (LSC). The radioactivity of liquid media was determined by a LSC after mixing with Ultima Gold M cocktails.

Plant Extraction and Transformation Products. The freeze-dried plant materials were extracted with 20 mL of methanol by sonication for 1 day. Samples (500 μL) of filtrates following filtration with Whatman 934-AH glass fiber filters were used for LSC analysis of extractable radioactivity. The extracts were evaporated under a fume hood and analyzed by a Packard 505 Flow Scintillation counter in series with the HPLC system for analysis of the transformed products of 2,4-DNT by plants. The residues after the filtration were combusted for bound radioactivity in the plants.

^{14}C Distribution in Hydroponic Systems. Plants grown in half-strength Hoagland solutions *(29)* were used to investigate the distribution of the radioactivity in different

plant tissues. Hydroponic systems were set up with modifications as described *(30)*. Microcentrifuge tubes (0.5 mL) were filled with melted 0.6% agar and the caps and bottom ends of the tubes were cut off after the agar solidified. The bottoms of the tubes were wrapped with a piece of fiberglass window screen to prevent leaching of agar and then the tubes were inserted into the holes cut into the caps of 50 mL disposable centrifuge tubes. One plant per tube was grown from seeds for 21 days before being transferred to 50 mL half-strength Hoagland solutions (pH 5.8) amended with about 2 μCi of [U-^{14}C]2,4-DNT. The plants were harvested at different times and divided into roots, leaves, and stems, followed by air-drying over 3 days at room temperature. The dry plant tissues were combusted by a biooxidizer and analyzed as described above.

Glutathione Measurement. Glutathione was quantified as described in Xiang and Oliver *(26)*. Fifty seeds were grown for 14 days before 2,4-DNT was spiked into the MS liquid media. Root tissues of the plants exposed to 10 mg/L of 2,4-DNT for 1 day were collected and ground under liquid nitrogen. After adding 2 volumes of 0.15N HCl, the plants were ground more until they were homogenized. The homogenates were centrifuged at 12000 rpm (13400 *g*) for 10 minutes at 4 ^{o}C. The free glutathione in the supernatant was derivatized with monobromobimane (Sigma-Aldrich). Glutathione standards were derivatized under the same conditions. The glutathione conjugates were analyzed by a Hewlett Packard 1100 HPLC system with a HP 0146A fluorescence detector. A Prevail C18 column from Alltech (Deerfield, IL) and gradient mobile phases (methanol and water with 0.25% acetic acid, pH 3.3) were used for separation. The elution conditions were as follows: a liner increase of methanol from 15% to 20% for 15 min, from 20% to 100% of methanol for 0.1 min, and then 100% of methanol for 3 min followed by a linear decrease from 100% to 15% for 3 min.

Real Time PCR. After *Arabidopsis* plants grown in MS liquid media for 14 days from seeds were exposed to 10 mg/L of 2,4-DNT for 1 day, about 100 mg of fresh root tissues were used for RNA extraction. After RNA extraction with Triazol (Invitrogen, Carlsbad, CA), first strand cDNA was synthesized using SuperScript II reverse transcriptase from Invitrogen according to the manufacturer's protocols. Genomic DNA was destroyed with DNase I from Invitrogen before cDNA synthesis. Serial dilutions of concentrated cDNA were used as relative standards. We selected genes (*GSH1* and *GSH2*) involved in glutathione synthesis, and genes (At1g17170 and At3g28740) which are highly induced by TNT exposure. Based on gene sequences at The Arabidopsis Information Resource (TAIR, http://www.arabidopsis.org), primer sets were designed by using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) with parameters of product sizes (80-150 bp), and optimum melting temperatures (T_m) at 60 ^{o}C. The sequences of primers, loci of genes, and PCR product sizes are listed in Table 1. The *Actin-8* gene was used as a control gene for both treated and untreated plants. Quantitative gene expressions were determined by using iCycler iQ real-time PCR detection system and iQ SYBR Green Supermix reagents (Biorad, Hercules, CA).

Results and Discussion

Toxicity of 2,4-DNT. For phytotoxicity studies, seedlings were transferred on solid media in Petri plates or liquid media in flasks amended with different concentrations of 2,4-DNT. The root lengths of the seedlings grown on the plates amended with 1 mg/L and 5 mg/L of 2,4-DNT were only 34% and 3% of the root length of untreated plants, respectively (Figure 2). The seedlings on the plates with over 10 mg/L of 2,4-

DNT did not grow at all during the 7-day exposure. In liquid cultures, the biomass of the plants exposed to 10 mg/L of 2,4-DNT corresponded to 43% of the biomass in untreated plants after 10 days. The plants did not grow and bleached at concentrations over 10 mg/L of 2,4-DNT. The root growth assay was more sensitive than the liquid cultures for toxicity tests presumably because the larger amount of biomass and the congregation of seedlings in liquid cultures may help tolerance at the higher concentrations of the contaminant. 2,4-DNT was toxic to root growth at concentrations as low as 1 mg/L.

Regarding toxicity of 2,4-DNT to other plants, alfalfa did not grow at 100 mg/kg 2,4-DNT in soil *(31)*. According to Adema and Henzen *(32)*, EC_{50} values of lettuce, tomato, and oats for 2,4-DNT were lower when tested in nutrient solution than in the soils suggesting the bioavailability of 2,4-DNT to the plants in soils is limited due to adsorption of 2,4-DNT to the soils. The highest non-observed adverse effect concentrations (NOAEC) for the growth were 5 mg/kg for lettuce, 10 mg/kg for wheat and mustard, and 20 mg/kg for lentil indicating that phytotoxic effects of nitroaromatic explosives depended on plant species *(33)*.

Uptake and Mass Balance. *Arabidopsis* seedlings grown from 50 seeds in 50 mL MS media for 15 days were exposed to various concentrations of 2,4-DNT to investigate the uptake rates by the plants. Only 5% of the initial 2,4-DNT remained in the liquid media after 120 hours (5 days) and there was no effect of different concentrations on uptake rates, as shown in Figure 3. The pseudo first order rate constants at different concentrations were 0.62 ± 0.03 day^{-1} for 8 mg/L, 0.59 ± 0.02 day^{-1} for 15 mg/L, and 0.58 ± 0.03 day^{-1} for 30 mg/L 2,4-DNT. The differences among rate constants were insignificant ($p>0.05$) by t-test. About 30% of the initial concentrations were adsorbed to the autoclaved plant materials after 24 hours (1 day). In the case of the glass controls, the concentrations did not change significantly.

Several studies on the uptake of 2,4-DNT by plants have been reported. Best *et al.* *(34)* applied a wetland system to remove explosives from the groundwater at the Volunteer Ammunition plant, resulting in an average 58% and 61% removal of 2,4-DNT and 2,6-DNT in a 115-day operation. It was observed that 67% of 2,4-DNT from soil was removed in a phytoremediation system using parrot feather *(35)*.

The plants survived at higher concentrations in the uptake study than in the toxicity tests resulting from the ages of the plants. Younger seedlings (4-5 day old) were used for the toxicity study while 15-day-old seedlings were used for the uptake study. In the application of phytoremediation in the field, the age of plants is an important design parameter.

When radiolabeled 2,4-DNT was used to obtain a mass balance, the radioactivity in the media gradually decreased and only 6% of the initial radioactivity remained in the solution after 10 days. The overall mass balances for live plants exposed to [U-^{14}C]2,4-DNT up to 14 days were over 86% (Table 2). Recovery for glass controls and autoclaved plants was over 90%. The amount of radioactivity adsorbed to autoclaved plant materials was about 5% due to the small biomass compared to that in the uptake study above. Less than 1% of the initial radioactivity was recovered from the CO_2 traps indicating that the mineralization of 2,4-DNT by the plants was insignificant (data not shown).

Transformation Products in Plant Extracts. Several unknown peaks having shorter retention times and peaks for aminonitrotoluenes were detected in tissue extracts from plants exposed to 2,4-DNT (Figure 4). Aminonitrotoluenes were identified by comparing to retention times with the standards in both radiochromatograms and UV absorbance chromatograms (Figure 4). The peak for 2,4-diaminotoluene was not

detected in radiochromatograms from plant extracts. When the percentage of peak areas in radiochromatograms was normalized to the fraction of extractable radioactivity, 22-40% and less than 3% of the intracellular radioactivity were accounted for by the unknown transformation products and aminonitrotoluenes, respectively (Figure 5). The percentage of 2,4-DNT decreased from 8.4% at 3 days to below detection limit (0.001 μCi/mL) at 14 days based on the radioactivity in the plant tissues.

In Figure 5, about a half (48%) of the radioactivity in the plants was extractable at 3 days while 23% of the radioactivity was extractable at 14 days. The percentage of the bound radioactivity increased from 49% at 3 days to 72% at 14 days suggesting transformed products of 2,4-DNT may be incorporated into plant tissues such as lignin and cellulose.

Unlike the situation with bacterial systems, little information is available on the transformation of 2,4-DNTs by plants. According to Todd and Lange *(35)*, only monoamino isomers, 2-amino-4-nitrotoluene (2A4NT) and 4-amino-2-nitrotoluene (4A2NT), were reported as reductive transformation products by plants under soil culture. They detected 4A2NT first in the plant tissues after 90 hours and then 2A4NT after 190 hours of exposure. Hydroxylaminotoluenes and dihydroxylaminotoluenes were produced in bacterial cell cultures and the further transformed products, aminohydroxylaminotoluenes and diaminotoluenes, were observed in the cell extracts *(36)*. *Hydrogenophaga palleronii* and *Burkholderia cepacia* produced intermediates and mineralized DNTs into CO_2 by mono- or dioxygenases *(18)*.

Since 2,4-DNT has a similar molecular structure to TNT and reductive transformation products by plants are observed, the unknown peaks with shorter retention times may be glucose conjugates. Bhadra *et al*. *(15)* characterized four conjugates of TNT metabolites with 6 carbon moieties produced by *C. roseus*. They found two of them have similar molecular structure to 2ADNT (labeled TNT-1 and 2A-1) and the rest of them are similar to 4ADNT (TNT-2 and 4A-2) indicating that the monoamines were precursors to the conjugates. Recent studies have elucidated these TNT conjugates. The conjugates of TNT metabolites by tobacco cell cultures are formed by conjugation of glucose on the hydroxylamine group of either 2HADNT or 4HADNT, and various diglycosides conjugates with gentiobioside or sophoroside forms were identified, including monoglycosides *(37)*.

The unknown metabolites could be potentially hazardous when they are leached from the senescence of leaves or roots. The annual death of fine roots ranges from 40% to 86% *(38)*, and 45.5% to 1.2% of intercellular radioactivity of explosives is leached as mostly transformed products from hybrid poplar leaves while less than 2% is leached from the root tissues *(39)*.

Distribution of ^{14}C in Plants. Hydroponic cultures were used to investigate the distribution of radioactivity in different tissues under nonsterile conditions. The radioactivity taken up by the plants from the nutrient solutions increased from 21% at 7 days to 46% at 25 days. As shown in Figure 6, most radioactivity taken up by the plants was in the root tissues (64% in the roots, 31% in the leaves, and 5% in the stems on average (n=14)) during 25-day exposure. The distribution of ^{14}C in different plant tissues did not change as the plants grew and the radiolabel accumulated in the plants.

The fate of 2,4-DNT in the plant after uptake was similar to that of TNT rather than nitramine explosives such as hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). Most of the radiolabel from [U-^{14}C]RDX was located in the leaves unlike TNT where the radiolabel was in the roots, suggesting that RDX was translocated into leaves readily,

but TNT was not. Over 60% of the radioactivity of RDX taken up was found in the aerial parts of hybrid poplars after 2 days *(40)*. In contrast, 78% of radioactivity of [U-^{14}C]TNT taken up by the poplars remained in roots after the same exposure time *(22)*. In addition, it is reported that poplar nodule cultures mineralized RDX significantly under sterile conditions *(21)*. In the case of TNT, mineralization by plants is insignificant *(22)*.

The mass balance from the hydroponic systems ranged from 78% (7 days) to 61% (25 days) for live plants and was 70% for the controls (without plants) while the mass balance for the sterile liquid cultures was over 86%. The losses are presumably due to microbial degradation or adsorption to the plastic tubes. Degradation of 2,4-DNT by microorganisms under aerobic conditions has been reported. Over 45% of the initial radiolabel from 2,4-DNT was mineralized by aerobic microorganisms in soil within 48 hours *(18)*. In another study, 28% of the initial radiolabel from 2,4-DNT was recovered as $^{14}CO_2$ by aquifer microorganisms after 28 day incubation in aquifer sediment *(41)*.

Glutathione and Gene Expression. Glutathione levels as well as the expression of the genes involved in glutathione synthesis in response to TNT treatment were quantified after the plants were exposed to 10 mg/L of 2,4-DNT for 1 day. The concentration of glutathione in root tissues of *Arabidopsis* exposed to 2,4-DNT increased significantly. The glutathione levels of 83.0 ± 3.7 μM (n=3) were measured in the root tissue extracts compared to 63.2 ± 2.4 μM (n=3) for the extracts from the unexposed plants. The concentrations of glutathione between treated and untreated plants were statistically significant based on the t-test ($p<0.05$). From the results of real time PCR analysis (Figure 7), both genes involved in glutathione synthesis, *GSH1* and *GSH2*, were induced 1.7-fold on average. Genes of a putative glutathione *S*-transferase (At1g17170) and a putative cytochrome P450 (At3g28740) which had been shown to respond to TNT treatment *(24)* were upregulated in the root tissues of 2,4-DNT-treated plants. The genes of At1g17170 and At3g28740 were induced 10-fold and 8-fold compared to the control plants.

No genomic DNA contamination was confirmed by PCR product sizes from agarose gels using primers designed in the exon regions of tubulin alpha-1 chain (At1g64740). The PCR product sizes were 0.4 k for cDNA and 1.2 k for genomic DNA (Table 1). Lack of non-specific amplifications were confirmed with single peaks in the melting curves from real-time PCR analysis.

The increase of glutathione levels in plant tissues in the presence of nitroaromatics has been noted earlier. According to Mezzari *et al. (23)*, the concentration of derivatives of glutathione with monochlorobimane, which produce fluorescence, increased in *Arabidopsis* roots when measured using a multiphoton microscope when the plants were exposed to TNT. However, they suggested that the conjugation of glutathione with the explosive does not occur since the intensity of fluorescence did not decrease showing that no competition for glutathione between monochlorobimane and TNT. In the case of herbicides such as metolachlor and acetochlor, the intensity of fluorescence (glutathione derivatives) decreased because of competition for glutathione between monochlorobimane and the herbicides indicating that glutathione conjugates with the herbicides were produced. According to Nepovim *et al. (42)*, the concentration of glutathione increased in the horseradish hairy roots treated with 0.1 mM 2,4-DNT by HPLC analysis.

The increase of glutathione *S*-transferases (GSTs) in *Arabidopsis* seedlings was reported when the plants were exposed to aromatic explosives. When 14-day-old *Arabidopsis* plants that were exposed to 15 mg/L of TNT were compared to untreated

plants after 24 hours, a glutathione *S*-transferase (At1g17170) which is categorized as Tau class in the GST family *(43)* was highly induced (up to 27 times) as measured by the serial analysis of gene expression (SAGE) *(24)*. In a different study, induction of three genes for GSTs in *Arabidopsis* was observed after PCR reactions of cDNA with the specific primers when the plants were exposed to 0.6 mM TNT *(23)*.

The genes for glutathione synthesis and GSTs were induced by other organic chemicals and heavy metals. According to Deridder *et al*. *(44)*, safeners (chemicals added to increase the tolerance of crops to herbicides) increased the level of glutathione and GST. The molecular structure of 2,4-DNT is similar to safeners which are aromatic compounds with functional groups containing nitrogen. The transcription of the genes for glutathione synthesis increased in *Arabidopsis* when the plants were exposed to cadmium or copper *(26)* and three genes for different classes of GST were induced from exposure to herbicides as well as explosives *(23)*. Thus, the induced expression of *GSH1*, *GSH2*, and a gene for GST (At1g17170) in this study may be the result of a generalized stress response to 2,4-DNT.

Expression of various genes in response to TNT has been reported. The highly induced genes from SAGE analysis were a GST (At1g17170), a cytochrome P450 (At3g28740), an ABC transporter which is known to expend ATP energy to transport hydrophobic molecules into or out of the cytoplasm, and a 12-oxophytodienoate reductase having high homology to nitroreductases of the bacteria, *Enterobacter* sp. *(24)*. According to Mentewab *et al*. *(45)*, the induction of 52 genes and the repression of 47 genes in total were observed from Arabidopsis seedlings exposed to 1 μM and 10 μM TNT for 10 days. They also confirmed the induction of genes of pathogenesis-related protein 1 precursor, DNA-binding proteins, and ABC transporter-like protein, in shoots exposed to 10 μM TNT for 10 days by real-time PCR analysis.

Conclusions

2,4-DNT was toxic to *Arabidopsis* at low concentrations and was transformed by the plants after uptake. About a half of the radiolabel from 2,4-DNT remained in the root tissues and the percentage of the bound radiolabel from 2,4-DNT increased over time presumably due to the incorporation to plant tissues. Reductive transformation products, monoaminotoluenes, and unknown metabolites were observed. Glutathione and the genes involved in its synthesis were induced significantly in response to 2,4-DNT. In addition, induction of gene expression for a glutathione *S*-transferase (At1g17170) and a cytochrome P450 (At3g28740) was observed. From the similar characteristics between mechanisms of 2,4-DNT and TNT metabolism by plants, it seems reasonable to assume that both 2,4-DNT and TNT share a common metabolic pathway, possibly including the same enzyme steps. Therefore, the phytoremediation processes and the development of engineered plants for 2,4-DNT degradation may be extrapolated from our more extensive knowledge of TNT metabolism due to the similarity in the fate of these compounds in plants and of gene expressions induced by TNT and 2,4-DNT. In addition, further study on the toxicity and identification of the unknown metabolites is required since they might be leached by water from the fallen or dead plant tissues by senescence and end up potential risks to human beings.

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Table 1. Primer sets used for real-time PCR and genomic DNA contamination.

Name	Locus	Primer sets	Product size (bp[a])
Actin-8	At1g49240	L[b]: TCCGGTTACAGCGTTTGGAGA R[b]: CGCGGATTAGTGCCTCAGGT	84
Tubulin α-1 chain (*TUA1*)	At1g64740	L: ATGAGGGAGATCATTAGCATTCATAT R: CTGAGGAGAAGGGTAGATGGTG	1191[c] 445[c]
GSH1	At4g23100	L: CCGTGTTCGAAGAGCTGCTG R: TTCCGGAGACTCGAATTCTTCA	106
GSH2	At5g27380	L: TGGATAACATTCACAACCATCTTGA R: GCTTTGCGGTCCTGTGGAAA	143
Putative GST	At1g17170	L: GTCGCTAAAGCCCTGCCTGA R: GAACAAAGCAACAACAGATCAACA	117
Putative cytochrome P450	At3g28740	L: CGACGATCTTGCCCTGGTTC R: GCTTTTCGCATTGTGGTTCC	146

[a] bp represents base pair.
[b] L and R represent left primers and right primers
[c] PCR product sizes are 1191 bp for genomic DNA and 445 bp for cDNA.

Table 2. Mass balance results at different harvest times from biooxidation and LSC analysis.

	Plant tissues (%)	Media (%)	Recovery (%)
Glass control (14 days, n[a]=3)	-	89.8 ± 0.9	89.8 ± 0.9
Autoclaved plants (14 days, n=3)	4.49 ± 0.55	89.1 ± 3.1	93.5 ± 2.6
Live plants (3 days, n=3)	35.9 ± 9.2	51.9 ± 6.6	87.7 ± 3.6
Live plants (5 days, n=3)	73.6 ± 5.4	21.0 ± 4.5	94.6 ± 1.8
Live plants (7 days, n=3)	76.1 ± 8.6	10.2 ± 0.9	86.3 ± 8.9
Live plants (10 days, n=4)	89.9 ± 1.8	5.81 ± 0.83	95.7 ± 1.8
Live plants (14 days, n=4)	92.5 ± 2.4	4.46 ± 0.49	97.0 ± 2.4

[a] n represents the number of flasks sacrificed.

Figure Legends

Figure 1. Pathway of glutathione synthesis and detoxification of xenobiotics by plants.

Figure 2. Root growth (top) and dry biomass (bottom) in response to different concentrations of 2,4-DNT. Four-day-old seedlings grown on plates were transferred to other plates amended with various concentrations of 2,4-DNT for the root growth assay and seedlings grown on plates for 4-5 days were transferred to MS liquid media containing different concentrations of 2,4-DNT for the biomass assay. The biomass and root length were measured at 10 days and 7 days after the transfer. Twenty-five seedlings per flask were prepared in triplicate for the biomass assay and 24 seedlings in total were used for the root growth assay. Error bars represent standard deviations.

Figure 3. Removal of 2,4-DNT from liquid media by *Arabidopsis* seedlings grown for 15 days from seeds. The initial concentrations were 8 mg/L for Live plant 1, 15 mg/L for Live plant 2, 30 mg/L for Live plant 3, and 15 mg/L for glass controls and autoclaved-plant controls. Each group was prepared in triplicate. Error bars represent standard deviations. The pseudo first order rate constants at different concentrations were 0.62 ± 0.03 day^{-1} for 8 mg/L, 0.59 ± 0.02 day^{-1} for 15 mg/L, and 0.58 ± 0.03 day^{-1} for 30 mg/L 2,4-DNT. The differences among the constants were insignificant by t-test ($p>0.05$).

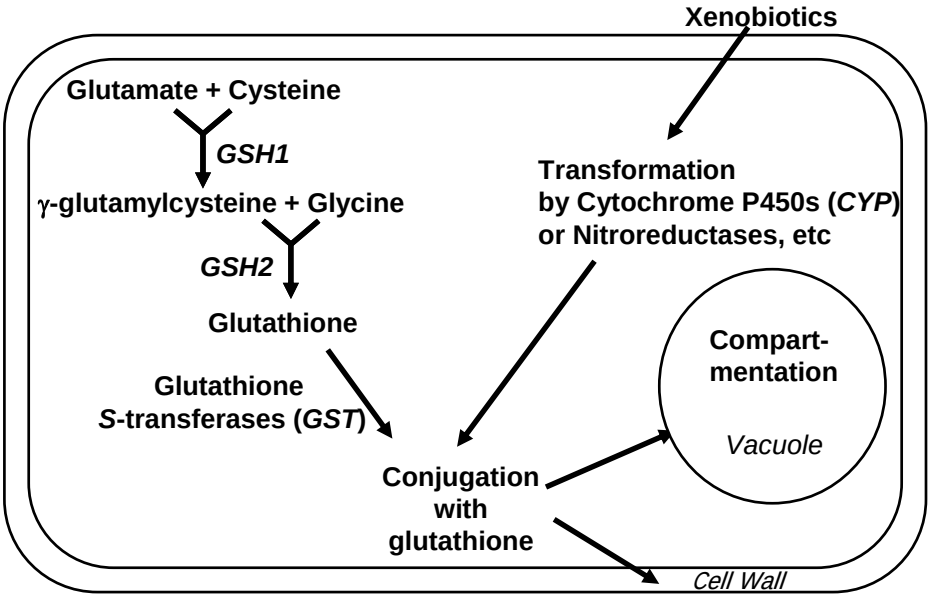
Figure 4. Radiochromatograms (bottom) of extracts from plant tissue exposed to 2,4-DNT at different times and UV chromatograms (top) of standards extracted at 240 nm. Peaks for monoaminonitrotoluenes and unknown metabolites with short retention times were detected, but the peak for 24DAT was not detected by comparing retention times of standards. The retention times: 2-amino-4-nitrotoluene (2A4NT): 8.35 min; 4-amino-2-nitrotoluene (4A2NT): 8.85 min; 2,4-dinitrotoluene (2,4-DNT): 14.41 min; 2,4-diaminotoluene (24DAT): 21.67 min.

Figure 5. Percentage of extractable radioactivity and transformation products from tissue extracts over harvest time based on the uptaken radioactivity. Error bars represent standard deviations.

Figure 6. Distribution of ^{14}C in plant tissues sacrificed after different exposure times. Most (68%) of the radioactivity in plants was in the root tissues. Error bars represent standard deviations.

Figure 7. Expressions of genes in root tissues (n=3) exposed to 10 mg/L of 2,4-DNT for 1 day compared to those of untreated plants (n=3). GST and CYP represent the genes of At1g17170 and At3g28740 which were induced by 10 and 8 times more than those of untreated plants, respectively. Error bars represent standard deviations.

Figure 1.


Xenobiotics
Glutamate + Cysteine
GSH1
γ-glutamylcysteine + Glycine
GSH2
Glutathione
Glutathione
S-transferases (GST)
Transformation
by Cytochrome P450s (CYP)
or Nitroreductases, etc
Compart-
mentation
Vacuole
Conjugation
with
glutathione
Cell Wall

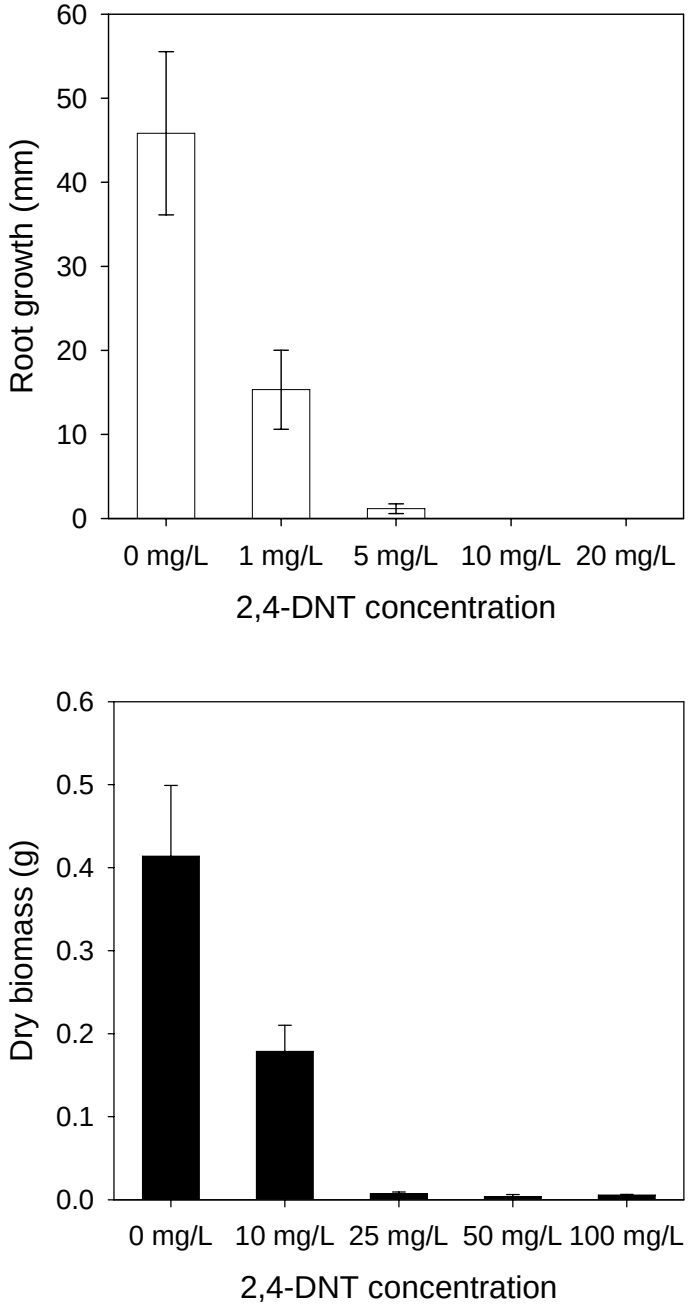

60
50
40
30
20
10
0
Root growth (mm)
0 mg/L
1 mg/L
5 mg/L
10 mg/L
20 mg/L
2,4-DNT concentration
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Dry biomass (g)
0 mg/L
10 mg/L
25 mg/L
50 mg/L
100 mg/L
2,4-DNT concentration

Figure 2.

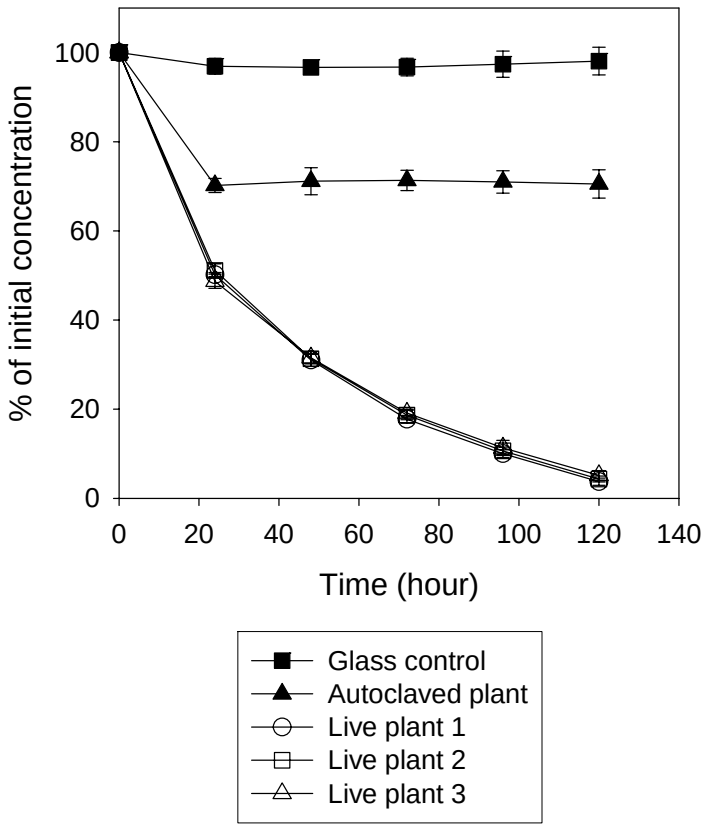

% of initial concentration
100
80
60
40
20
0
0
20
40
60
80
100
120
140
Time (hour)
Glass control
Autoclaved plant
Live plant 1
Live plant 2
Live plant 3

Figure 3.

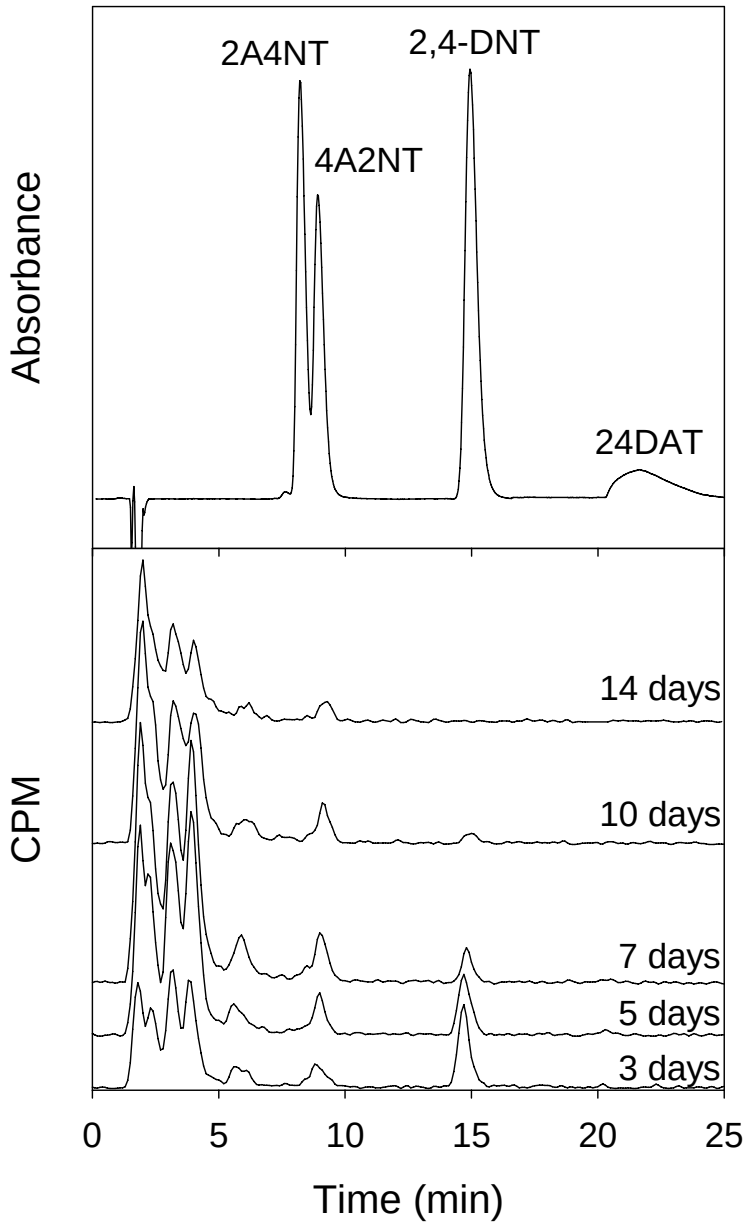

2A4NT
2,4-DNT
4A2NT
24DAT
Absorbance
14 days
10 days
7 days
5 days
3 days
CPM
0
5
10
15
20
25
Time (min)

Figure 4.

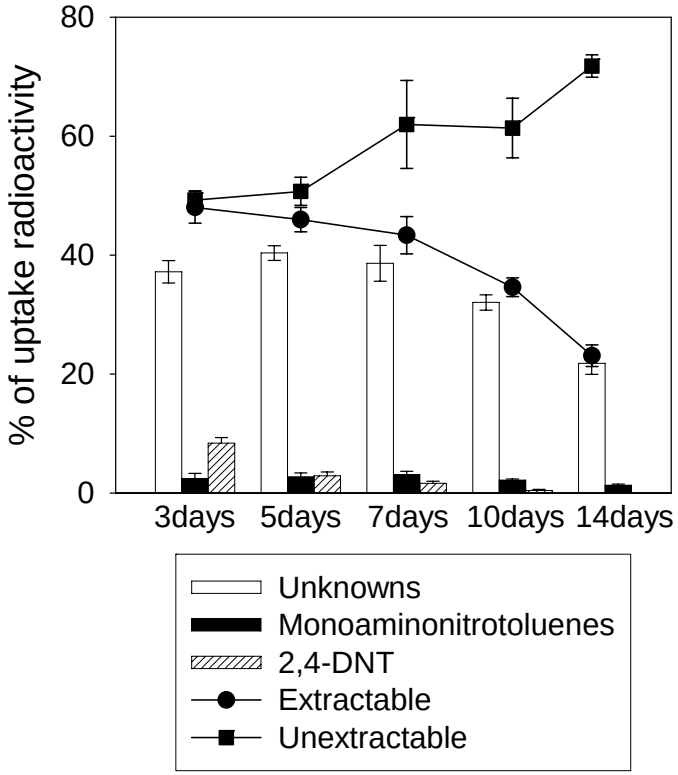

80
60
40
20
0
% of uptake radioactivity
3days
5days
7days
10days
14days
Unknowns
Monoaminonitrotoluenes
2,4-DNT
Extractable
Unextractable

Figure 5.

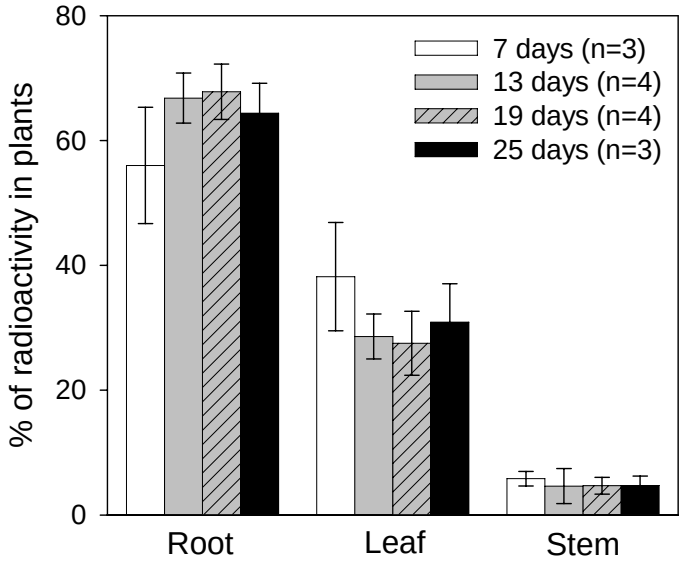

80
60
40
20
0
% of radioactivity in plants
7 days (n=3)
13 days (n=4)
19 days (n=4)
25 days (n=3)
Root
Leaf
Stem

Figure 6.

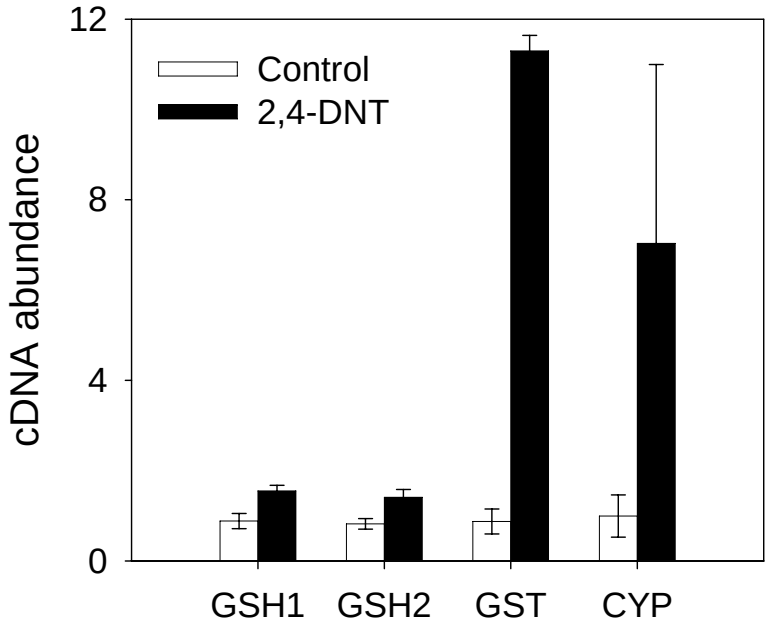


Figure 7.

Yoon, J. M., Oliver, D. J., Shanks, J. V., "Phytotransformation and Phytoremediation of 2,6-Dinitrotoluene using a Model Plant, Arabidopsis thaliana", Chemosphere, accepted, 2007.

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Phytotransformation and Phytoremediation of 2,6-Dinitrotoluene Using a Model Plant, *Arabidopsis thaliana*

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Abstract
Biochemical and genetic studies of xenobiotic metabolism in the model plant Arabidopsis have significant potential in providing information for phytoremediation. This paper presents the toxicity of 2,6-dinitrotoluene (2,6-DNT) to Arabidopsis under axenic conditions, the fate and transformation of 2,6-DNT after uptake by the plant, and the effect of a putative glutathione *S*-transferase (GST), which is highly induced by TNT in the previous study, on the detoxification of 2,6-DNT. 2,6-DNT had toxic effects on the growth of Arabidopsis based on whole seedling as well as root growth assays. Using [U-^{14}C]2,6-DNT, the recovery was over 87% and less than 2% accounted for the mineralization of 2,6-DNT in axenic liquid cultures during the 14 days of exposure. About half (48.3%) of the intracellular radioactivity was located in the root tissues in non-sterile hydroponic cultures. 2-amino-6-nitrotoluene (2A6NT) and two unknown metabolites were produced as transformation products of 2,6-DNT in the liquid media. The metabolites were further characterized by proton NMR spectra and the UV-chromatograms when the plant was fed with either 2,6-DNT or 2A6NT. In addition, polar unknown metabolites were detected at short retention times from radiochromatograms of plant tissue extracts. The GST gene of the wild-type of Arabidopsis in response to 2,6-DNT was induced by 4.7-fold. However, the uptake rates and the tolerance at different concentrations of 2,6-DNT and TNT were not significantly different between the wild-type and the *gst* mutant indicating that induction of the GST gene is not related to the detoxification of 2,6-DNT.

Keywords: phytoremediation, uptake, toxicity, real time PCR, glutathione *S*-transferase

1. Introduction

Dinitrotoluenes (DNTs) are produced as by-products of 2,4,6-trinitrotoluene (TNT) and as intermediates for polyurethane synthesis (Zhang, et al., 2000). Contamination of soil and groundwater with DNTs results from improper treatment of wastewater produced in the manufacturing sites and from military activities in firing ranges. Commercial grade DNT consists of 76% 2,4-dinitrotoluene (2,4-DNT), 20% 2,6-dinitrotoluene (2,6-DNT), and 4% other isomers (Rickert, et al., 1984). DNTs increase the risk of hepatobiliary cancer in an epidemiologic study (Stayner, et al., 1993), and 2,6-DNT is more toxic than 2,4-DNT in the Microtox test while 2,4-DNT is more toxic to a green alga (*Selenastrum capricomutum*) than 2,6-DNT, indicating that the level of toxicity of dinitrotoluenes is species-dependent (Dodard, et al., 1999). Most studies regarding the toxicity of dinitrotoluenes to plants were limited to non-sterile soils (Picka and Friedl, 2004; Rocheleau, et al., 2006). According to the Drinking Water Health Advisory, 2,6-dinitrotoluene (2,6-DNT) is classified as a possible human carcinogen and the health advisory guideline value is limited to 0.4 mg l^{-1} for the one day and 10 days of exposure to a 10-kg child (EPA, 2004).

Phytoremediation of explosives is an alternative for in situ remediation of contaminated soil and groundwater because it is a cost-effective and environmentally-friendly treatment (Schnoor, et al., 1995). The phytoremediation of TNT and nitramine explosives has been studied extensively (Rivera, et al., 1998; Van Aken, et al., 2004), but not dinitrotoluenes. The scarcity of the research on phytoremediation of dinitrotoluenes may come from the speculation that the application of phytoremediation of dinitrotoluenes can be based on TNT studies due to the similar chemical structures between TNT and DNTs. However, the nitroaromatic explosives showed different fates in bioremediation. For example, DNTs are mineralized by aerobic bacteria (Nishino, et al., 1999) while TNT is rarely mineralized by bacteria (Esteve-Nunez, et al., 2001).

Arabidopsis is commonly used as a tool in biochemical and genetic studies in the field of plant science because its genome is completely sequenced and seeds of T-DNA insertion mutants for specific genes are publicly available for research on gene expression. Gene expression studies, such as microarray analysis and SAGE analysis (Ekman, et al., 2003; Mentewab, et al., 2005), in response to explosives may help the development of transgenic plants with the abilities of enhanced tolerance and faster removal rates at high concentrations of the contaminants. According to the "green liver model" (Sandermann, 1994; Schroder and Collins, 2002), one of crucial detoxification mechanisms is the conjugation of glutathione and xenobiotics by glutathione *S*-transferases (GSTs). In previous studies, the GST gene (At1g17170) is highly expressed when Arabidopsis was exposed to TNT (Ekman, et al., 2003; Mezzari, et al., 2005), and thus could be hypothesized that the GST gene is involved in the detoxification of 2,6-DNT by Arabidopsis.

The objectives of this study are to determine the phytotoxicity, to investigate the fate and transformation of 2,6-DNT after uptake under axenic conditions, and to explore the effect of the GST gene (At1g17170), which is highly induced in response to TNT, in the detoxification of 2,6-DNT by the comparison between the wild-type and a *gst* T-DNA mutant.

2. Materials and Methods

2.1. Chemicals

2,6-dinitrotoluene (purity 99%) and 2,4,6-trinitrotoluene (TNT, purity 98%) were purchased from Chemservice (West Chester, PA). Uniformly labeled [U-^{14}C]2,6-DNT

(radioactivity 6.23 mCi/mmol, purity 99%) and unlabeled 2-amino-6-nitrotoluene (2A6NT, purity 99.9%) were obtained from PerkinElmer Life and Analytical Sciences (Boston, MA) and Sigma-Aldrich (St. Louis, MO). Other chemicals and solvents were reagent grade and better.

2.2. Analytical methods

A reverse-phase HPLC (Waters, Milford, MA) was used for monitoring the concentrations of nitroaromatics in the media, and was equipped with a 996 photodiode-array (PDA) detector, 510 pumps, and a 717 plus autosampler. An isocratic mobile phase of 82/18 (water/2-propanol, v/v%) and a flow rate of 1 ml min^{-1} were used. A Nova Pak C8 column (5 μm, 3.9x150 mm, Waters) was used for analyte separation. Chromatograms were extracted at 240 nm from spectra scanned between 200 and 400 nm for quantification.

The radioactivity in the plant biomass was determined by combusting a portion of the biomass in an OX700 Harvey Biological Biooxidizer. A sample (500 μl) out of 15 ml CarboSorb E cocktail trapping $^{14}CO_2$ produced from oxidation of the plant biomass was mixed with 3 ml PermaFluor E^+ cocktail before analysis by a Packard 2900 TR Liquid Scintillation Counter (LSC). The radioactivity of liquid media and plant extracts as well as the mineralization of [U-^{14}C]2,6-DNT was determined by the LSC after mixing with the cocktail solutions. Ultima Gold M cocktail was used for liquid media and extracts and Hionic-Fluor cocktail was used for the 1N NaOH solution. The ratio of the samples to cocktails was 1:5. Radiochromatograms of plant extracts were obtained from a Packard 505 flow scintillation counter serially attached to the PDA detector. The ratio of the mobile phase of HPLC to Ultima Flo M cocktail was 1: 2.

2.3. Cultivation of plants

Wild-type *Arabidopsis thaliana* (Columbia) seeds were obtained from the Arabidopsis Biological Resource Center (ABRC) (Columbus, OH). Seeds were sterilized as described (Xiang and Oliver, 1998), and then the seeds were mixed with a 0.1% sterile agar solution before being placed in plates containing solid half-strength Murashige and Skoog (MS) media with 1% agar. The seedlings were grown in the vertically placed plates for 4 days and then transferred to 250-ml wide-mouth Erlenmeyer flasks containing 50 ml MS liquid media (half-strength MS salts, 0.5 g of MES, 20 g of sugar, and 3 ml of a 6% KH_2PO_4 solution per liter, pH 5.8) for liquid cultures, or to different plates amended with explosives for the root growth assay.

2.4. Phytotoxicity tests

For the biomass assay, twenty five seedlings grown in vertically placed plates for 4 days were transferred to each flask containing liquid MS media (pH 5.8) in order to assure the same initial biomass. Quadruplicates for each treatment were prepared. A day was allowed for adjustment before different volumes of 2,6-DNT stock solution were added into the liquid media. After 14 days, the plants were rinsed with deionized (DI) water, and were dried at 65 ^{o}C in an oven or freeze-dried before the dry biomass was measured gravimetrically.

The root growth assay was used as an alternative toxicity test. Twelve seedlings grown in vertically placed plates containing solid MS media (pH 5.8) for 4 days were transferred to each 1% agar plate amended with various concentrations of 2,6-DNT. The plates were prepared in duplicate and the number of seedlings for each treatment

ranges from 23 to 24. The ends of root tips were marked on the back side of the plates after transfer, and the root elongation was measured after 7 days.

The concentration of 2,6-DNT where 50% of its effect is observed (EC_{50}) for the biomass assay and 50% inhibition concentration (IC_{50}) for the root growth assay were determined from a non-linear regression (SigmaPlot 8.0, Systat Software, Richmond, CA) based on the 3-parameter logistic model in Adema and Henzen (1989).

2.5. Uptake and mass balance

2,6-DNT stock solutions in methanol were added to sterile MS liquid media where twenty-five seedlings were grown for an additional 11 days after transfer from the plates. The plants were exposed to 20, 40, and 80 mg l^{-1} of 2,6-DNT for 7 days. Samples from the liquid media were taken periodically and were mixed with acetonitrile (1:1 vol) before the liquid samples were filtered through Xpertek syringe filters (0.2 μm) from P.J. Cobert Associates (St. Louis, MO) for HPLC analysis.

About 2 μCi [U-^{14}C]2,6-DNT were added into the liquid media after 5 days of additional growth following the transfer from the plates to flasks for the mass balance study. Glass controls and autoclaved-plant controls were prepared also. The initial volumes of the liquid media were measured with 50 ml sterile pipettes for determining the initial radioactivity. The flasks were plugged with foam caps to prevent microbial and fungal contamination. Nine flasks were sealed with No.8 rubber stoppers for the measurement of $^{14}CO_2$ captured in 6 ml serum vials containing 1 ml of 1N NaOH. The vials were installed under the rubber stoppers. The plants sacrificed at different times (3, 5, 7, 10, and 14 days) were freeze-dried for 2 days, and then, were pulverized into fine powders by spatula. The powders of freeze-dried plant materials were sonicated for 1 day after adding methanol (20 ml). Samples filtered with Whatman 934-AH glass fiber filters were used for LSC analysis of extractable radioactivity. The residues after the filtration were combusted for the bound intracellular radioactivity in the plant biomass. The extracts were concentrated via evaporation under a fume hood and reconstituted in methanol and DI water (1:1 vol%). Reconstituted extracts were filtered and analyzed by the radiodetector in series with the HPLC system for the transformed products of 2,6-DNT by plants.

Hydroponic systems were set up as described in Tocquin et al. (2003) in order to investigate the distribution of the radioactivity in different plant tissues. Four or five seeds were sown in microcentrifuge tubes (0.5 ml) filled with 0.6% agar. The microcentrifuge tubes were wrapped with a piece of fiberglass window screen to prevent the leaching of agar were inserted into holes in the caps of 50 ml disposable centrifuge tubes. A healthy plant per tube was selected and grown for 21 days before being transferred to half-strength Hoagland solution (pH 5.8) amended with 2 μCi of [U-^{14}C]2,6-DNT. The plants were harvested at 7, 13, 19, and 25 days, and divided into roots, leaves, and stems. The plant tissues were air-dried in a hood for over 3 days. The radioactivity in different plant tissues was determined by combustion and LSC analysis as described above.

2.6. Characterization of metabolites

The media after 5 days of exposure to either 2,6-DNT or 2A6NT were collected and concentrated by freeze-drying and reconstitution in DI water. The eluents after the separation column in the HPLC were manually collected at different time windows according to the retention time of metabolites-2.8 to 3.1 min for a metabolite of 2,6-DNT (labeled as 26DNT-2.9); 3.4 to 3.7 min for the other metabolite of 2,6-DNT (labeled as 26DNT-3.5) and a metabolite of 2A6NT (labeled as 2A6NT-3.5). The

fractions of the eluents were further freeze-dried and reconstituted with CD_3OD for NMR analysis. The proton NMR spectra of 2,6-DNT and its metabolites were obtained by a Bruker DRX500 or DRX400 spectrometer. The values of chemical shifts were reported relative to CD_3OD (^1H: δ, 3.31 and 4.87 ppm) and coupling constants are given in hertz (Hz).

2.7. Comparison of wild-type and gst mutant

The SALK_034472 line, which has T-DNA insertions at the exon region of At1g17170 and the 5'UTR region of At5g47780, is described on the SIGnAL (Salk Institute Genomic Analysis Laboratory) website (http://signal.salk.edu/cgi-bin/tdnaexpress) and the seeds were provided by the ABRC. Homozygous mutant plants were isolated from the result of gel electrophoresis after PCR from young leaves taken from seedlings grown in soil for 1 week. The left primer (LP, GCGTACAACACTCATTTCTCACA) and the right primer (RP, CACCTTTGACCGCCCAAATC) were used for the wild-type while the left border primer (LBb1, GCGTGGACCGCTTGCTGCAACT) and the right primer (RP) were used for the T-DNA insertion. Other wild-type and heterozygous plants were pulled out and discarded. Seeds from the homozygous mutant were harvested when the siliques of the mutant plants were mature.

For real-time PCR analysis, the wild-type and the *gst* mutant (25 seedlings per flask) were grown for 15 days from seeds (4 days in the plates and an additional 11 days in the MS liquid media) as described previously, and then the 2,6-DNT stock solution in methanol was added to the media to make 50 mg l^{-1} 2,6-DNT and equal amounts of methanol were added for the controls. After 1 day of exposure, RNA was extracted with Triazol (Invitrogen, Carlsbad, CA) from about 100 mg of fresh root tissues. First strand cDNA was synthesized by using SuperScript II reverse transcriptase (Invitrogen) after destroying genomic DNA by DNase I enzyme (Invitrogen) treatment. Given the gene sequence of the GST from The Arabidopsis Information Resource (TAIR, http://www.arabidopsis.org), the primer set for the gene (LP: GTCGCTAAAGCCCTGCCTGA RP: GAACAAAGCAACAACAGATCAACA) was designed by using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) based on of the product sizes (100-150 bp) and the optimum melting temperature (60 ^oC). The *Actin-8* gene (At1g49240) was used as a control gene for both treated and untreated plants and the sequences of the primer set are TCCGGTTACAGCGTTTGGAGA for the left primer and CGCGGATTAGTGCCTCAGGT for the right primer. Quantitative gene expression was calculated from calibration curves of serial dilutions of concentrated cDNA after running PCR with 45 cycles by using iCycler iQ real-time PCR detection system and iQ SYBR Green Supermix reagents (Biorad, Hercules, CA).

The wild-type and mutant plants were exposed to various concentrations of 2,6-DNT and TNT for 7 days for the comparison of uptake rates. The kinetic constants for the uptake rate were determined based on the pseudo first order kinetics. In addition, the wild-type and mutant plants were placed on the plates amended with 2,6-DNT and TNT for the root growth assay. The statistical difference of the root lengths between the *gst* mutant and the wild-type was performed by using the data analysis tool in the Microsoft Excel.

3. Result and Discussion

3.1. Phytotoxicity and uptake study

When Arabidopsis was exposed to different concentrations of 2,6-DNT in liquid culture and on agar plates under axenic conditions, 2,6-DNT has toxic effects on dry biomass and root elongation of the plant (Figure 1). The growth of the biomass was severely inhibited at the higher concentrations than 20 mg l^{-1} in liquid culture and the EC_{50} value determined from the biomass assay was 29.0 mg l^{-1} (Figure 1(a)). The root growth of 4-day-old seedlings was stunted at 20 mg l^{-1} of 2,6-DNT and the IC_{50} from the root growth assay was 15.7 mg l^{-1} (Figure 1(b)).

Other studies have determined the phytotoxicity of 2,6-DNT on plants grown in soil systems. Rocheleau et al. (2006) determined the EC_{50} values of 2,6-DNT from dry biomasses of three different plants grown in aged or fresh amended soils ranging from 2.8 to 26 mg kg^{-1}. Our EC50 value (15.7 mg l^{-1}) of 2,6-DNT in the root growth assay was within the ranges of the values and slightly higher in the biomass assay even though our experiments were performed in vitro under axenic conditions. In another study, the EC_{50} values of 2,6-DNT determined by shoot dry mass of four terrestrial plants ranged from 26 to 61 mg kg^{-1} (Picka and Friedl, 2004). Both studies showed that 2,6-DNT is more toxic to the tested terrestrial plants in the freshly amended soil than TNT (Rocheleau, et al., 2006; Picka and Friedl, 2004) although TNT is more toxic than dinitrotoluenes to mammalian cell cultures (Mitchell and Burrows, 1995; Tchounwou, et al., 2001) and Arabidopsis from the root growth assay shown later in this study (section 3.4).

In the uptake study, plants in liquid cultures removed 95%, 83%, and 56% after 7 days when they were exposed to 20, 40, and 80 mg l^{-1} 2,6-DNT (Figure 2), respectively. The reduced uptake rate of the plants exposed to 40 mg l^{-1} and 80 mg l^{-1} compared to that of the plants exposed to 20 mg l^{-1} likely results from the toxicity of 2,6-DNT or its metabolites. In the case of controls, 18% and 30% of the initial concentration was removed for glass controls and autoclaved plants, respectively. Regarding the uptake of 2,6-DNT by plants, only one paper has been published. Best et al. (2001) reported that 60% of the inlet concentration of 2,6-DNT was removed after 115-day operation in wetland.

3.2. Mass balance and distribution of radiolabel in plant tissues

[U-^{14}C]2,6-DNT was used for mass balances in axenic liquid cultures and for the distribution of ^{14}C label in plants grown in non-sterile hydroponic solutions. In axenic liquid cultures, less than 10% of the initial radioactivity remained in liquid media of live plants while over 80% remained in the media for the controls after 14 days (Table 1). The average recovery was 87% for live plants and the controls from biooxidation and LSC analysis. The extractable portion for the plants sacrificed at 14 days (30.8% of the radioactivity in the plants) decreased by half compared to that for the plants harvested after 3 days (59.5% of the intracellular radioactivity). Less than 2% of the initial radioactivity accounted for CO_2 production in both live plants and controls indicating the mineralization of 2,6-DNT by the plants is insignificant (Figure S1 in Supplementary Information).

In hydroponic systems, about a half of the radioactivity taken up by the plants (48.3%) was located in the roots while 40.7 % was in the leaves and 11.0 % was in the stems after 25 days of exposure (Figure 3). The recovery for plants decreased from 70.4% (7 days) to 58.8% (25 days), based on the initial radioactivity, while that for controls (without plants) was 66.0% after 25 days. The decrease of recovery might be caused by microbial degradation or adsorption to the plastic tubes (Table S1 in Supplementary Information).

The results of the fate and distribution of [U-^{14}C]2,6-DNT in the plants from this study are similar to those of TNT. Most of the radiolabel of [U-^{14}C]TNT taken up by hybrid poplars was located in the root tissue and the mineralization of TNT by the plants was not observed (Thompson, et al., 1998). Also, the intracellular bound portion accumulated; 30-40% of the applied [U-^{14}C]TNT after 8 days for *Catharanthus roseus* and 74% after 7 days for Arabidopsis (Bhadra, et al., 1999a; Subramanian, et al., 2006). The results show that the fate of 2,6-DNT in plants after uptake is similar to that of TNT and thus phytoremediation of 2,6-DNT can be based from the studies on TNT.

3.3. Phytotransformation of 2,6-DNT

Arabidopsis transformed 2,6-DNT into monoaminonitrotoluenes and other unknown polar compounds. From the radiochromatograms of plant extracts, unknown polar metabolites with short retention times were detected (Figure 4). The relative percentage of the unknown transformed products ranged from 72% to 100% based on the total peak areas. The relative percentage of peak areas for 2,6-DNT from the plant extracts was 19% after 3 days, and then decreased below detection limit (0.001 μCi ml^{-1}). 2A6NT was hardly detected in the radiochromatograms of the plant extracts because the quantity is below the detection limit, but it was detected from liquid media by the UV-detector at concentrations of 1-2% of the initial mole of 2,6-DNT.

In the media, two unknown metabolites of 2,6-DNT transformed by the plants were detected at retention times of 2.9 min and 3.5 min (on the shoulder of the peak whose retention time was 2.9 min) from UV chromatograms (Figure 4). These metabolites of 2,6-DNT are labeled as 26DNT-2.9 and 26DNT-3.5. When 2A6NT was fed to Arabidopsis liquid cultures, a peak at 3.5 min was observed in the media and is labeled as 2A6NT-3.5. The peak corresponding to 26DNT-2.9 was not detected in the 2A6NT feeding study, indicating the metabolite, 26DNT-2.9, was converted from 2,6-DNT, not via 2A6NT.

The proton NMR chemical shifts of 2,6-DNT, 26DNT-2.9, 26DNT-3.5, 2A6NT-3.5, and other possible candidates from literatures are listed in Table 2. The peak for the methyl group was not observed in 26DNT-2.9, indicating the methyl group is modified while the presence of the peak of the methyl group for 26DNT-3.5 and 2A6NT-3.5 suggests that the methyl group is intact. In addition, the chemical shift for 26DNT-3.5 was more upfield than those of the parent compound, 2,6-DNT and multiple peaks were observed from all of three metabolites (26DNT-2.9, 26DNT-3.5, and 2A6NT-3.5) between 3.3 and 4.7 ppm (data are not shown).

From the results of HPLC chromatograms and NMR analysis of the liquid media, conjugation after reduction and oxidation is a possible pathway in the detoxification of 2,6-DNT. Oxidative metabolites, 2,6-dinitrobenzoic acid, 2,6-dinitrobenzyl alcohol glucuronide, and 2-amino-6-nitrobenzoic acid, have been reported from urinary excretion of rats and workers at a manufacturing facility as metabolites of 2,6-DNT (Long and Rickert, 1982; Turner, et al., 1985). In plant systems, there has been no information about the transformation products of 2,6-DNT. In the case of TNT, which has a similar structure to 2,6-DNT, both oxidative and reductive metabolites of TNT by plants are found (Bhadra, et al., 1999b). Recently, Subramanian et al. (2006) reported that hydroxylamines transformed from TNT by plants play central roles in conjugation and reduction pathways. The hydroxylamines are converted into conjugates in Arabidopsis (Subramanian, et al., 2006) and into monoglycoside and diglycoside conjugates by tobacco suspension cultures (Vila, et al., 2005).

3.4. Effect of GST gene in comparison of the wild type and the gst mutant

After a homozygous *gst* mutant line was isolated out of 9 seedlings from the result of gel electrophoresis following PCR reactions (Figure S2 in Supplementary Information), the seeds were harvested to be used for real-time PCR, and the comparison of uptake rates and root growth between the wild-type and the *gst* mutant. From the real-time PCR analysis, the GST gene of the wild-type exposed to 50 mg l^{-1} 2,6-DNT for 1 day was induced by 4.7 times compared to the wild-type (control) which were not exposed to 2,6-DNT (Figure 5). In the case of the mutant, the GST gene was barely expressed when the mutant line was exposed to both controls (without 2,6-DNT) and 50 mg l^{-1} 2,6-DNT confirming that the mutant line has a T-DNA insertion at At1g17170.

When the uptake of 2,6-DNT and TNT by the *gst* mutant and wild-type was considered, the plants took up TNT faster than 2,6-DNT. Over 99% of the initial concentration of TNT was removed by the plants after 1 day while 30% was removed for 2,6-DNT at the same period of time. When the uptake rates were compared, the kinetic constants of the wild-type exposed to 50 mg l^{-1} 2,6-DNT and 100 mg l^{-1} TNT were slightly greater than those of *gst* mutants (Table S2 in Supplementary Information). However, the uptake rate of the wild-type was slightly less than or similar to that of the *gst* mutant at other concentrations. There was no dramatic difference in the uptake rates between the mutant and wild-type when the level of GST gene induction was considered. As for the root growth assay, the root elongation of both the mutant and wild-type was inhibited at lower concentrations of TNT than 2,6-DNT (Figure 6). In addition, the difference of the root elongation between the *gst* mutant and wild-type exposed to different concentrations of 2,6-DNT and TNT was statistically insignificant from t-test (two-tail, $p>0.05$).

Expression of GST genes in plants exposed to nitroaromatics has been reported elsewhere (Ekman, et al., 2003; Mezzari, et al., 2005). AtGSTU24 (At1g17170) is induced by 40 times when Arabidopsis was exposed to 0.6 mM TNT for 6 hr treatment (Mezzari, et al., 2005) and 27.5 times from the SAGE analysis with exposure of 15 mg l^{-1} TNT to Arabidopsis for 1 day (Ekman, et al., 2003). The GST activity of a hairy root culture of horseradish is induced at 0.1 mM TNT for 27 hr exposure by using an enzymatic assay (Nepovim, et al., 2004). In our study, the gene did not affect the uptake rate and the tolerance of Arabidopsis to the explosives, 2,6-DNT and TNT even though the GST gene (At1g17170) was overexpressed when the wild-type was exposed to 2,6-DNT. Genes that are induced in response to xenobiotics from the quantitative gene expression study, such as SAGE analysis and real-time PCR does not necessarily warrant the involvement of these genes in the detoxification pathway or in their involvement in tolerance to the xenobiotic by plants.

4. Conclusions

This study provides the basic knowledge on the phytotoxicity and fate of 2,6-dintrotoluene (2,6-DNT) after uptake by Arabidopsis as well as the effect of a GST gene on the detoxification of nitroaromatic explosives for the application of phytoremediation of 2,6-DNT.

The uptake and the transformation of 2,6-DNT by the plants prove that phytoremediation is a feasible treatment for groundwater or soil contaminated with 2,6-DNT. Also, the application of phytoremediation of 2,6-DNT can be based on studies of phytoremediation of TNT due to the similarity of 2,6-DNT and TNT in the fate and transformation pathways. In the mutant study, induction of genes does not guarantee the involvement of the genes in the detoxification of xenobiotics by plants.

Further study on the identification of the unknown metabolites and their toxicity is required for the assessment of potential hazards.

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Figure Captions

Figure 1. The toxicity of 2,6-DNT to Arabidopsis by the biomass assay (a) and the root growth assay (b). The EC_{50} for 2,6-DNT was 29.0 mg l^{-1} from the biomass assay and the concentration at which the root growth was inhibited by 50% of that of the control was 15.7 mg l^{-1}.

Figure 2. Percentage of the initial concentrations in the media amended with 2,6-DNT. Twenty-five 15-day-old Arabidopsis seedlings were exposed to various initial concentrations of 2,6-DNT for 7 days. The initial concentrations of 2,6-DNT were 20 mg l^{-1} for the controls and Live plant 1, 40 mg l^{-1} for Live plant 2, and 80 mg l^{-1} for Live plant 3. Each group was prepared in triplicate. Error bars represent standard deviations.

Figure 3. Distribution of ^{14}C in plant tissues harvested at different exposure times. About half of the intracellular radioactivity was located in the root tissues. Error bars represent standard deviations and n represents the number of replicates.

Figure 4. Radiochromatograms (top) of plant extracts at different harvest times after exposure to 2,6-DNT and UV-chromatograms (bottom) from the media after 2 days of exposure to 2,6-DNT (solid line) and 2A6NT (dotted line). The retention times for 2A6NT and 2,6-DNT were 7.86 min and 17.28 min, respectively. Several unknown polar metabolites form the plant extracts were detected from the radiochromatograms. 26DNT-2.92 and 26DNT-3.38 and 2A6NT were detected in the media as transformation products from 2,6-DNT. In 2A6NT feeding study, the peak labeled as 2A6NT-3.38 was observed at 3.38 min in the media suggesting 26DNT-3.38 may be transformed by way of 2A6NT, but the peak corresponding to 26DNT-2.92 was not detected, indicating 26DNT-2.92, was converted from 2,6-DNT, not via 2A6NT.

Figure 5. Expression of the GST gene in the roots of the wild-type and the *gst* mutant from the real-time PCR analysis. The plants were exposed to 50 mg l^{-1} of 2,6-DNT for 1 day. The GST gene in the root of the wild-type was induced by 4.7-times in response to 2,6-DNT while the GST gene was barely expressed in both the treated and untreated (control) *gst* mutants. The samples were prepared in triplicate. Error bars represent standard deviations.

Figure 6. Comparison of the tolerance to nitroaromatic explosives between the wild-type and the *gst* mutant by the root growth assay. The difference of the root elongation between the wild-type and the *gst* mutant exposed to different concentrations of 2,6-DNT and TNT was statistically insignificant from t-test (two-tail, $p>0.05$). Error bar represent standard deviations (n=27).

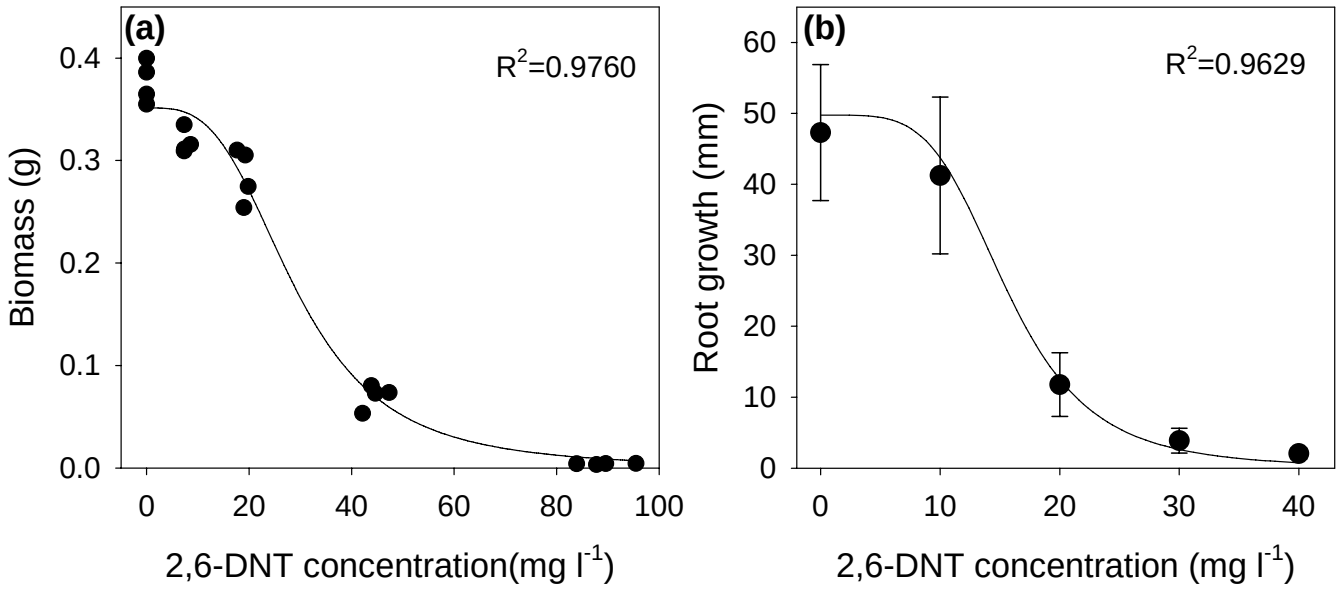

(a)
R2=0.9760
Biomass (g)
0.0
0.1
0.2
0.3
0.4
0
20
40
60
80
100
2,6-DNT concentration(mg l-1)
(b)
R2=0.9629
Root growth (mm)
0
10
20
30
40
50
60
0
10
20
30
40
2,6-DNT concentration (mg l-1)

Figure 1.

Figure 2.

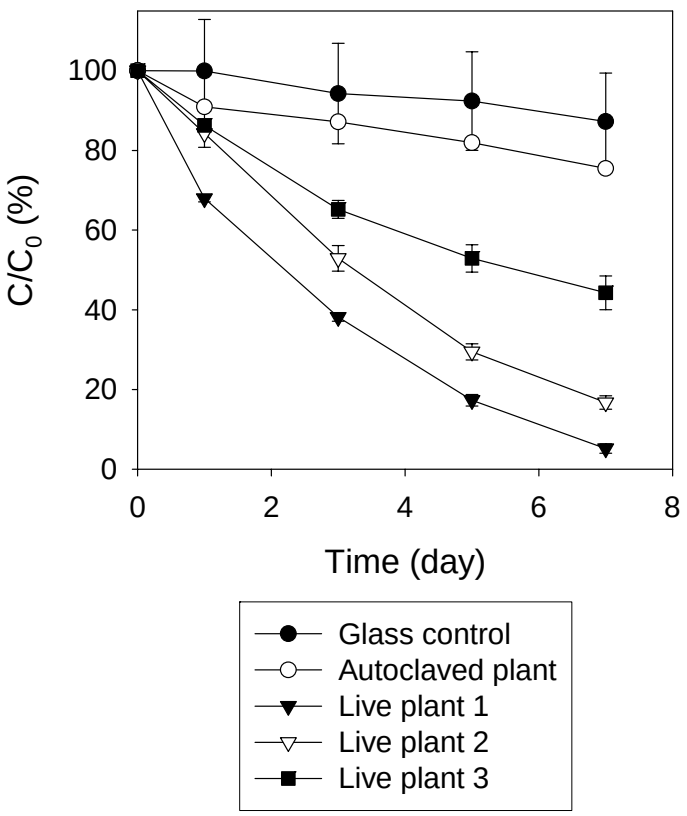

100
80
60
40
20
0
C/C0 (%)
0
2
4
6
8
Time (day)
Glass control
Autoclaved plant
Live plant 1
Live plant 2
Live plant 3

Figure 3.

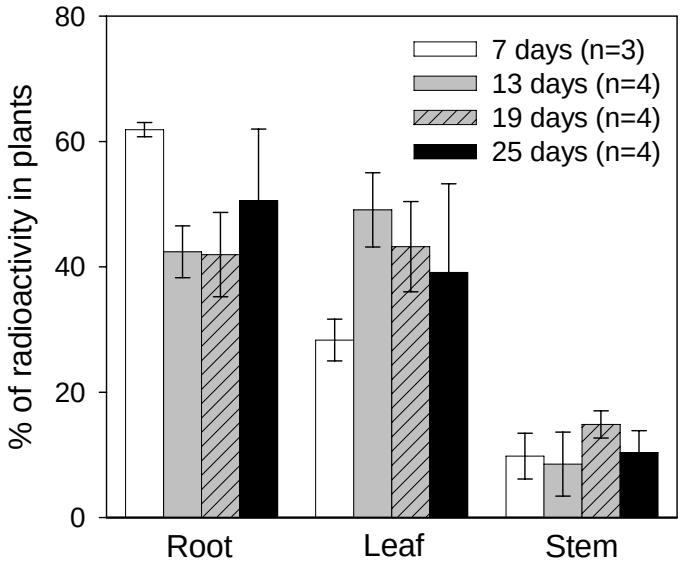

80
60
40
20
0
% of radioactivity in plants
7 days (n=3)
13 days (n=4)
19 days (n=4)
25 days (n=4)
Root
Leaf
Stem

Figure 4.

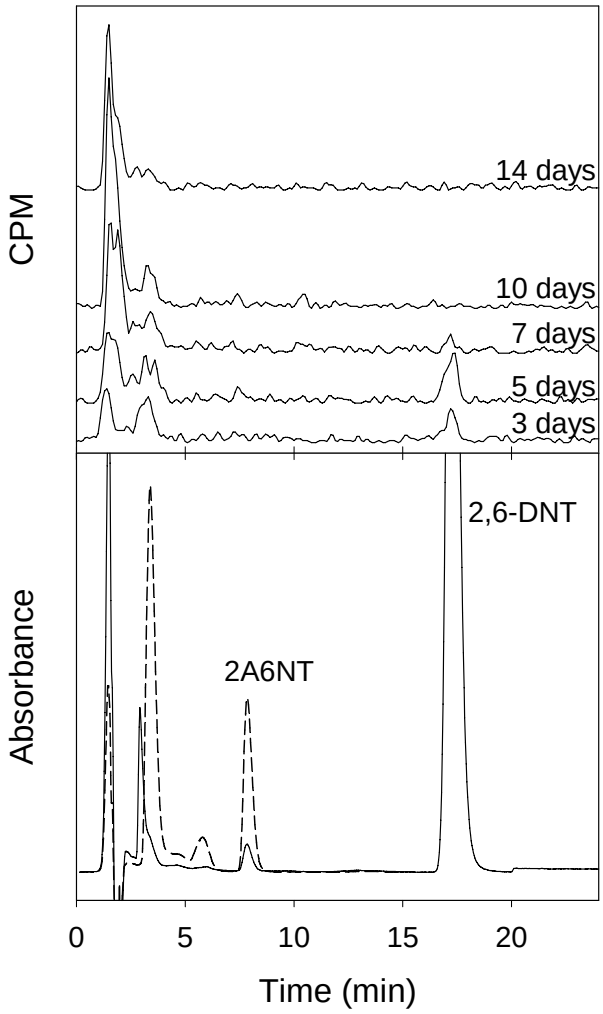
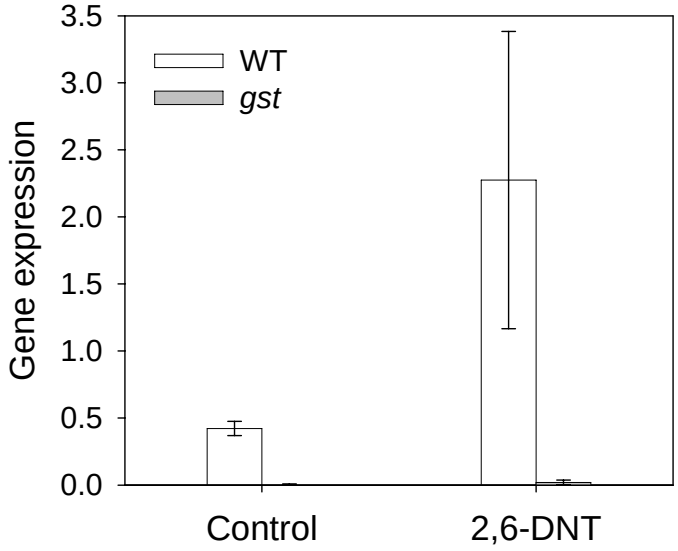

CPM
14 days
10 days
7 days
5 days
3 days
Absorbance
2,6-DNT
2A6NT
0
5
10
15
20
Time (min)

Figure 5.



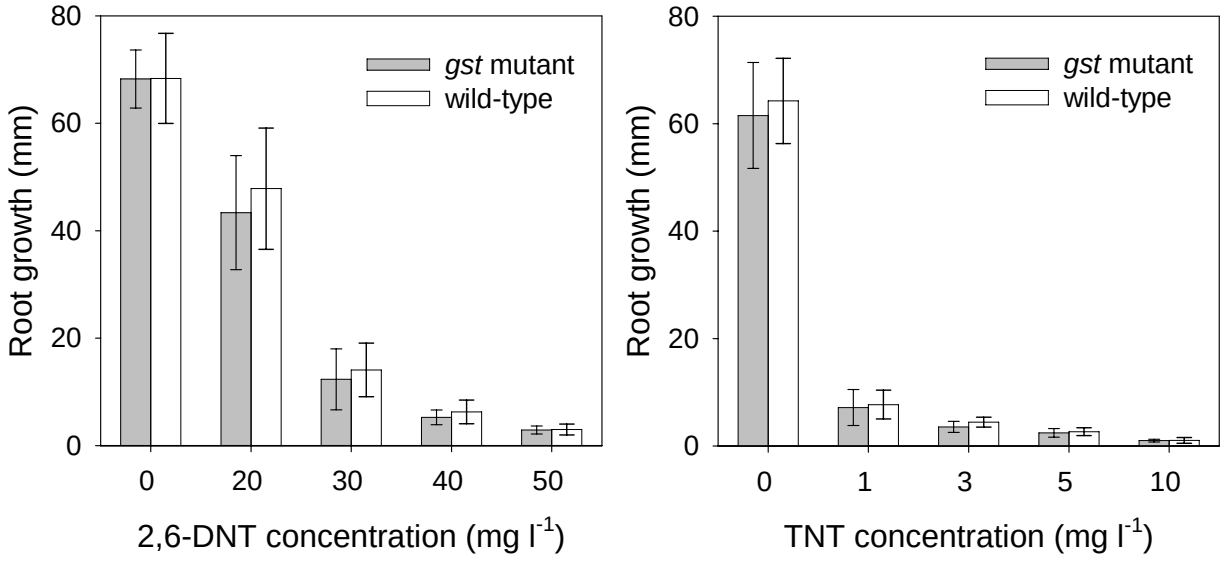

80
60
40
20
0
Root growth (mm)
gst mutant
wild-type
0
20
30
40
50
2,6-DNT concentration (mg l-1)
80
60
40
20
0
Root growth (mm)
gst mutant
wild-type
0
1
3
5
10
TNT concentration (mg l-1)

Figure 6.

Table 1. Mass balance at different harvest times based on the initial radioactivity of [U-^{14}C]2,6-DNT from biooxidation and LSC analysis.

	Plant tissues (%)		Liquid media (%)	Recovery (%)
Glass control (14days, n[a]=3)	-		83.6 ± 1.7	83.6 ± 1.6
Autoclaved plants (14days, n=3)	4.47 ± 0.44		79.6 ± 1.3	85.7 ± 0.8
3 days (n=3)	28.9 ± 3.6	(59.5 ± 1.8)[b]	60.2 ± 5.7	89.1 ± 2.3
5 days (n=3)	53.4 ± 2.1	(42.7 ± 0.5)	35.8 ± 1.3	89.3 ± 1.5
7 days (n=2)	72.9 ± 3.8	(44.2 ± 1.8)	15.8 ± 2.0	88.7 ± 1.8
10 days (n=4)	82.3 ± 0.9	(36.9 ± 2.0)	6.65 ± 1.28	88.9 ± 0.6
14 days (n=4)	75.1 ± 10.2	(30.8 ± 5.4)	9.75 ± 2.56	84.8 ± 8.5

a: n represents the number of flasks sacrificed
b: the parentheses are extractable portions of the intracellular radioactivity.

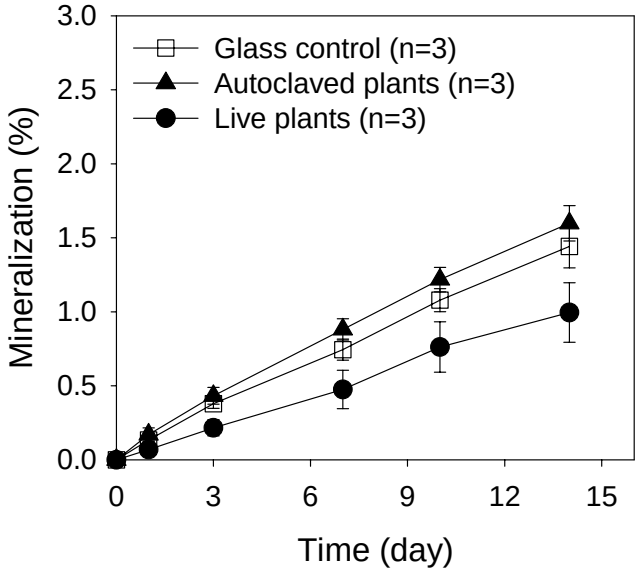
Table 2. Summary of proton NMR chemical shifts of metabolites and standards.

	Methyl group	Aromatic protons		
2,6-dinitrotoluene[a]	2.51(s)	8.09 (d, 2H, *J*=8.0)	7.63 (t, *J*=8.0)	
26DNT-2.92 Metabolite 1 of 2,6-DNT[a]	-	8.08 (d, 2H, *J*=8.0)	7.70 (t, *J*=8,0)	
26DNT-3.38 Metabolite 2 of 2,6-DNT[a]	2.27(s)	7.11 (d, 2H, *J*=8.0)	7.21 (t, *J*=8.0)	
2A6NT-3.38 Metabolite of 2A6NT[a]	2.24(s)	7.11 (d, 2H, *J*=8.0)	7.22 (t, *J*=8,0)	
2-hydroxylamino-6-nitrotoluene[b]	2.25(s)	7.56 (d, *J*=7.5)	7.45 (d, *J*=7.5)	7.34 (d, *J*=7.5)
2-amino-6-nitrotoluene[b]	2.24(s)	7.18 (dd, *J*=1.0, 7.5)	7.10 (d, *J*=7.5)	6.87 (dd, *J*=1.0, 7.5)
2,6-diaminotoluene[b]	1.96(s)	6.21 (dd, 2H, *J*=2.5, 7.5)	6.83 (d, *J*=7.5)	

[a] Chemical shifts relative to CD_3OD (^1H: δ, 3.31 and 4.87) and coupling constants (*J*) are given in ppm and hertz (Hz), respectively. [b] The data were taken from Hughes et al. (1999). Abbreviation-s: singlet, d: doublet, dd: double doublet, t: triplet.

Supplementary Information

Figure S1.



Cumulative mineralization of [U-^{14}C]2,6-DNT. The mineralization of dinitrotoluenes by plants was insignificant compared to the controls (less than 2 % of the initial radioactivity). Error bars represent standard deviations.

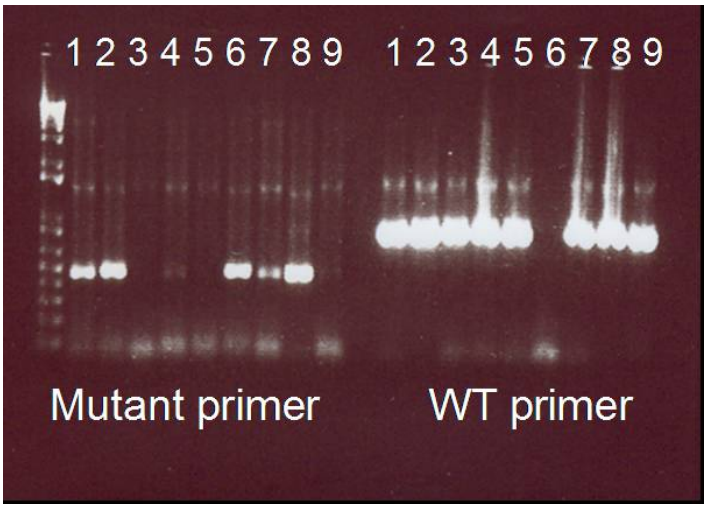


Figure S2. Gel electrophoresis after PCR using two-set primers for isolation of homozygous mutants. The 6th lane has a band using the primer set for the T-DNT insertion, but no band is observed in the 6th lane using the wild-type primer set. This indicates the plant is a homozygous *gst* mutant.

Table S1. Percentage of the initial radioactivity (^{14}C-2,6-DNT) at different harvest times in non-sterile hydroponic systems.

	7 days (n=3)	13 days (n=4)	19 days (n=4)	25 days (n=4)
Root	6.54 ± 0.49	5.86 ± 1.25	13.4 ± 1.97	19.4 ± 4.22
Leaf	3.00 ± 0.54	6.81 ± 1.49	14.1 ± 4.29	15.4 ± 7.04
Stem	1.02 ± 0.34	1.10 ± 0.50	4.82 ± 1.16	3.92 ± 1.09
Liquid medium	52.3 ± 5.00	45.7 ± 4.19	27.3 ± 4.11	17.0 ± 3.69
Methanol rinse	7.47 ± 0.71	6.86 ± 3.90	3.87 ± 0.76	3.04 ± 0.97
Recovery	70.4 ± 4.98	66.3 ± 7.12	63.5 ± 3.90	58.8 ± 3.97

Table S2. Summary of pseudo-first order constants and correlation coefficient for the gst mutant and the wild-type exposed to various concentrations of 2,6-DNT and TNT for 7 days.

		gst mutant		wild-type	
		$-k$ (day^{-1})	R^2	$-k$ (day^{-1})	R^2
2,6-DNT	50 mg/L	0.2827	0.9980	0.3340	0.9958
	75 mg/L	0.2794	0.9925	0.2625	0.9992
	100 mg/L	0.2516	0.9945	0.2565	0.9833
TNT	25 mg/L	9.686	0.9984	9.002	0.9994
	50 mg/L	9.026	0.9748	9.274	0.9889
	100 mg/L	5.448	0.9840	6.490	0.9438

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Enhanced transformation of TNT by tobacco plants expressing a bacterial nitroreductase

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Abstract

The manufacture, disposal and detonation of explosives have resulted in the pollution of large tracts of land and groundwater. Historically, 2,4,6-trinitrotoluene (TNT) is the most widely used military explosive and is toxic to biological systems and recalcitrant to degradation. To test the feasibility of altering the ability of plants to detoxify the explosive TNT, we created transgenic tobacco constitutively expressing a bacterial (*Enterobacter cloacae)* nitroreductase. The product of TNT reduction of this enzyme was found to be 4-hydroxylamino-2,6-dinitrotoluene (4-HADNT). Characterization of these plants in sterile, aqueous conditions amended with TNT demonstrated that the transgenic tobacco plants were able to remove all of the TNT from medium at an initial concentration of 0.5mM (113 mg L^{-1}) TNT. In contrast, growth was suppressed in wild type plants at 0.1mM (23 mg L^{-1}). Following uptake, transgenic seedlings transformed TNT predominantly to 4-HADNT and its high levels appeared to correlate with enhanced tolerance and transformation of TNT. The wild type seedlings produced both isomers of the further reduced product of TNT, the amino-dinitrotoluenes (2-ADNT and 4-ADNT). Transformation products of TNT in wild type and transgenic tobacco were subsequently conjugated to plant macromolecules. The observation of this process indicates that the phytodetoxification of TNT follows the "Green liver model of xenobiotic metabolism" established for other compounds such as herbicides.

Introduction

Phytoremediation - the use of green plants to remediate environmental contamination- is currently being investigated as a treatment method for soil and groundwater polluted by explosives. The high-explosive 2,4,6-trinitrotoluene (TNT) is a primary concern for remediation due to its toxicity to humans (it is designated a class C carcinogen), and extent of environmental contamination. In the U.S. alone there is an estimated 0.82 million cubic metres of soil contaminated with TNT and its degradation products at former manufacturing and testing sites (USATHAMA, 1989). TNT enters the environment through the manufacture, packaging, military use and decommissioning of outdated explosives.

The process by which plants remove TNT from their environment, although observed in many species, is hampered by the acute phytotoxicity of TNT (Pavlostathis et al. 1998; Thompson et al. 1998a). In contrast, whilst bacterial enzymes have been shown to have activity against TNT, bacteria do not generate a high enough biomass to remove significant amounts of TNT and its transformation products from heavily contaminated sites (Rosser et al. 2001). We previously demonstrated how the expression of bacterial enzymes in plants could improve their ability to detoxify explosives (French et al. 1999, Hannink et al. 2001). In particular, expression of nitroreductase (NR) from *Enterobacter cloacae* in *Nicotiana tabacum* (tobacco) was found to enhance tolerance to TNT phytotoxicity at germination and during vegetative growth when compared to wild type plants.

The most common TNT transformation products, of plant origin, are the monoamino-dinitrotoluenes (ADNTs), which have been observed in the tissue of aquatic and terrestrial plants, monocotyledons and dicotyledonous plants (Best et al. 1997;

Schneider et al. 1996; Scheidemann et al. 1998) (Figure 1). The hydroxylamino dinitrotoluenes (HADNT) are rarely observed (Pavlostathis et al. 1998, Burken et al. 2000) and oxidation products of TNT have only been observed in one study (Bhadra et al. 1999a). TNT transformation products are also observed in soil and sterile growth media associated with plants (Scheidemann et al. 1998, Hughes et al. 1997). Following transformation of a toxic compound by endogenous enzymes, plants have been found to conjugate these transformation products to plant macromolecules such as sugars and glutathione (Coleman et al. 1997; Sandermann et al. 1992). This makes the toxic compound more soluble such that it can be mobilised and stored away from sensitive parts of the cell in regions such as the vacuole or bound to cell wall constituents (Coleman et al. 1997). These processes are the final stages of the "Green liver model of xenobiotic transformation" in plants. Conjugation of TNT transformation products to plant macromolecules has been observed with root cultures of *Catharanthus roseus* in previous work, as shown in Figure 2 (Bhadra et al., 1999b; Wayment et al. 1999). The nature of TNT-conjugation products was investigated using axenic root cultures of *Catharanthus roseus* (Bhadra et al. 1999b). Four TNT-conjugates were observed- two with UV spectra similar to 2-ADNT and another two with spectra similar to 4-ADNT. The first were designated TNT-1 and 2A-1 that were spectroscopically similar to 2-ADNT. Two other metabolites designated TNT-2 and 4A-1 were found to be spectroscopically similar to 4-ADNT. Chemical or enzymatic hydrolysis was observed to return the conjugate to the monoamino-dinitrotoluene. The mass spectral evidence indicated that at least a 6-carbon molecule of plant origin was involved in conjugate formation to the amine group of the respective monoaminodinitrotoluene.

It was established by Hannink et al. (2001) that expression of the *Enterobacter cloacae* (*E.cloacae)* nitroreductase gene (*nfsI*) enhanced tolerance to TNT in the transgenic seedlings (designated NR 3-2) at germination and as plantlets. NR from *E.cloacae* strain 96-3 (ATCC43560) was found to have type I, also known as oxygen-insensitive nitroreductase activity which involves a two-electron reduction of TNT (Bryant and DeLuca, 1991) (Figure 1); however, the reduction products of TNT have not previously been established. Given that transformation of TNT by native plants has been frequently observed, the studies presented herein were to establish what benefit a bacterial NR may provide in enhancing tolerance to TNT phytotoxicity.

Results

Transformation of TNT by *Enterobacter cloacae* Nitroreductase

In order to ascertain which of the TNT transformation products from the transgenic plant line were the products of *E. cloacae* nitroreductase activity, NR was purified to homogeneity and the products of TNT reduction by NR were studied using an HPLC-based, time-course assay. During the assay TNT was rapidly consumed with subsequent formation of a product with an identical retention time to that of an authentic standard of 4-hydroxylamino-2,6-dinitrotoluene (4-HADNT) (Figure 3). There was no observed production of the nitroso compound. A second, minor product was formed, which was observed to slightly increase in peak size as HADNT levels decreased over the course of the assay. This peak had a retention time and UV/vis spectra consistent with descriptions of 2,4-dihydroxylamino-6-dinitrotoluene (2,4-DHANT (Hughes et al. 1998). Characteristics of 2,4-DHANT include a maximum

absorbance peak at 350nm and a minor absorbance peak at 240nm. Initially, reduction of TNT and formation of 4-HADNT followed a 1:1 stoichiometric conversion. Small amounts of the 4-HADNT products then appeared to be reduced further to 2,4-DHANT which correlated with a reduction in 4-HADNT concentration. As there was no authentic standard available for the 2,4-DHANT quantification of this compound was not possible.

Phytotoxic Effects of TNT During Vegetative Growth

First reference to Table I

Wild type tobacco and homozygous transgenic tobacco NR 3-2 seeds (50 per flask) were surface-sterilised, germinated and grown for 18 days in flasks of non-TNT amended media. At this time flasks were normalized for weight and plants were incubated in the presence of TNT for a further five days. Without TNT amendment the wild type plants were found to have a very similar growth index of 1.87 compared with the NR 3-2 line that had an index of 1.82 (Table I). While wild type plants increased in weight on average at 0.1mM TNT (1.24 growth index), the transgenic line was observed to gain thrice as much biomass (1.75). At a concentration of 0.25 mM TNT, wild type plants lost weight slightly (0.97 growth index) and were seen to exhibit severe chlorosis and were necrotic at the end of the five-day study (Figure 4). In contrast, the transgenic line, NR 3-2 had gained a significant amount of weight compared to the wild type plants (P=0.05) (1.72 growth index) and did not appear chlorotic after five days in 0.25mM TNT-amended media (Figure 4). Wild type plants lost considerable weight (0.46 and 0.47 growth index respectively) at 0.38 and at 0.5mM. In contrast, the transgenic line, NR 3-2 gained weight at both 0.38mM and 0.5mM TNT concentrations (index of 1.39 and 1.53 respectively). (Table I).

Wild type plants exhibited severe chlorosis and eventually necrosis at 0.5mM (113mg L^{-1}) TNT (the aqueous solubility limit of TNT is 132mg L^{-1} (0.58mM) at 25^0C (Lynch et al., 2001)) whereas NR 3-2 plants were not chlorotic but did appear to have changed colour to a darker green. At the extremely high TNT concentration of 0.75mM (170mg L^{-1}) TNT both wild type and transgenic plants appeared to be chlorotic and necrotic (Figure 4). At these high TNT concentrations (0.5 and 0.75mM TNT), precipitation of TNT was observed. Control plants grown without TNT in the medium exhibited no visible signs of toxicity such as chlorosis, an indication that the toxic effects were due to the presence of TNT and not submersion in the growth medium.

Transformation of TNT by Tobacco in Axenic culture

Wild type plants incubated with 0.1mM (23mg L^{-1}) TNT removed all of the explosive by 120 hours, just before the end of the study (Figure 5), compared to the transgenic line, NR 3-2, which had removed all TNT from the medium by 25 hours. At 0.25mM (56 mg L^{-1}) TNT, wild type plants removed approximately 48% of the initial TNT added by the end of the five-day study, at which time the seedlings were necrotic. In contrast, NR 3-2 seedlings removed 50% of the TNT within 10 hours and all of the TNT was removed within 60 hours. Control flasks of TNT without plant biomass showed no significant loss of TNT (data not shown). However, at the higher TNT concentrations of 0.38mM (86mg L^{-1}) and 0.5mM (113mg L^{-1}) there was a drop in TNT concentration at the start of the experiment (Figure 5 C and D). Although suffering severe effects of toxicity, wild type plants removed 31% and 25 % of TNT from media initially containing 0.38mM (86mg L^{-1}) and 0.5mM (113mg L^{-1}) respectively. At 0.38mM TNT, NR 3-2 seedlings removed all TNT in solution within

50 hours. At a higher biomass, and highest TNT concentration, NR 3-2 plants at 0.5mM TNT displayed even faster removal of TNT with all of the explosive taken up from the media within 50 hours.

Products of TNT Transformation Identified from Seedling Growth Medium

The distinguishing feature of the metabolite profile in NR 3-2 was the high levels of 4-hydroxylamine-2,6-dinitrotoluene (4-HADNT) formed, and an almost complete absence of 2-substituted metabolites. The transgenic plant line transformed 28, 17, 11 and 14% of the initial TNT to 4-HADNT at 0.1, 0.25, 0.38 and 0.5mM TNT, respectively. Production of 4-HADNT was followed by its rapid decline and a subsequent increase in levels of 4-ADNT and 4-substituted conjugates (Figure 6, and Table II). 4-HADNT had completely disappeared from the transgenic system within 12 hours, while 4-ADNT was present until the end of the experiment at less than 5% of the initial TNT added. The NR 3-2 line only produced the 2-ADNT and 2-HADNT isomers at the two highest concentrations of TNT studied (0.38 mM; 86mg L^{-1} and 0.5mM; 133mg L^{-1} TNT). However, at all concentrations, the 4-isomer was the most abundant of the monoamino-dinitrotoluenes produced by NR 3-2 plant line.

First reference to Table II

In contrast, the wild type seedlings did not produce either of the hydroxylamine isomers and produced 2-ADNT and 4-ADNT in almost equal amounts at both initial TNT concentrations. In the wild type growth medium a maximum of 3.4, 1, 1.8 and 2% of the initial TNT added was found to be transformed to 2ADNT at 0.1, 0.25, 0.38 and 0.5mM TNT respectively (Figure 6). Similarly, a maximum of 3.9, 2, 1.2 and 0.5% of the initial TNT added was converted to 4-ADNT. Overall, there was a much higher concentration of transformation products produced by the transgenic line compared to wild type.

Products of TNT Transformation Isolated from Plant Tissue

Plant tissue was extracted with methanol to determine whether TNT and its transformation products were present in the seedling biomass as well as in the growth medium. Extraction of TNT and transformation products from whole plant tissue was performed at 10 and 120 hours. The trends of metabolite production observed in the growth medium (Figure 6) also appear to be occurring in extractions from plant tissue itself (Figure 7 and 8). In wild-type seedlings, the 2 and 4 isomers of the ADNTs were the only products observed in the plant tissue extracts, with the 4 isomer at slightly higher concentrations. Concentrations of ADNTs were highest at 120 hours (compared to 10 hours) at 0.1mM TNT. Levels of 2-ADNT reached maximum concentration of 264 $\mu g\ g^{-1}$ dried tissue (0.25mM TNT) and 4-ADNTs at 538 $\mu g\ g^{-1}$ dried tissue (0.25mM) in these samples. In the tissue of wild-type seedlings, TNT was found to the compound of greatest abundance at 0.25, 0.38 and 0.5mM TNT with only trace amounts of the transformation products. At 0.5mM TNT (120 hours), a maximum of 7.5mg TNT g^{-1} dry weight plant tissue was extracted.

In contrast, no TNT was found in any tissue sample from the transgenic line, indicating that TNT was fully transformed or unavailable for extraction (Figure 7 and 8). As in samples from the growth medium, the significant products were 4-ADNTs and 4-HADNTs at all concentrations of TNT studied. The most abundant product at 10 hours in NR 3-2 tissue was 4-HADNT at a concentration of 378 $\mu g\ g^{-1}$ dried tissue (0.1mM). The levels of 4-HADNT were as high as 894 mg g^{-1} (0.5mM). The levels of 4-HADNT in NR-32 were also higher than that of any product isolated from wild-

type tissue. However, 4-HADNT was not present in tissue extractions at 120 hours, but was replaced by high levels of 4-ADNTs The levels of 4-HADNT in NR 3-2 were at all TNT concentrations, again at higher concentrations than any wild-type TNT product at 120 hours. The maximum concentration of 4-ADNTs was 1411 $\mu g\ g^{-1}$ at 10 hours at 120 hours.

Identification of conjugates between TNT and plant macromolecules.

In order to determine whether the expression of bacterial NR in tobacco also had an effect on the subsequent conjugation of TNT transformation products, the conjugates were also investigated in plant growth medium and from extractions of plant tissue (at the end of the five day study). As illustrated in Table II, wild type tobacco seedlings were observed to produce all four previously characterised conjugates of TNT in the 0.1 and 0.25mM TNT amended-medium (Bhadra et al. 1999b; Wayment et al. 1999). These conjugates were found to be composed of the 2-isomer reduction products of TNT and plant macromolecules(s) (designated 2A-1 and TNT-1) and the 4-isomer reduction products of TNT (designated 4A-1 and TNT-2) (Figure 2). These previously isolated conjugates were used here as standards to identify compounds of TNT transformation from tobacco. However they were not adequate for reliable quantification between the different conjugates, but relative levels of a given conjugate are valid. TNT-1 and TNT-2 were isolated from the wild type plant tissue extractions at the end of the study (five days) at 0.1, 0.25 and 0.38mM TNT (Table II). The media in which wild type plants were grown was also found to contain 4A-1, TNT-1, TNT-2 at all concentrations of TNT and 2A-1 was isolated from media initially containing 0.1 and 0.25mM TNT.

In contrast the NR 3-2 transgenic line only produced conjugates at the 4 position in 0.1, 0.25, 0.38 and 0.5mM TNT amended media The conjugates TNT-2 and 4A-1 were isolated from growth media and the conjugate TNT-2 was the only conjugate observed in the tissue extractions from transgenic seedlings (Table II).

Discussion

We have demonstrated that the transgenic plant line expressing the *E.cloacae* NCIMB10101 NR gene (*nsf*I) possessed a dramatic ability to tolerate concentrations of TNT that are highly toxic to wild type tobacco plants. To gain further insight into the mechanism of detoxification, NR was purified to homogeneity and the reduction products of TNT were investigated. A time-course HPLC-based assay revealed that the major product of TNT reduction was the 4-isomer of hydroxylamino-dinitrotoluene. *E.cloacae* (strain 96-3) NR has also been shown to reduce nitrobenzene to hydroxylamino benzene by Koder and Miller (1998); however, the TNT transformation products had not been previously identified. The *E.cloacae* NR is known to belong to a family of oxygen-insensitive nitroreductases including nitroreductases from *Escherichia coli* B (Anlezark et al. 1992) and *Salmonella typhimurium* (Watanabe et al. 1990). It shares over 80% amino acid sequence identity with both of these enzymes (Bryant et al. 1991; Koder and Miller, 1998). Interestingly, the TNT reduction products of these bacteria are different. *E. coli* strain B reduces TNT to the hydroxylamino derivative whilst TNT is reduced further to the amino derivative by *S typhimurium*. The physiological role of the bacterial nitroreductases has still to be established. What aspect of the nitroreductase protein

structure determines whether the final reduction product will be a hydroxyl or an amino derivative is currently not known (Spain, 1995). The hydroxylamino derivative is rarely reported in plant studies of TNT transformation and when observed, the specific isomer has not been reported (Pavlostathis et al. 1998).

Having established that the nitroreductase from *E.cloacae* produced the 4-hydroxylamino dinitrotoluenes, the effect of its over expression on TNT metabolism in transgenic plants was investigated. TNT phytotoxicity was measured according to the gain or loss of weight by the plant lines in the presence of a range of TNT concentrations. At the higher TNT concentrations of 0.38mM (86mg L^{-1}) and 0.5mM (113mg L^{-1}) there was a drop in TNT concentration at the start of the experiment (Figure 5 C and D). which may be due to adsorption of TNT to the plant biomass. At 0.5mM (113mg L^{-1}) TNT, crystals were also observed in the flasks indicating that insufficient mixing or heterogeneity may have occurred as this concentration is close to the aqueous solubility limit of TNT (132 mg L^{-1} at 25^0C).

Growth suppression and chlorosis have been observed as symptoms of TNT phytotoxicity in Eurasian watermilfoil (*Myriophyllum spicatum*) between concentrations of 5.9 μM (1.3mg L^{-1}) and 23 μM (5.22 mg L^{-1}) and higher (Pavlostathis et al., 1998). In this study, the transgenic plant line NR 3-2 was not chlorotic and gained biomass at levels of TNT that were toxic to wild type (0.25mM TNT). In contrast to wild type, the NR 3-2 plants were also found to remove all TNT from 0.5mM TNT (a concentration close to the aqueous solubility limit of TNT at 0.58mM).

To our knowledge, this level of TNT tolerance has not been previously reported of any plant species. The observed growth suppression in this study may correlate with results of decreased respiration, as observed in other work on mature poplar plantlets (Thompson et al. 1998b). The cytotoxic effects of TNT on plant cells have not been established, but such studies have been undertaken for other aromatics and may provide some insight into the effects of TNT. Nitrobenzene has been observed to completely destroy the ultrastructure of the lower and upper parts of the leaf. The first signs of destruction were noticeable in the nuclei. The nuclear membrane configuration was significantly changed and eventually the nucleus became invaginated. Increases in concentrations of benzo[a]pyrene and benzo[a]anthracene resulted in chromatin coagulation indicating disruption in DNA synthesis. Mitochondria were observed to lose their contents and complete destruction of the cell was observed (Korte et al. 2000).

As with most toxicants, the cytotoxic effects of TNT may be dependent on the amount the plant is exposed to. The wild type seedlings were able to gain weight at 0.1mM (23 mg/L) TNT but when this concentration was more than doubled to 0.25mM (56 mg/L) TNT the wild type seedlings were necrotic by the end of the study (five days). In the light of the different phytotoxic effects at germination (primarily poor root development or complete failure to germinate; Hannink et al. 2001) and as mature plantlets, the mode of TNT toxicity may depend on the developmental stage of the plant.

The two striking features of TNT transformation by the NR 3-2 transgenic tobacco were the high levels of 4-HADNT observed, and the near complete absence of 2-

HADNTand related metabolites (2-ADNT, 2A-1, TNT-1). The high levels of 4-HADNT have not been observed in any other study, and appear to be characteristic of NR 3-2. In contrast, wild type tobacco plants did not show any atypical behaviour. No hydroxylamines were isolated, and low levels of all monoamines and conjugates were observed. The NR 3-2 transgenic line also showed highly enhanced TNT transformation characteristics, evidenced by the near complete TNT removal within 20 and 50 hours for 0.1 mM and 0.25 mM initial TNT added, respectively. The wild type tobacco, in contrast, took 120 hours to remove the TNT from 0.1 mM system and could not transform a significant amount of TNT in the 0.25, 0.38 or 0.5 mM systems.

Interestingly, there was no direct observation of HADNT production in wild-type tobacco. Hydroxylamines have rarely been detected in TNT transformation studies, and have never been observed in non-aquatic plant species (Pavlostathis et al. 1998, Wang et al. 2003). A recent study by Wang et al. (2003) observed the transformation of TNT to the hydroxylamines (both 2-HADNT and 4-HADNT) in the aquatic species, *Myriophyllum aquaticum.* However, the plants were exposed to a relatively low concentration of 25mg L^{-1} TNT (0.11mM) TNT and an initial high peak of HADNTs was not observed. The ratio of 2-HADNT to 4-HADNT production was not reported. In previous work with *Myriophyllum spicatum* (Eurasian water milfoil) 4-HADNT was observed at low concentrations in the first 60 hours after TNT amendment (Pavlostathis et al., 1998). A ferrodoxin $NADP^+$-dependent reductase was isolated from spinach leaves that was found to transform TNT to 4-hydroxylamino-2,4-dinitrotoluene (Goheen et al. 1999). However, in spite of the very rare detection of hydroxylamines it has been speculated that they are formed during TNT transformation even in terrestrial species (Burken et al. 2000). Monoamines are seen in almost all TNT phyto-transformation studies to date and are presumably formed from the further reduction of hydroxylamines. In addition, the conjugates TNT-1 and TNT-2 are formed by direct conjugation of hydroxylamines to a six-carbon plant macromolecule (Bhadra et al. 1999b). The non-observance of hydroxylamines are probably because of their unstable nature in aquatic, aerobic media, and their high turnover rate (Wang et al. 2000). As the analytical methods used here did detect production of 4-HADNT in the transgenic seedlings, it would be expected that HADNTs would also be detectable in wild type plants if produced at a high enough level.

The wild type plants appeared to produce both 2- and 4- isomers of the monoamino-dinitrotoluene (ADNT) in approximately equal amounts in the growth medium. Both 2- and 4-ADNT have been observed in various plant species in numerous studies (Harvey et al. 1990; Gorge et al. 1994; Scheidemann et al. 1998). These trends were also reflected in results of the plant tissue extractions. Wild type plants again produced both 2-ADNT and 4-ADNT transformation products of TNT and as in the growth medium samples, no HADNT products were observed. The ADNT transformation products were found in plant tissue at higher amounts in 120 hour samples (compared to the 10 hour samples) only at 0.1mM (23mg L^{-1}). This may be due to the observation that at concentrations higher than this, wild type plantlets were necrotic by 120 hours and presumably unable to transform TNT to any degree. At 0.25, 0.38 and 0.5mM TNT, wild type plant tissue was also found to contain TNT in much greater quantities than any of the reduced transformation products. This indicated that although the wild type tobacco appeared to be suffering effects of TNT toxicity such as chlorosis and growth suppression, TNT was still being taken up into

the biomass at early stages of the study but the plants were unable to transform it to a degree in order to reduce phytotoxicity. This has previously been observed in other studies (Pavlostathis et al. 1998) and suggests that TNT uptake is indiscriminate. The reduction of TNT to monoamino-dinitrotoluenes by wild type tobacco correlates with observations of these transformation products in many other plant species (Burken et al. 2000; Hannink et al. 2002) and indicated that wild type tobacco does possess endogenous reductive activity against TNT. The presence of reducing enzymes in plants have been found using antibodies to detect native nitroreductases in plants such as poplars, grasses and aquatic plants (Watanabe, 1997).

Relatively large amounts of TNT and low levels of transformation products were found in wild type plant tissue, which raises the question of whether it is TNT itself or its transformation products that are eliciting the phytotoxic effects. Numerous studies have produced a range of results on TNT toxicity to mammalian and bacterial systems. It has been suggested that toxicity of TNT was due to its reduced transformation products (Whong and Edwards, 1984). However, later work has found that 2-ADNT and 4-ADNT are mutagenic, but the TNT induced a greater degree of mutagenicity. *Salmonella typhimurium* mutagenicity studies indicate that TNT also induced frameshift mutations (Whong and Edwards, 1984). Studies also with *Salmonella* found that 2-ADNT and 4-ADNT were less mutagenic than TNT itself in strains TA98 and TA100 (George et al. 2001). Other TNT reduction products were also investigated with this system revealing that TNT and monohydroxylamino derivatives were all found to be mutagenic. However, the HADNT transformation products were less mutagenic than TNT itself. The hydroxylamino group at C-2 (2-HADNT) was found to be more mutagenic than at the C-4 position (4-HADNT) (Padda et al. 2000).

One major difference between the bacterial and mammalian detoxification systems is that plants have the ability to conjugate transformed toxic compounds to a plant macromolecule and store them away from sensitive cellular constituents (Coleman et al. 1997). This has been observed in studies of TNT detoxification with *Catharanthus roseus* hairy root cultures (Bhadra et al., 1999b; Wayment et al., 1999). The 4-HADNT produced here by NR 3-2 had a very short life and was quickly transformed to 4-ADNT and TNT-2. The 4-HADNT was rapidly removed, presumably by endogenous plant enzymes and possibly also due to its reactivity. Hence, not only does the transgenic plant line remove TNT faster from the growth medium, the 4-HADNT produced is also turned over very rapidly. This result is manifested in the formation of the conjugate, TNT-2, which is formed from the direct conjugation of 4-HADNT. The observation of the TNT-2 conjugate appears to be directly correlated to the reduced phytotoxicity observed in NR 3-2. At 120 hours TNT-2 was still present intracellularly at all concentrations of TNT and would presumably undergo polymerisation and irreversible binding to the biomass, thus further reducing phytotoxicity (Figure 9). In addition, the high levels of 4-ADNT observed in transgenic tissue suggest that native tobacco enzymes may be further reducing the 4-HADNT (produced by the bacterial NR) to 4-ADNT, which is then conjugated to form 4A-1. The metabolites observed in NR 3-2 indicated the progress of the TNT transformation pathway occurs predominantly along the 4-substituted branch (4-HADNT, 4-ADNT, TNT-2), possibly because of the initial step wherein TNT was partially reduced to 4-HADNT.

It is perhaps not surprising that wild type tobacco was found to produce both 2 and 4 isomer conjugates as it produced both 2 and 4 isomers of the monoamino-dinitrotoluenes in almost equal amounts. The production of conjugates TNT-1 and TNT-2 indirectly identifies the production of 2-HADNT and 4-HADNT by wild type plants (Table II and Figure 9). It is possible that the HADNT transformation products were produced in amounts too low for direct detection as they are known to be unstable in aerobic conditions (Wang et al. 2000). The HADNTs may have also been directly conjugated to an endogenous plant molecule before they could be detected.

These conjugates do appear to be "gateways" to bound residues (Fellows, 1992) that are presumed to contribute to the difficulty in analyzing the transformation of TNT in plants. The TNT transformation products reported in this paper do not constitute a mass balance of the fate of TNT in the plant systems and even when using ^{14}C labeled TNT a full mass balance on identified transformation metabolites has not yet been reported (Burken et al. 2000). The presence of such bound transformation products has been suggested from the "Green liver model of xenobiotic transformation" (Coleman et al. 1997; Sandermann et al. 1992). The "bound" transformation products are defined as those that are non-extractable from plant tissue with the use of conventional solvents (Komoβa et al. 1995). Following transformation (presumably reduction in this case) the compound is conjugated to a plant macromolecule and stored away from cellular constituents that are susceptible to phytotoxicity. Studies with ^{14}C labeled TNT support this theory with the finding that 33% of TNT was found in a bound state in *Myriophyllum aquaticum* (Bhadra et al. 1999b). Other work with *Phaseolus vulgaris* (bush bean) has shown that 50% of labeled TNT was found in the cytoplasm, whilst the remaining TNT was found to be mostly associated with a lignin fraction in the cell wall in a "fixed" form which was suggested to be a result of covalent bonding (Sens et al. 1999).

In summary, the production of the 4-hydroxylamino-2,6-dinitrotoluene TNT metabolite was observed only in transgenic tobacco. This correlated with the production of this product in pure NR assays. The specific importance of 4-HADNT production is currently unknown, but it is likely that the high reductive activity of the bacterial nitroreductase is transforming TNT at a faster rate than endogenous tobacco enzymes, and thus enabling greater conjugation and sequestration of TNT and hence decreasing its phytotoxicity.

Materials and Methods

Chemicals and Materials

Reagents were purchased from Sigma (Poole, Dorset, UK and St. Louis, US), Aldrich Chemical Company Ltd. (Gillingham, UK), Fisher Scientific (Loughborough, UK, Chicago, US or Melford Laboratories (Ipswich, UK); TNT was either donated by the Defence Science and Technology Laboratory (Fort Halsted, UK) or purchased from Chem Service (West Chester, PA). Authentic standards of TNT and its transformation products were purchased from AccuStandards (New Haven, US).

Purification of nitroreductase

The purification method was modified from Bryant and Deluca (1991). In brief, the vector pART7 carrying the nitroreductase gene (*nfsI*) from *Enterobacter cloacae*

(*E.cloacae*) was transformed into the *Escherichia coli (E. coli)* host BLR (DE3) (Novagen) (French et al. 1998). A single colony was used to inoculate a 20mL starter culture (terrific broth media [Sigma] containing 100 μg mL^{-1} of carbenicillin) and grown at 30 °C, 250 rpm overnight. The starter culture was then used to inoculate the main culture (12 x 0.5 L volumes) which was grown at 30°C at 250 rpm until the OD_{600} had reached 1. The culture was then induced with 1mM IPTG and left to grow for a further 18 h. Cells were pelleted by centrifugation at 8,000 x *g* for 20 minutes and stored at –20 °C until required. The frozen pellet was resuspended in 100 mL of 50mM Tris pH 7.5 containing 5mM DTT. The bacteria were lysed using a French Press cell disrupter (SLM instruments) by two consecutive passes through the vessel at 12,000 psi. The lysate was clarified by centrifugation (50,000 x *g* for 30 minutes) and the supernatant containing the soluble nitroreductase was retained for further purification.

All chromatography steps were carried out at 4 °C. The clarified supernatant was applied to a DEAE sepharose column at [Amersham Pharmacia] (200 mL bed volume packed into an XK50 column) which had previously been equilibrated with 2 column volumes of start buffer (50 mM Tris pH 7.5 containing 5mM DTT). A further 3 column volumes of start buffer was used to wash the column to remove any unbound protein. A linear salt gradient (0-1M NaCl, 50 mM Tris pH 7.5 over 4 column volumes) was used to elute the protein. Fractions containing nitroreductase activity as determined using the standard assay were then pooled prior to acetone precipitation. The nitroreductase was further purified using acetone precipitation as described in Bryant and Deluca (1991). Following the acetone cuts, the pellets were dissolved in the smallest possible volume of 50 mM Tris pH 7.5, 5 mM DTT and then dialysed (5 kDa M_r cut-off dialysis tubing) against 5 L of the same buffer.

Protein from the dialysed acetone precipitation step was purified using hydrophobic interaction chromatography (HIC). To the dialysed sample, solid ammonium sulphate was added to give a final saturated salt concentration of 30% and allowed to dissolve at 4 °C with gentle stirring for 3 minutes. Precipitated protein was removed by centrifugation (25,000 x *g* for 20 minutes) before being applied to a 50 mL butyl agarose column [Amersham Pharmacia] that had previously been equilibrated with 40% saturated ammonium sulphate, 50mM potassium phosphate, 5mM DTT pH 7.0. The column was then washed with 4 column volumes of equilibration buffer before eluting the bound protein by linearly reducing the salt concentration to zero over 5 column volumes. Fractions containing nitroreductase activity were identified using the standard assay and then pooled prior to gel filtration chromatography. The nitroreductase containing fractions from the HIC column step were concentrated down to 10 mL before being applied to a Hi Prep Sephacryl S-200 HR 26/60 column (Amersham Phamacia) which had been equilibrated with 50mM Tris pH 8.0, 5mM DTT and 150 mM NaCl. The sample was loaded and eluted at a flow rate of 1mL/minute. Fractions containing the highest purity nitroreductase were identified by SDS-PAGE and pooled. Glycerol was added to the pooled enzyme to a final concentration of 20 % and stored at -20 °C until required. The standard nitroreductase enzyme assay used was as described in Bryant and Deluca (1991). Protein concentration was assayed using the method of Bradford (1976) using the Coomassie Protein Assay Reagent from Pierce (Rockford, IL, USA) according to the manufacturer's instructions. Bovine serum albumin (Pierce) was used for construction of the standard curve.

Reduction of TNT by *E.cloacae* nitroreductase

Reactions were performed in 50mM phosphate buffer at pH 7.0, at 25^0C. The final concentrations of the components of the assay were 150 μM NADH, 30 μM TNT, and 10μg purified NR. NADH was continually recycled by including 5 units/mL *T. brockii* $NADP^+$-dependent secondary alcohol dehydrogenase (Sigma) and 2.5% (v/v) isopropanol in the reaction mixture. Time-course TNT transformation measurements were performed in 2mL HPLC vials, in the thermostatically regulated autosampler chamber of the Waters Alliance HPLC system. Aliquots of 20 μL were automatically removed and analysed every 30 minutes by HPLC as described below.

TNT Toxicity to Vegetative Growth

Quadruplicate batches of 50 seeds from both wild type and NR 3-2 transgenic tobacco (*Nicotiana tabacum* cv. Xanthi) were surface-sterilized and aseptically added to flasks containing 100mL liquid GM media. Seeds were germinated and grown for 18 days. Seedlings were rinsed in UHP autoclaved water, weighed and equal mounts of biomass were aseptically transferred to sterile flasks containing 100 mL water, except 0.5mM TNT where more biomass was added for NR3-2 due to contamination problems with the WT line. The flasks were then amended with a range of TNT concentrations (solubilized in methanol). Seedlings were grown in flasks at 25^0C in constant light with rotary shaking at 100rpm. Wet weight of seedlings was then determined after a further five days of growth. The seedlings used in this study were concurrently used to study the transformation of TNT (next section).

Transformation of TNT by Plants

Plants were grown in sterile media for 18 days, as described above, at which time TNT was added to a final concentration of 0.1mM (23 mg L^{-1}), 0.25mM (56 mg L^{-1}), 0.38mM (86 mg L^{-1}) and 0.5mM (113 mg L^{-1}). Samples of the seedling growth media were taken at regular intervals of 2, 6 and 12 hours and then every 12 hours for five days (120 hours). At hours 10 and 120 of the study, plant tissue extractions were also performed whereby plant tissue was frozen at -80^0C and lyophilized for up to 72 hours, then ground into a fine powder and sonicated in 10 mL methanol for 12 hours in a cooled water bath. The sonication process was repeated again with fresh methanol, and extracts from both sonications combined and evaporated to a known volume. A correction factor was used to account for the evaporation of water during this study. Samples were filtered (0.22 μM) and 50 μL of the extract was analysed by HPLC.

Analysis of TNT Transformation Products

Samples were analysed by reverse-phase HPLC (Waters, Milford, MA) with PDA detection (model 996) as described in Hughes *et al*. (1997) with a Nova-Pak C8 column. TNT and transformation products were identified and quantitated by comparison to UV spectra and retention time of authentic standards of 2,4,6-trinitrotoluene, 2-hydroxylamino-4,6-dinitrotoluene, 4-hydroxylamino-2,6-dinitrotoluene, 2-amino-4,6-dinitrotoluene 4-amino-2,6-dinitrotoluene, 2,4-diamino-6-nitrotoluene and 2,6-diamino-4-nitrotoluene. Previously extracted samples of TNT conjugates (TNT-1, TNT-2, 2A-1 and 4A-1) were used as standards for conjugate identification (Bhadra *et al.*, 1999b).

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Figure Captions and Legends

Figure 1. General pathway of nitroaromatic compound reduction by nitroreductases

Figure 2. TNT-derived conjugates from *Catharanthus roseus* hairy root cultures (Burken at el., 2000). Conjugates were isolated from "feeding studies" where root cultures were amended only with TNT (resulting in production of conjugates TNT-1 and TNT-2) or 2-ADNT (resulting in production of 2A-1) or 4-ADNT (resulting in production of 4A-1). R_1, R_2, R_3 and R_4 are six carbon sugars.

Figure 3. Reduction of TNT to 4-HADNT by *E.cloacae* nitroreductase. TNT was incubated with *E.cloacae* NR, NADH and cofactor recycler in phosphate buffer at 25^0C. Samples were taken every 30 min and analysed by HPLC. Results are the mean and standard error of the mean of duplicated results.

Figure 4. Phytotoxicity of TNT exhibited by wild type and transgenic seedlings. Fifty seedlings of each plant line were grown for 14 days, at which time TNT was added and remained in growth medium for a further five days. Pictures are wild type seedlings (flasks on left) and transgenic NR 3-2 seedlings (flasks on right) at A: Without TNT amendment; B: 0.25mM (56mg L-1) TNT; C: 0.5mM (113mg L-1) TNT and D: 0.75mM (170mg L-1) TNT (this concentration was not used for TNT transformation studies).

Figure 5. Removal of TNT from liquid media by wild type (WT) and transgenic tobacco plants (NR). Initial concentrations were A: 0.1mM (23 mg L-1) TNT; B: 0.25mM (56 mg L-1), C; 0.38 mM (86mg L-1), D: 0.5mM (113mg L-1) TNT. Eighteen day-old seedlings were incubated with TNT for five days during which samples of growth medium were routinely taken and analysed for TNT removal by HPLC. Results presented are the mean and standard error of the mean of duplicated results except 0.38 and 05mM which are individual results after six hours.

Figure 6. Products of TNT transformation isolated from the plant growth medium of wild type (WT) and transgenic (NR) plants. A: Wild type and transgenic NR 3-2 seedlings at 0.1mM (23 mg L-1) TNT; B: Wild type and transgenic NR 3-2 seedlings at 0.25mM (56 mg L-1) C; 0.38mM (86mg L-1)TNT, D: 0.5mM (113mg L-1)TNT. Eighteen day-old seedlings were incubated with media amended with TNT for five days during which samples of growth medium were routinely taken and analysed for products of TNT transformation by HPLC. Results presented are the mean and standard error of the mean of duplicated results except C and D which are individual results after six hours.

Figure 7. Products of TNT transformation isolated from tissue extractions of wild type (WT) and transgenic (NR) plants. A: Wild type seedlings at 0.1mM (23 mg L-1) TNT; B: Transgenic NR 3-2 seedlings at 0.1mM (23 mg L-1), C: Wild type seedlings at 0.25mM (56 mg L-1) TNT; D: NR 3-2 seedlings at 0.25mM (56 mg L-1) TNT. TNT was added to the growth medium of eighteen day-old seedlings for 10 hours or 120 hours at which time transformation products of TNT were extracted from plant tissue. Results presented are the mean and standard error of the mean of duplicated results.

Figure 8. Products of TNT transformation isolated from tissue extractions of wild type (WT) and transgenic (NR) plants. A: Wild type seedlings at 0.38mM (86 mg L-1) TNT; B: Transgenic NR 3-2 seedlings at 0.38mM (86 mg L-1), C: Wild type seedlings at 0.5mM (113 mg L-1) TNT; D: NR 3-2 seedlings at 0.5mM (113 mg L-1) TNT. TNT was added to the growth medium of eighteen day-old seedlings for 10 hours or 120 hours at which time transformation products of TNT were extracted from plant tissue. Results presented are of an individual flask.

Figure 9. Overview of the main products of TNT transformation by wild type and transgenic plants (NR 3-2), as observed from results in this paper. Enzymes proposed to conduct TNT transformations are included to clarify discussion.

Table I. Effects of TNT toxicity on the vegetative growth of wild type and transgenic plants. Eighteen day-old seedlings were normalized for weight and incubated in the presence of TNT for five days when wet weights were measured to establish gain or loss of plant biomass. Results are the mean and standard error of the mean from duplicated results except 0, 0.38 and 0.5mM that are individual results. Growth index was calculated as final/ initial weights.

Initial TNT [mM]	Wild Type Plants			NR 3-2 Plants			Null Value Hypothesis
	Initial Weight (g)	Final Weight (g)	Growth ratio	Initial Weight (g)	Final Weight (g)	Growth ratio	$P_{NR\text{-}WT}$
0	2.3 ± NA	4.3 ± NA	1.8 ± NA	3.3 ± NA	6.0 ± NA	1.8	-
0.1	3.7 ± 0	4.6 ± 2.26	1.2 ± 0.5	3.9 ± 0.28	6.8 ± 1.2	1.8 ± 0.27	0.12
0.25	3.4 ± 0.14	3.35 ± 0.49	0.9 ± 0.1	3.75 ± 0.07	6.5 ± 0.7	1.7 ± 0.16	0.00
0.38	3.3 ±NA	1.6 ± NA	0.5 ± NA	4.1 ±NA	5.7 ±NA	1.4	-
0.5	3.2 ±NA	1.5 ±NA	0.5 ± NA	8.9 ±NA	13.6 ±NA	1.5	-

Table II. Conjugation products produced by wild type and transgenic seedlings in intracellular samples and growth medium. Eighteen day-old seedlings were incubated with TNT for five days during which the media was sampled and after which the products of TNT conjugation were extracted from plant tissue and analysed by HPLC. Results are taken from two independent samples (results recorded where both samples contained the conjugate) and individual results from 0.38 and 0.5mM TNT amendment to media.
* TNT-2 for WT taken as 1 mg L^{-1} in order to calculate ratio.

Initial TNT Concentration	Conjugates identified in intracellular samples (120 hours after TNT amendment)		
	Wild Type Plants	NR 3-2 Plants	TNT-2 mass ratio (NR3-2/ WT)
0.1 mM 23 mg/L	TNT-1 TNT-2	TNT-2	1.6 ± 0.95
0.25 mM 56 mg/L	TNT-1 TNT-2	TNT-2	115 ± 51.4
0.38 mM 86 mg/L	TNT-1 TNT-2	TNT-2	144
0.5 mM 113 mg/L	None observed	TNT-2	>180*

Initial TNT Concentration	Conjugates identified in growth medium (120 hours after TNT amendment)	
	Wild Type Plants	NR 3-2 Plants
0.1 mM 23 mg L^{-1}	TNT-1 TNT-2 2A-1 4A-1	TNT-2 4A-1
0.25 mM 56 mg L^{-1}	TNT-1 TNT-2 2A-1 4A-1	TNT-2 4A-1
0.38 mM 86 mg L^{-1}	TNT-1 TNT-2 4A-1	TNT-2 4A-1
0.5 mM 113 mg L^{-1}	TNT-1 TNT-2 4A-1	TNT-2 4A-1

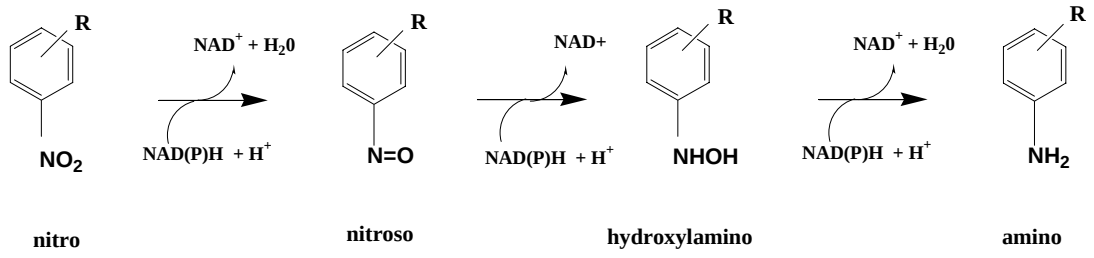


Figure 1. General pathway of nitroaromatic compound reduction by nitroreductases

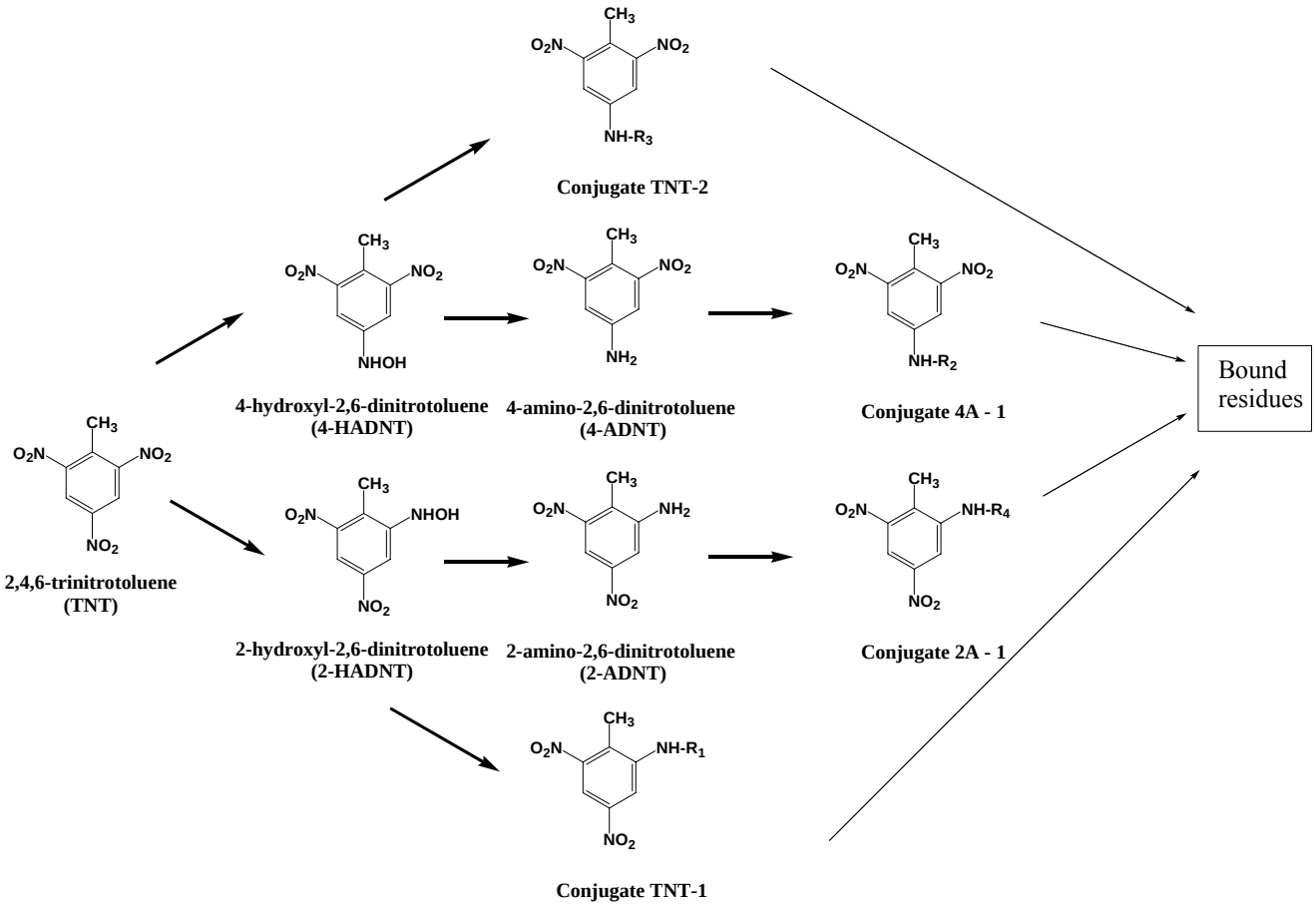
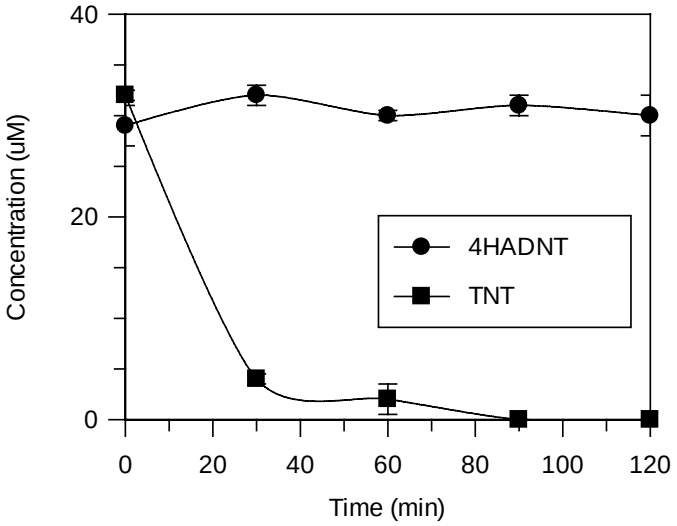


Figure 2. TNT-derived conjugates from *Catharanthus roseus* hairy root cultures (Burken at el. 2000).



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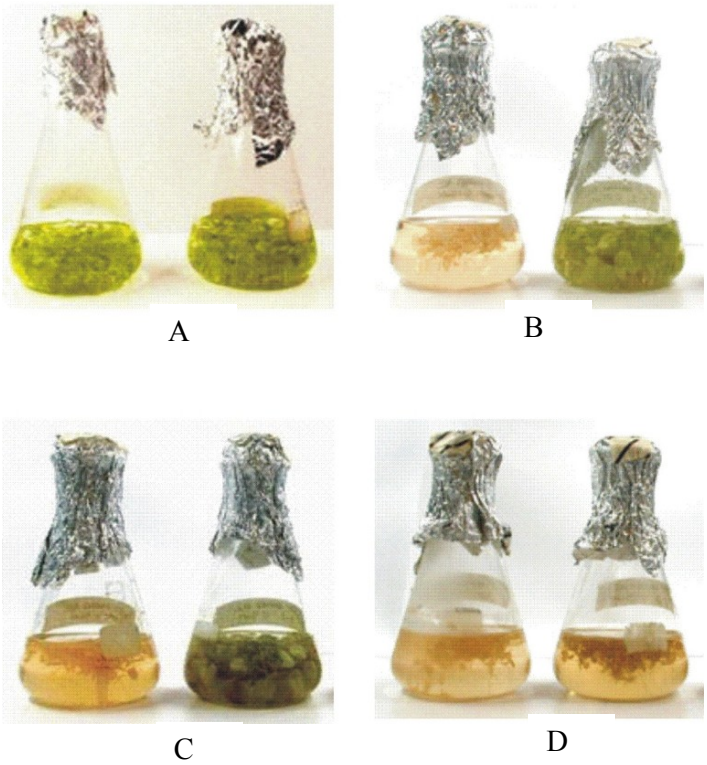

Figure 4. Phytotoxicity of TNT exhibited by wild type and transgenic seedlings.

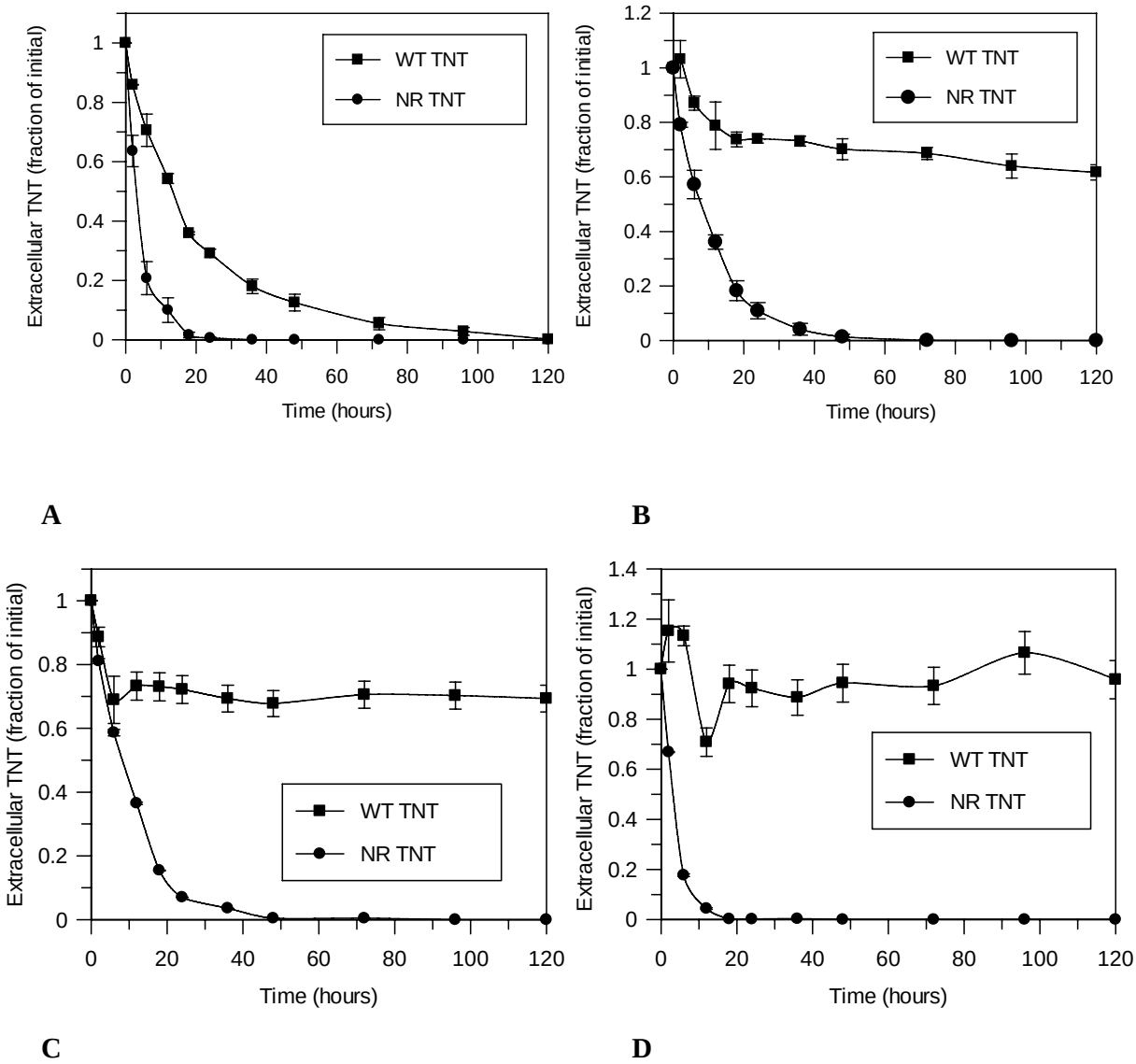

Figure 5. Removal of TNT from liquid media by wild type (WT) and transgenic tobacco plants (NR).

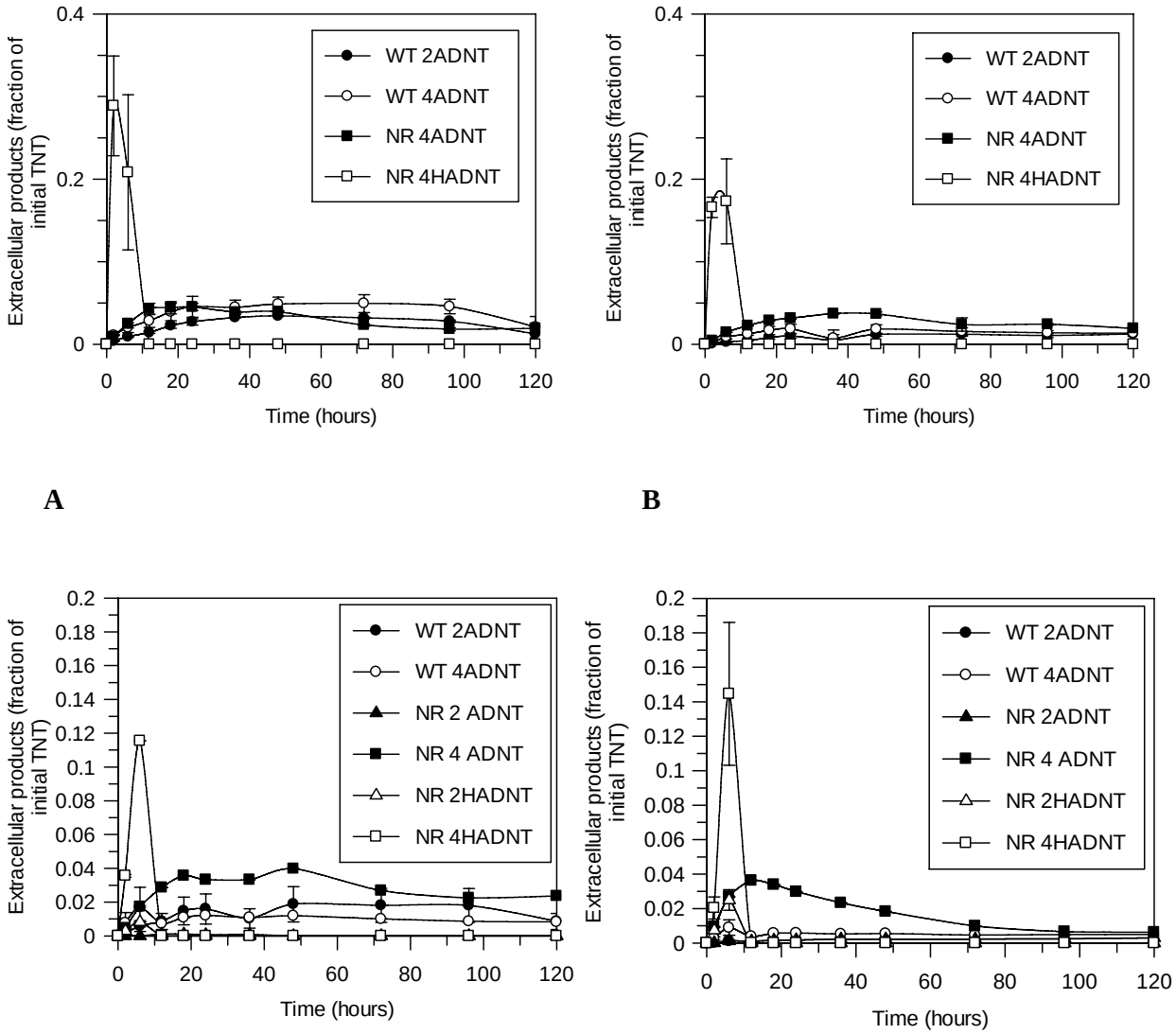

Figure 6. Products of TNT transformation isolated from the plant growth medium of wild type (WT) and transgenic (NR) plants.

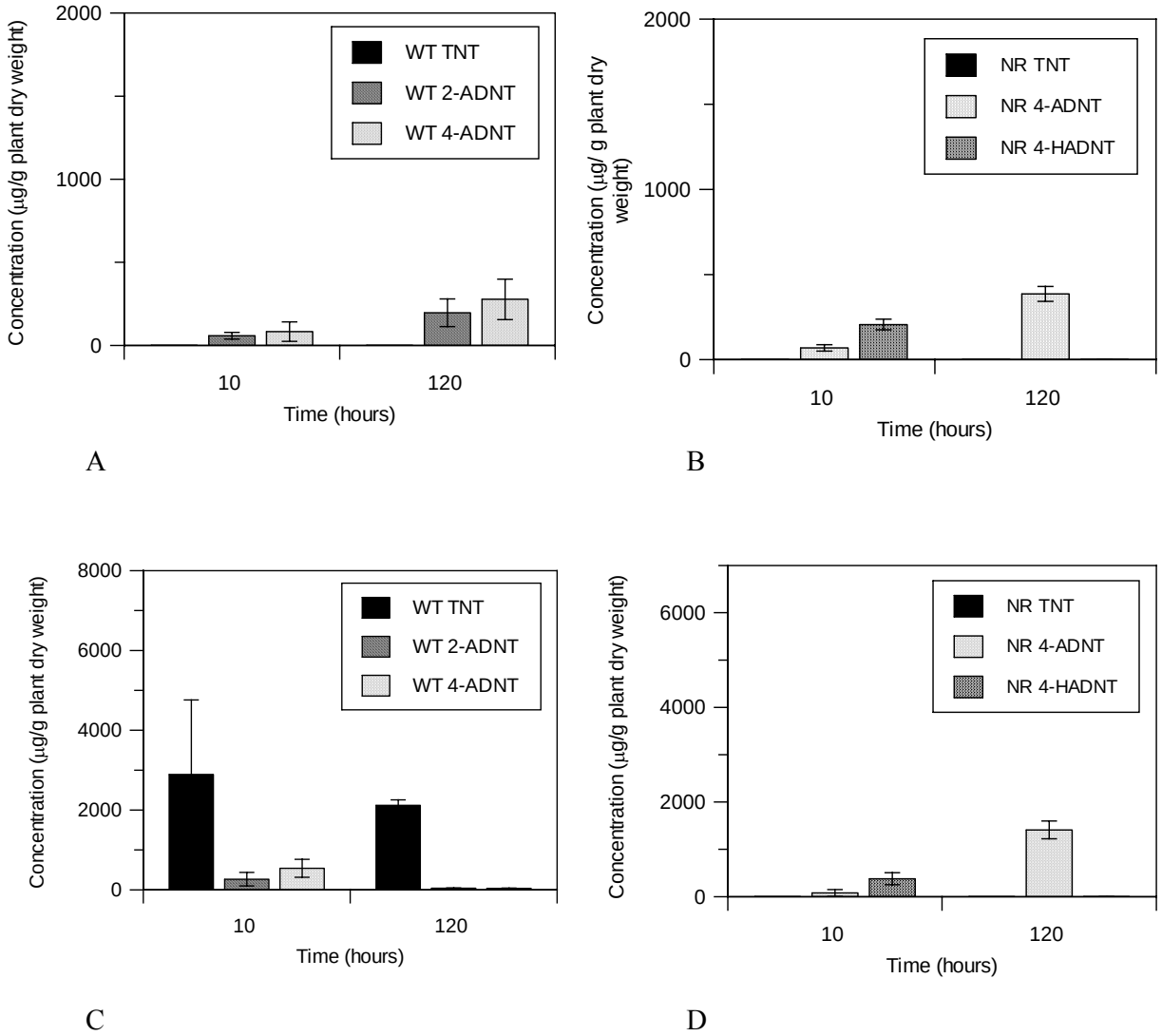

Figure 7. Products of TNT transformation isolated from tissue extractions of wild type (WT) and transgenic (NR) plants.

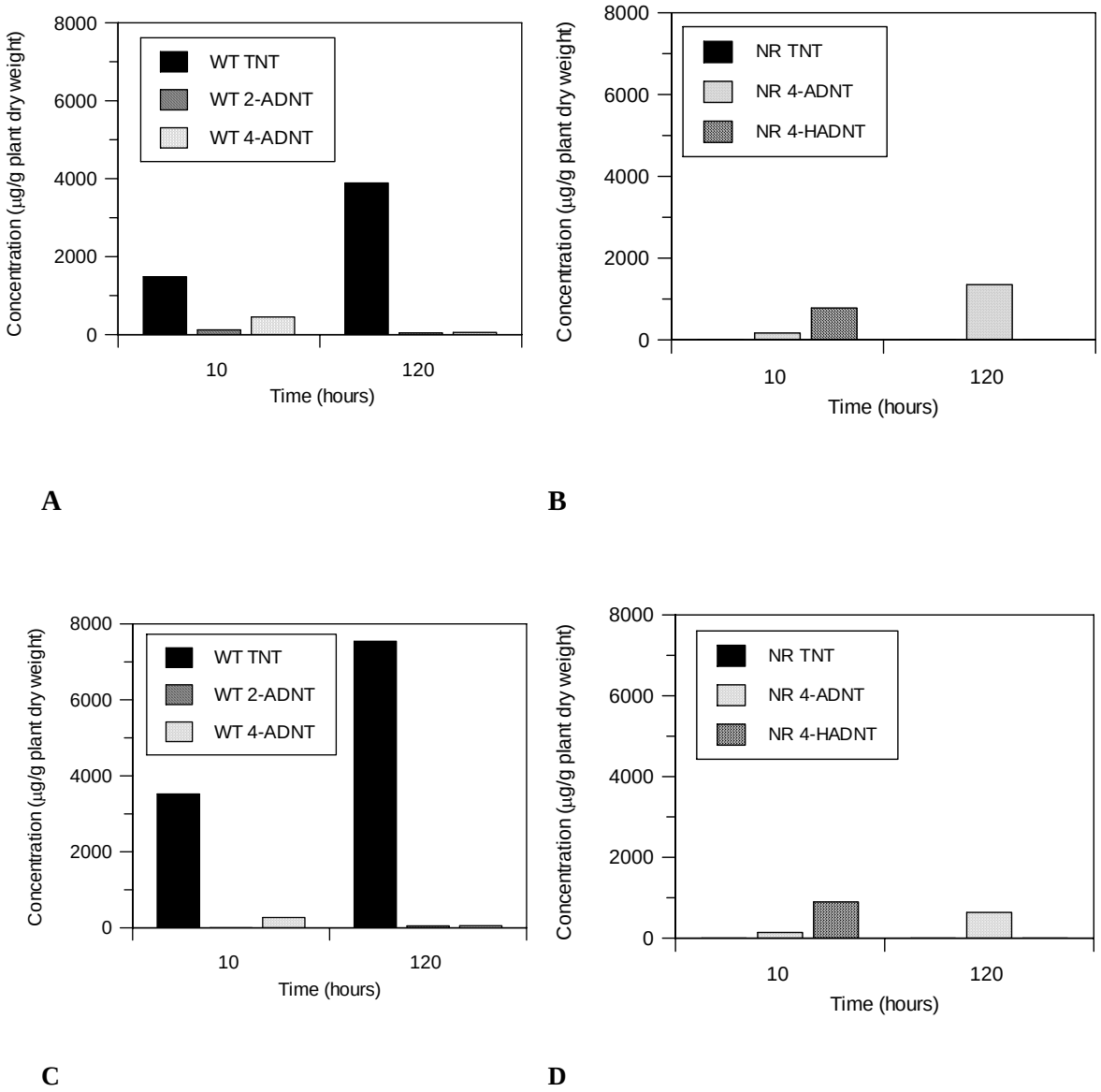

Figure 8. Products of TNT transformation isolated from tissue extractions of wild type (WT) and transgenic (NR) plants.

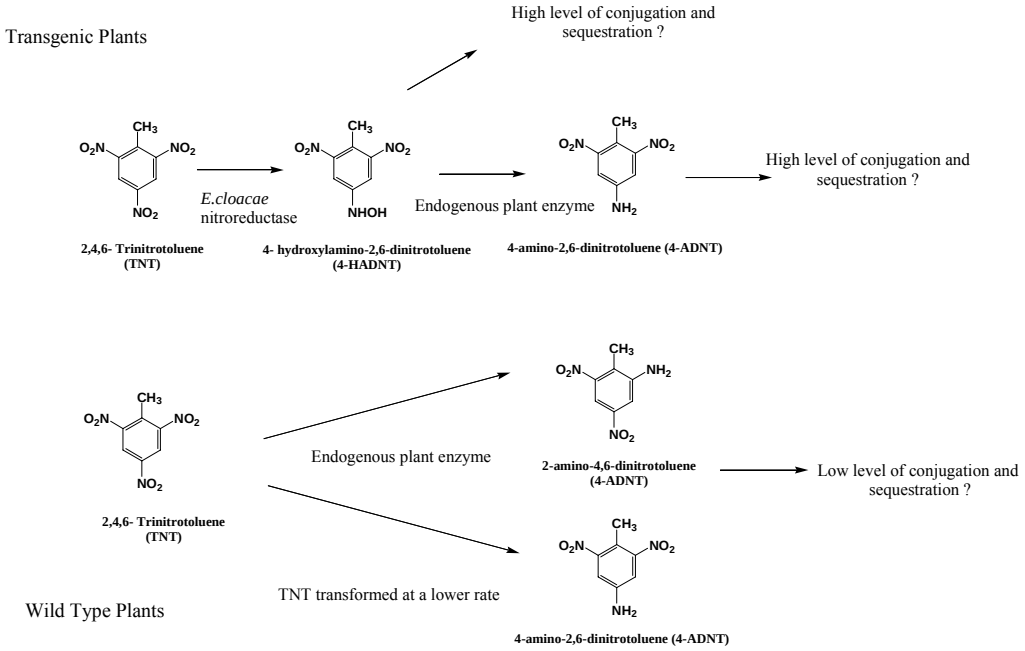

Figure 9. Overview of the main products of TNT transformation by wild type and transgenic plants (NR 3-2), as observed from results in this paper.

Shanks, J.V., Oliver, D.J., Moon H., Rollo, S., Subramanian, M., "Genetic and Biochemical Basis for the Transformation of Energetic Materials (RDX, TNT, DNTs) by Plants," Annual Meeting of the American Institute of Chemical Engineers, November 2003, San Francisco, CA

Genetic and Biochemical Basis for the Transformation of Energetic Materials (RDX, TNT, DNTs) by Plants

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Abstract

The energetic materials (RDX, HMX, TNT, DNTs) are possible sources of groundwater and surface soil (<1 ft) contamination at DoD training and testing sites. Phytoremediation is an inexpensive, self-sustaining treatment technology that may be suitable for prevention of contamination. However, a basic knowledge of the transformation pathways of the energetic materials is needed. Our objective is to construct a genetic and biochemical knowledge base for the transformation pathways of energetic materials (RDX, TNT, DNTs) by exploiting the fact that these chemicals are phytotoxic. We are using *Arabidopsis thaliana* as a model plant system. Our technical approach has three basic aims: (1) screening mutagenized populations of the model plant, *Arabidopsis thaliana*, to isolate mutants resistant to RDX, TNT, and the DNTs, due to under or over-expression of individual genes; (2) using transcriptome and proteome analyses to identify genes and proteins involved in plant response to these chemicals; and (3) using metabolite analyses to select final mutants and link gene to function. Metabolic analyses indicate that TNT transformation pathways for wild-type Arabidopsis seedlings are similar to those observed in *Catharanthus roseus*. Arabidopsis seedlings grown in liquid culture under photoheterotrophic conditions transform TNT at the same pseudo-first order rate constant ($k = 4 \times 10^{-4}$ L/g FW*h) up until a TNT initial concentration of 50 mg/L. In contrast, seedings exposed to a higher initial TNT concentrations (110 – 125 mg/L) had a rate one order of magnitude lower k= (2-4 $\times 10^{-5}$ L/g FW*h).

Keywords: Explosives, phytoremediation, nitroaromatics,

1. Introduction

Energetic materials (RDX, HMX, TNT, DNTs) are significant soil and groundwater contaminants (1). Contamination at decommissioned munitions production and handling facilities is well documented (1-4). These materials also are potential sources of groundwater and surface soil contamination at active DoD training and testing sites. The toxicity and mutageneticity of these compounds are an ongoing concern and the subject of continuing research (5-10). Because of the scope of the problem and the economic and environmental costs, there has been great interest in developing alternative, low-cost, efficient treatment technologies. A substantial literature base exists on the remediation of soils and groundwater contaminated by energetic materials. Bioremediation and phytoremediation are inherently cost-effective methods that hold promise for solving these contamination problems (11-13). Phytoremediation may be well suited to explosives contamination, since the contamination is spread over large areas in near-surface soils (12).

Studies with RDX, TNT, and the DNTs have shown the potential of phytoremediation of these materials, although to varying degrees. TNT, the most widely studied of all energetic materials, can be transformed to non-toxic forms by a variety of plant species (12-20) and we have a good understanding of the basic transformation processes. Tobacco plants engineered with a bacterial nitroreductase show enhanced resistance to TNT (21) – thus showing the potential of transgenic plants to revegetate and possibly remediate TNT-contaminated sites. While pathway and metabolite information for RDX is scarce, RDX can taken up by aquatic and agronomic plants and tissue cultures and RDX accumulates in plant tissue with apparently little or no transformation (22-24). Interestingly, Schnoor and co-workers show the disappearance of RDX in terrestrial plants – perhaps sunlight plays a role (12, 24). HMX transformation is a much greater challenge since it is not taken up at significant rates by plants and transformation is slow (12).

Ongoing case studies with poplar trees and lagoons at Iowa Army Ammunition Plant (IAAP) in Middletown, Iowa (12) provide some lessons on the application of phytoremediation in the field.

Areas of IAAP were determined to be too grossly contaminated to support plant life or any phytoremediation processes. Thus, approximately 100,000 cubic yards of soil were excavated. Residual contamination is being remediated by using phytoremediation with poplars as a final polishing step for soil and with wetland to treat residual contamination in surface and ground waters. Wetlands appear effective at treating low-level contaminated ground and surface waters, with minimal threat to indigenous ecosystems.

This case study reveals some of the current limitations of phytoremediation. Phytoremediation of TNT and related compounds is a slow process. Phytoremediation of energetic materials is limited because these chemicals are poisonous to plants and thus inhibit plant growth at contaminated sites. Phytotransformation of TNT may result in compounds more toxic than the parent molecule. Indeed, the mechanisms of the toxicity of TNT and transformation products to plants are unknown. From an ecological approach, the fate of parent compound and the degradation products, and their toxicity is important. Efforts to attain solid confirmation of the degradation

byproducts have recently led to elucidation of the metabolites for TNT and insight to the mechanisms that produce them (reviewed in 12).

We premise that understanding the genetics, enzymology, and biochemical pathways by which plants metabolize energetic materials will allow us to tackle two main concerns. One is to effectively engineer (21,25) or breed new plants with enhanced energetic-material transformation capabilities, and another is to have the basis to determine toxicity of the transformation products to other forms of life. For the former, we hypothesize that lowering toxicity of energetic materials and their transformation products to plant growth will be the main avenue for improvement of transformation capabilities. *Arabidopsis* transforms TNT, thus opening up the possibility of molecular genetic approaches to identify genes and enzymes in the TNT transformation pathways.

Considerable progress has been made in the last six years in identifying plant processes for the transformation of TNT, yet we still have a significant fraction of the type of transformation processes from parent compound unidentified (14-16, 18). The current model (12) includes the four types of processes in TNT transformation: reduction, oxidation, conjugation, and incorporation into biomass. The chemical structures of the identified metabolites are given in the references (12).

The current model suggests that first one of the nitrate groups is reduced to a hydroxylamine (2-hydroxylamine 4,6-dinitrotoluene or 2HA 4,6DNT and 4-hydroxylamine 2,6-dinitrotoluene or 4HA 2,6-DNT). The hydroxylamine is then further reduced to the amine. This amine (or possibly the hydroxylamine from which it is derived) is then conjugated to a range of molecules and detoxified during the process. Mass balance studies (14-16, 18) concur with this representation for the "green liver" model for TNT. Some intermediates have been observed in the roots of aquatic and land plants where their concentrations exceed that of TNT and some (like the hydroxylamines) are postulated reaction intermediates that are unstable in the presence of oxygen and therefore more difficult to detect. More work is needed to complete the pathway structure. Mass balances show significant amounts of ^{14}C label are incorporated into unknown TNT transformation products (14-16, 18). In addition, none of the enzymes hypothesized in this pathway have been identified, although unpublished studies of spinach reductases by Pacific Northwest Batelle Labs and nitroreductases by EPA Athens (reviewed in 12) suggests that this class of enzymes are involved in initial steps of the pathways.

As stated earlier, *Arabidopsis thaliana is* a good model plant for genetic and biochemical approaches to elucidate pathway structure and function. However, to date, TNT transformation has not been explored in *Arabidopsis*. This paper reports on metabolic studies of *Arabidopsis* seedlings exposed to TNT.

2. Experimental

2.1 Growth studies

Arabidopsis thaliana plants were grown in the *Arabidopsis* Growth Medium in 125 mL flask for one week at 20°C under illumination, then TNT was added in concentrations between 0 - 70 mg/L. The growth of the seedlings was monitored visually and with dry-weight measurements for one week after amendment.

2.2 Kinetic studies

Arabidopsis thaliana plants were grown in 50 mL *Arabidopsis* Growth Medium for fourteen or seventeen days at 25°C under illumination, then TNT was added in concentrations ranging from 5 - 125 mg/L. Evaporation, heat-killed, and evaporation plus photodegradation controls were also performed. The fresh weight of these plants was measured prior to the start of the experiment, and the volume of media equalized to 50 mL. Intracellular and extracellular measurements were periodically made by sacrificing a plant or removing a portion of the media, respectively. Samples of the medium and biomass were analyzed by HPLC for presence of TNT and known pathway intermediates. Kinetic analysis of TNT disappearance from the medium are determined through linear regression of data fit to first-order kinetics with respect to TNT.

3. Results and Discussion

3.1 Growth studies

Previous work with other plant species indicated toxicity of TNT to plant growth. Figure 1 shows the appearance of *Arabidopsis* seedlings one week after TNT amendment in the concentrations from 0 – 30 mg/L. At 10 mg/L TNT, *Arabidopsis* growth was significantly affected with roots turned brown and its growth retarded. Starting at a concentration of 20 mg/L tested, plants turned brown and/or bleached and their growth was severely reduced. Figure 2 shows quantitatively the effect of TNT on plant biomass one week after amendment for TNT concentrations from 40 – 70 mg/L. Plant biomass is reduced by 40% at 40 mg/L and 80% at 70 mg/L.

For plants treated with 1 or 5 mg/L TNT, three days after TNT amendment, growth retardation of the plants was noticeable. This is shown in Figure 3, where the dry weight is plotted as a function of time for these concentration levels. As Figures 1 and 3 indicate, plants treated with 1 mg/L TNT remained healthy and caught up with the growth of the control plants after a week, whereas 5 mg/L treated plants showed signs of stress including some necrotic leaves and root growth significantly affected.

3.2 Kinetic studies

Arabidopsis transforms TNT rapidly for initial TNT concentrations up to approximately 60 mg/L, as shown in Figure 4. The pseudo-first order rate constants are given in Table 1. *Arabidopsis* seedlings grown in liquid culture under photoheterotrophic conditions transform TNT at the same pseudo-first order rate constant ($k = 4 \times 10^{-4}$ L/g FW*h) up until a TNT initial concentration of 58 mg/L. In contrast, *Arabidopsis* seedlings transform TNT one order of magnitude lower ($k = 2\text{-}4 \times 10^{-5}$ L/g FW*h) at high initial concentrations of TNT (110 – 125 mg/L). (TNT saturation level in media is approximately 150 mg/L).

All reduction products and conjugated compounds observed in previous studies with *C. roseus* and *Myriophyllum* (14-16, 18) are observed in these *Arabidopsis* studies, particularly at the higher TNT concentrations (data not shown). Figures 5 and 6 show the temporal profiles of TNT and 2-amino-4,6-dinitrotoluene in the medium at the

lower initial TNT concentrations of 6 mg/L and 15 mg/L. TNT disappears at a rapid rate, with 2-amino-4,6-dinitrotoluene as the main metabolite formed extracellularly in this time period. The 4- substituted transformation metabolites, 4-amino-2,6-dinitrotoluene and the conjugate of 4A-1, were identified extracellularly in the 15 mg/L TNT system (data not shown). They were absent in the lower initial TNT concentration system, likely due to the sensitivity of the HPLC measurements.

4. Conclusion

Arabidopsis thaliana exhibits phytotoxicity to TNT but also transforms it. Phytotoxicity is observed visually and by biomass measurements, beginning at low concentrations of TNT. Initial TNT transformation activity is not affected until much higher concentrations. *Arabidopsis thaliana* appears to be a good model plant to screen for mutants resistant to TNT. Mutants that can transform TNT at high levels of the parent compound are good candidates for further genetic studies.

Acknowledgements

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Table 1.

Initial [TNT] (mg/L)	Pseudo-first order rate constant, k (L/g*hr)
7.5	$4.5 * 10^{-4}$
19	$4.2 * 10^{-4}$
30	$4.2 * 10^{-4}$
42	$3.9 * 10^{-4}$
58	$3.5 * 10^{-4}$
110	$4.3 * 10^{-5}$
125	$2.3 * 10^{-5}$

Figure Captions

Figure 1. *Arabidopsis* seedlings one week after TNT addition to one-week-old liquid cultures

Figure 2. Biomass levels one week after TNT addition to one-week-old liquid cultures of *Arabidopsis* seedlings for concentrations of TNT from 40 – 70 mg/L.

Figure 3. Growth of *Arabidopsis* seedlings after TNT addition to one-week-old liquid cultures for concentrations of TNT from 0 – 5 mg/L.

Figure 4. Disappearance of TNT in the medium of two-week-old liquid cultures of *Arabidopsis* seedlings.

Figure 5. Disappearance of TNT in the medium of two-week-old liquid cultures of *Arabidopsis* seedlings exposed to low levels of TNT.

Figure 6. Temporal profiles of monoamine compounds in the medium of two-week-old liquid cultures of *Arabidopsis* seedlings exposed to low levels of TNT.

Figure 1

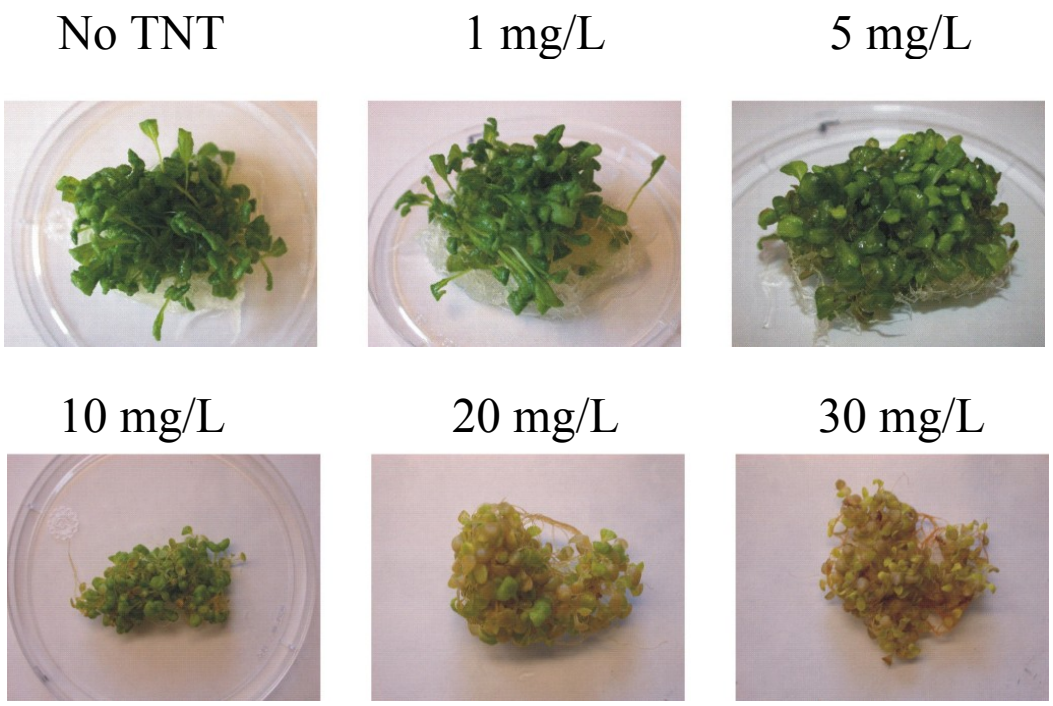


Figure 2

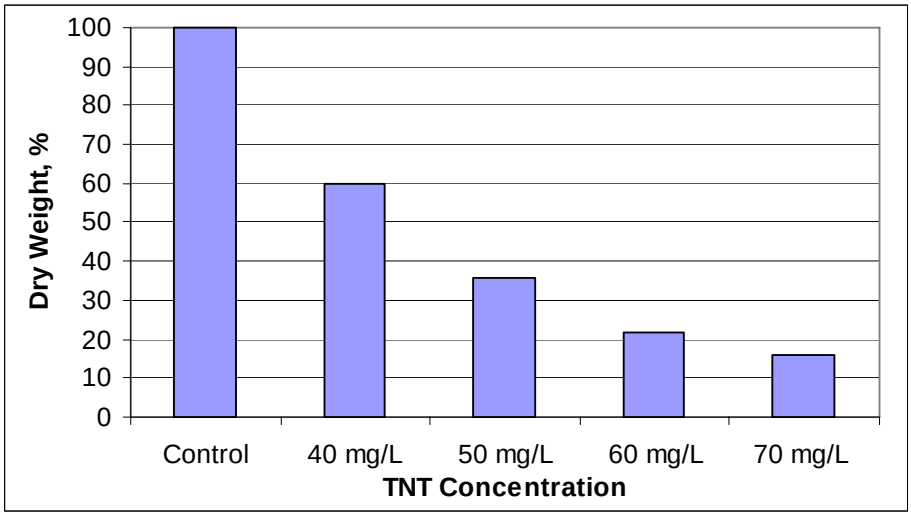


Figure 3

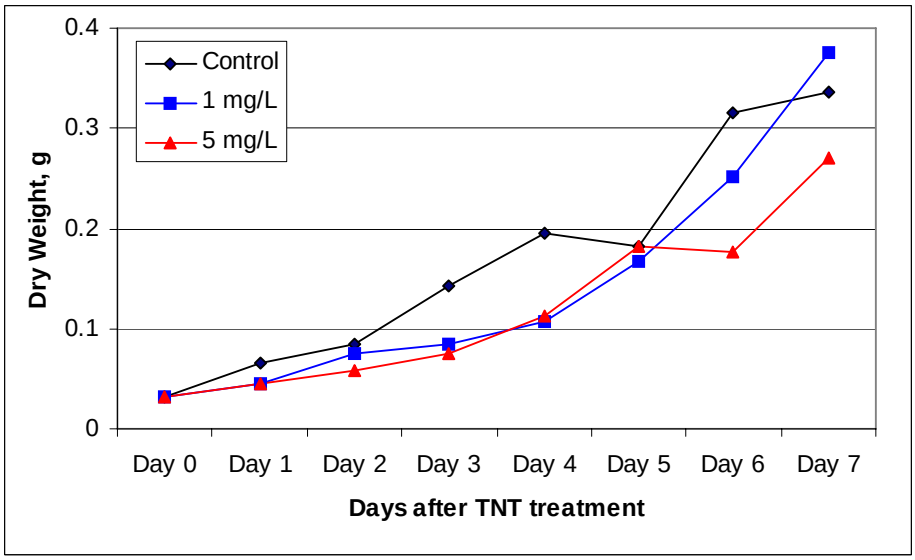


Figure 4

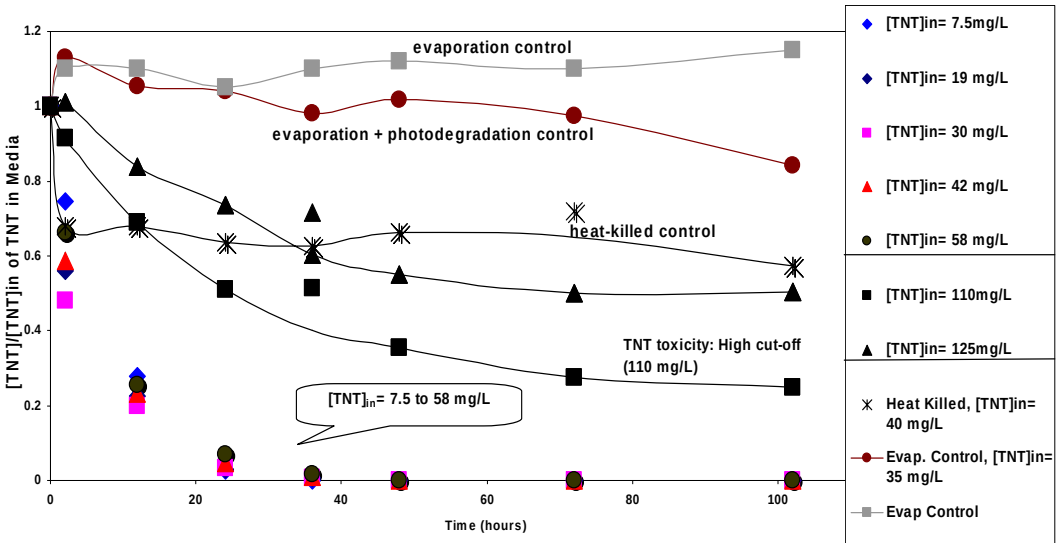


Figure 5

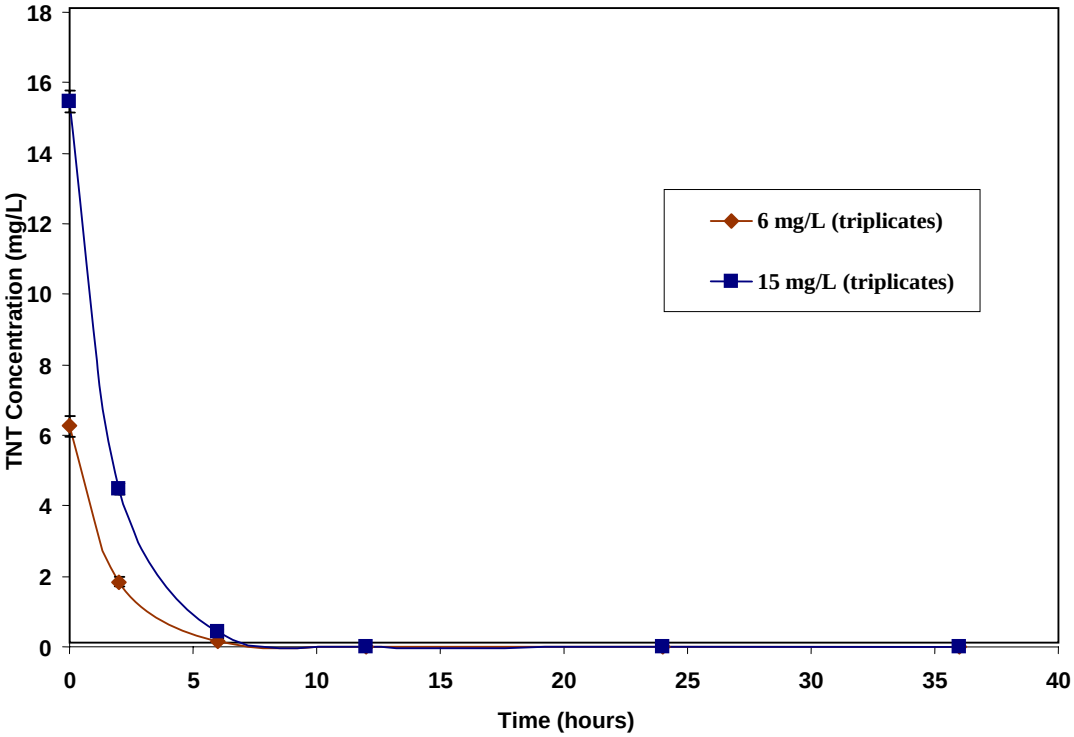
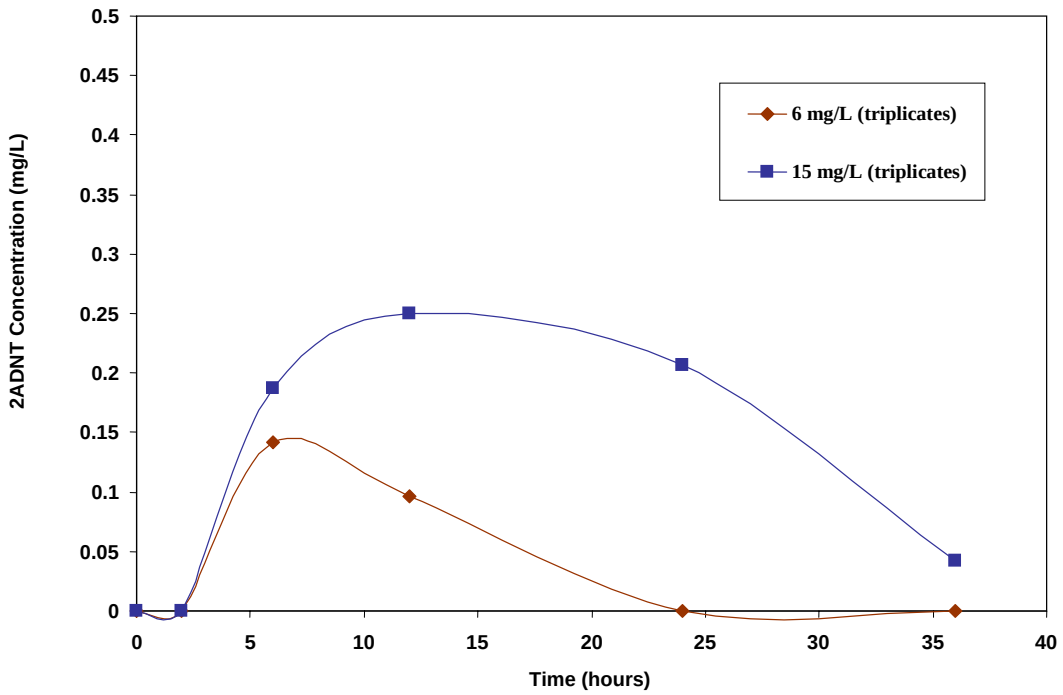


Figure 6


0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
2ADNT Concentration (mg/L)
0
5
10
15
20
25
30
35
40
Time (hours)
6 mg/L (triplicates)
15 mg/L (triplicates)

Moon, H., Subramanian, M., Rollo, S., Oliver, D.J. , Shanks, J.V., Hangsik Moon, "Use of Arabidopsis as a Model System for Genetic and Biochemical Studies of TNT Transformation in Plants," Battelle Conference on Sustainable Range Management, January 2004, New Orleans, LA.

USE OF *ARABIDOPSIS* AS A MODEL SYSTEM FOR GENETIC AND BIOCHEMICAL STUDIES OF TNT TRANSFORMATION IN PLANTS

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ABSTRACT: Energetic material contamination of ground water and surface soils (<1 ft) is of increasing concern, given their toxicity to the ecosystem. Phytoremediation, the use of plants in the field, may offer an inexpensive and self-sustaining procedure to alleviate the contamination problem. Knowledge about the genetic and biochemical pathway of 2,4,6-trinitrotoluene (TNT) uptake and transformation is beneficial for a successful application of the procedure in the field. Two types of *Arabidopsis thaliana* mutant libraries, T-DNA insertion and enhancer trap, were generated and screened to isolate mutants that are resistant to higher levels of TNT than the wild-type. From screening of approximately 250,000 seeds of T-DNA insertion mutant library and 300,000 seeds of the enhancer trap mutant library, 8 and 12 mutants, respectively, were selected for their greater TNT resistance. These mutants are being subjected to further genetic and metabolic analyses. Metabolic studies have been conducted on 2-week-old wild-type *Arabidopsis* seedlings. These studies indicate that TNT transformation pathways for wild-type *Arabidopsis* seedlings are similar to those observed in *Catharanthus roseus*. *Arabidopsis* seedlings grown in liquid culture under photoheterotrophic conditions transformed TNT at the same pseudo-first order rate constant ($k = 4 \times 10^{-4}$ L/g FW*h) up until a TNT initial concentration of 50 mg/L. In contrast, seedings exposed to a higher initial TNT concentrations (110 – 125 mg/L) had a rate one order of magnitude lower ($k= 2\text{-}4 \times 10^{-5}$ L/g FW*h).

INTRODUCTION: Energetic materials (RDX, HMX, TNT, DNTs) are significant soil and groundwater contaminants (Spain, 2000). Contamination at decommissioned munitions production and handling facilities is well documented (Etnier, 1989; Levsen *et al.*, 1993; Pennington, 2002; Spain, 2000). These materials also are potential sources of groundwater and surface soil contamination at active DoD training and testing sites. The toxicity and mutageneticity of these compounds are an ongoing concern and the subject of continuing research (Lachance *et al.*, 1999; Nipper *et al.*, 2001; Robidoux *et al.*, 2002; Thompson *et al.*, 1998). Because of the scope of the problem and the economic and environmental costs, there has been great interest in developing alternative, low-cost, efficient treatment technologies. A substantial literature base exists on the remediation of soils and groundwater contaminated by energetic materials. Bioremediation and phytoremediation are inherently cost-effective methods that hold promise for solving these contamination problems (Burken *et al.*, 2000; Hannink *et al.*, 2002; Subramanian and Shanks, 2003). Phytoremediation may be well suited to explosives contamination, since the contamination is spread over large areas in near-surface soils (Burken *et al.*, 2000).

TNT, the most widely studied of all energetic materials, can be transformed to non-toxic forms by a variety of plant species (Bhadra *et al.*, 1999b; Bhadra *et al.*, 1999a; Bhadra *et al.*, 2001; Burken *et al.*, 2000; Hughes *et al.*, 1997; Larson, 1997;

Larson *et al.*, 1999; Pavlostathis *et al.*, 1998; Wayment *et al.*, 1999) and there exists a good understanding of the basic transformation processes. The current "green liver" model of TNT transformation suggests the reduction of the nitro group to an amino group via hydroxylamine intermediates. The amino groups (and in many cases its hydroxylamine parent) are conjugated by a range of plant molecules that detoxifies the metabolite to a large extent. Knowledge gaps that exist in this pathway include the quantification of the hydroxylamines, other unidentified intermediates, and the lack of a complete mass balance. In addition, the enzymes involved in the transformation processes are largely unknown, although unpublished studies of spinach reductases by Pacific Northwest Battelle Labs and nitroreductases by EPA Athens (reviewed in Burken *et al.*, 2000) suggests that this class of enzymes are involved in initial steps of the pathways.

Although phytoremediation of TNT is a promising process, its slow nature and the lack of clear final fates of TNT are impediments to its field implementation (Burken *et al.*, 2000; Subramanian and Shanks, 2003). In addition, phytoremediation of energetic materials is further limited because these chemicals are poisonous to plants and thus inhibit plant growth at contaminated sites. Phytotransformation of TNT may result in compounds more toxic than the parent molecule. Indeed, the mechanisms of the toxicity of TNT and transformation products to plant are unknown. From an ecological approach, the fate of parent compound and the degradation products, and their toxicity are important. Efforts to attain solid confirmation of the degradation byproducts have recently led to elucidation of the metabolites for TNT and insight to the mechanisms that produce them (reviewed in Subramanian and Shanks, 2003).

Understanding the genetics, enzymology, and biochemical pathways by which plants metabolize energetic materials will allow us to select natural plants or breed new plants with enhanced energetic material transformation capabilities. This will hasten the process and produce more clearly identifiable end-points to the transformation procedure. Tobacco plants engineered with a bacterial nitroreductase have shown enhanced resistance to TNT (Hannink *et al.*, 2001). With relatively short life cycle and its whole genome sequenced, *Arabidopsis thaliana* is a good model plant to study TNT metabolism in plant and genes involved in the transformation of TNT. However, to date, TNT transformation has not been explored in *Arabidopsis*. This paper reports on metabolic and genetic studies of TNT transformation in *Arabidopsis*.

EXPERIMENTAL

Growth Studies of *Arabidopsis* Seedlings. *Arabidopsis thaliana* plants were grown in *Arabidopsis* growth medium (half-strength MS salts, 0.018% KH_2PO_4, B5 vitamins, 2% sucrose, 0.05% MES, pH 5.8) in 125 mL flasks for one week at 20°C under illumination, following which TNT was added in initial concentrations between 0 - 70 mg/L. The growth of the seedlings was monitored visually and with dry-weight measurements for one week after amendment.

Screening of *Arabidopsis* Mutants. Two kinds of *Arabidopsis* mutant pools were used in this study, T-DNA insertion mutants and enhancer trap mutants. The T-DNA insertion mutant lines were generated using a binary vector containing *NPTII* and *BAR* genes, which conferred transgenic plants with kanamycin and Basta resistance,

respectively. The enhancer trap lines were produced by the vector, pSKI015, which contained the *BAR* gene for selectable marker and 4x CaMV 35S enhancers.

Approximately 250,000 seeds of the T-DNA insertion lines and 300,000 seeds of the enhancer trap mutants were screened for TNT resistance. The seeds were sterilized in 20% commercial bleach for 15 min and washed with sterile water three times before plating on *Arabidopsis* growth medium containing 20 mg/L TNT. After cold treatment at 4°C for 3 days, the plates were incubated at 22°C under approximately 40 $\mu mol/m^2/s$ continuous light from cool white fluorescent bulbs. The surviving seedlings were transferred to soil and grown to maturity and seeds were harvested from individual plants. Those seeds were again challenged with 25 mg/L TNT in secondary and tertiary screenings.

Preparation of Genomic DNA and Southern Blot Analysis. Approximately 100 mg of rosette leaves were pulverized in liquid nitrogen and genomic DNA was extracted in an extraction buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 mM NaCl, 1% SDS, 10 mM β-mercaptoethanol). After treatment with 5M potassium acetate, DNA was precipitated with isopropanol, washed with 70% ethanol and then dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). DNA gel blot analyses were performed to determine numbers of T-DNA insertion in the mutant lines. Genomic DNA was digested with *Hind*III, DNA fragments were fractionated in agarose gel and transferred to a blotting membrane. *BAR* gene was used as a probe.

Cloning and Sequencing of Tagged Genes. Thermal asymmetric interlaced PCR (TAIL-PCR) was performed using genomic DNA as templates to clone DNA fragments flanking the left border of T-DNA in the selected mutant lines. The primers and PCR conditions were essentially same as described by Liu et al. (1995) except that the annealing temperature for high stringency cycles was 56°C and the concentrations of the degenerated primers and specific primers were the same. The specific primers, designed to anneal to near the left border, were LB1 (5'-ATACGACGGATCGTAATTTGTC-3'), LB2 (5'-TTATAATAACGCTGCGGACATCTAC-3'), and LB3 (5'-TTGACCATC-ATACTCATTGCTG-3'). The PCR products were gel purified and DNA sequencing reactions were carried out using LB3 primer. Resulting sequences were analyzed by Blast search.

Kinetic Studies on Wild-Type *Arabidopsis* Seedlings. *Arabidopsis thaliana* seedlings were grown in 50 mL *Arabidopsis* growth medium for seventeen days at 25°C under illumination, then TNT was added in concentrations ranging from 5 to 125 mg/L. Evaporation, heat-killed, and evaporation plus photodegradation controls were also included. The fresh weight of these plants was measured prior to the start of the experiment, and the volume of media equalized to 50 mL. Intracellular and extracellular measurements were periodically made by sacrificing the seedlings and removing a portion of the media. Samples of the medium and the tissue were analyzed by HPLC for presence of TNT and known pathway intermediates. Kinetic analyses of TNT disappearance from the medium were determined through linear regression of data assuming first-order kinetics with respect to TNT removal.

RESULTS AND DISCUSSION

Growth Studies. Previous work with other plant species indicated toxicity of TNT to plant growth. At 10 mg/L TNT, *Arabidopsis* growth was significantly affected with roots turned brown and its growth retarded. Starting at a concentration of 20 mg/L tested, plants turned brown and/or bleached and their growth was severely reduced. Figure 1 shows quantitatively the effect of TNT on plant biomass one week after amendment for TNT concentrations from 40 – 70 mg/L. Plant biomass is reduced by 40% at 40 mg/L and 80% at 70 mg/L.

For plants treated with 1 or 5 mg/L TNT, three days after TNT amendment, growth retardation of the plants was noticeable (data not shown). Plants treated with 1 mg/L TNT remained healthy and caught up with the growth of the control plants after a week, whereas 5 mg/L treated plants showed signs of stress including some necrotic leaves and root growth significantly affected.

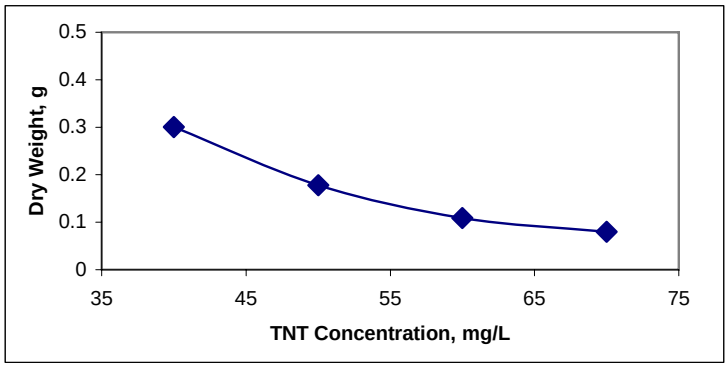


FIGURE 1. Biomass levels one week after TNT addition to one-week-old liquid cultures of *Arabidopsis* seedlings for concentrations of TNT from 40 – 70 mg/L.

Screening of Mutants and Genetic Studies. In the presence of TNT in the *Arabidopsis* growth medium, seed germination was substantially slowed. With 25 mg/L of TNT in the medium, it took 3 to 4 weeks for the seeds of mutant lines resistant to TNT to germinate but no wild-type seeds could germinate (Figure 2). Through tertiary screening, 8 and 12 of the T-DNA insertion and enhancer trap mutant lines, respectively, were selected that showed germination rate significantly higher than the wild-type in the presence of TNT in the medium.

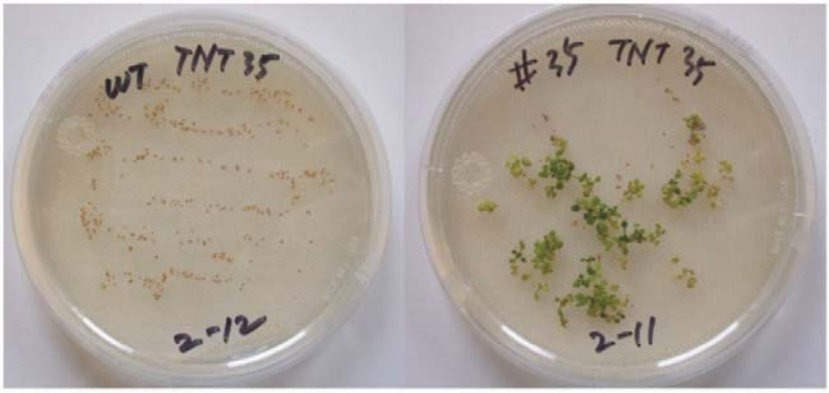
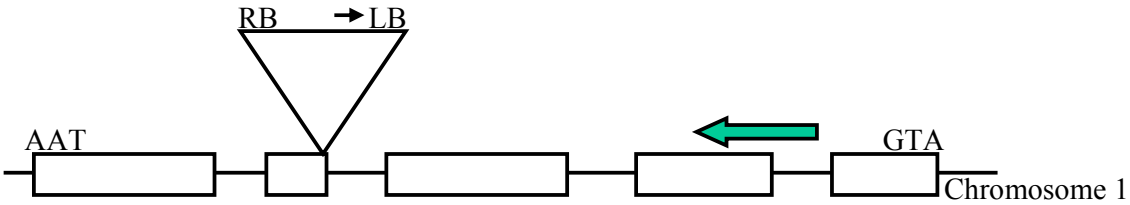


FIGURE 2. Comparison of germination between wild-type and a mutant line in the presence of 25 mg/L TNT 3 weeks after plating.

DNA gel blot analyses of the T-DNA insertion mutant lines indicated that the numbers of T-DNA insertion differed with mutant lines ranging from one to six (data not shown). Using genomic DNA of the enhancer trap mutant lines as templates, TAIL-PCR was performed with three successive reactions for each sample. Sequence analyses of the PCR products gave rise to isolation of genomic sequences flanking T-DNA insertions from the mutant lines that are resistant to TNT. The number of insertion varied with mutant lines with maximum of 3. In most cases, the T-DNA was inserted between two genes and in a few mutant lines the insertion occurred in an exon or an intron of a gene (Figure 3). Further studies are being performed to decipher which gene (or genes) are involved in giving the specific mutant TNT resistant.

Mutant line: ET212



Gene : At1g49560, myb family transcription factor.

FIGURE 3. An example of a map of the T-DNA insertion in an enhancer trap mutant line. T-DNA was inserted in the 4th exon of the gene in chromosome 1 in this mutant. The arrow indicates the orientation of the gene, and ATG and TAA are the start and stop codons of the gene, respectively.

Kinetic studies. *Arabidopsis* transforms TNT rapidly for initial TNT concentrations up to approximately 60 mg/L, as shown in Figure 4. *Arabidopsis* seedlings grown in liquid culture under photoheterotrophic conditions transform TNT at the same first order rate constant ($k = 4 \times 10^{-4}$ L/g FW*h) up until a TNT initial concentration of 58 mg/L. In contrast, *Arabidopsis* seedlings transform TNT one order of magnitude lower ($k = 2\text{-}4 \times 10^{-5}$ L/g FW*h) at high initial concentrations of TNT (110 – 125 mg/L). (TNT saturation level in media is approximately 150 mg/L). All reduction products and conjugated compounds observed in previous studies with *C. roseus* and *Myriophyllum* (Bhadra *et al.*, 1999b; Bhadra *et al.*, 1999a; Bhadra *et al.*, 2001; Hughes *et al.*, 1997) are observed in these *Arabidopsis* studies (data not shown). The 4-hydroxylamine isomer was observed only at the higher TNT concentration tested.

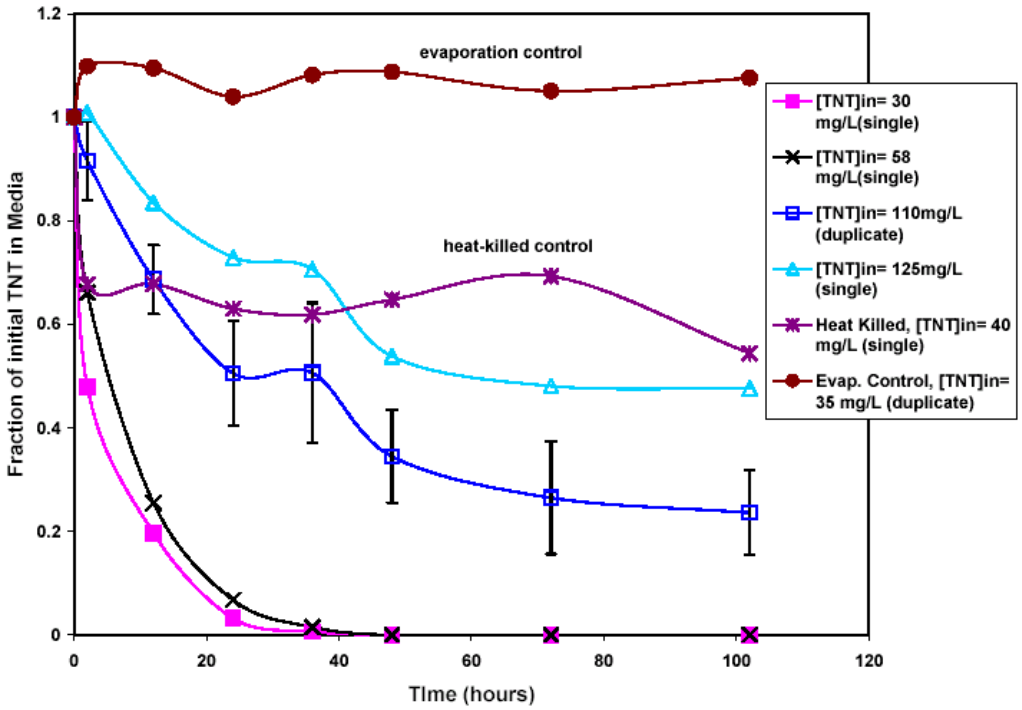


FIGURE 4. Removal of TNT from the extracellular TNT by 2 week old *Arabidopsis* seedlings, under sterile conditions.

CONCLUSIONS: *Arabidopsis thaliana* exhibits phytotoxicity to TNT but also transforms it. Phytotoxicity is observed visually and by biomass measurements, beginning at low concentrations of TNT. Initial TNT uptake rate from medium is not affected until much higher concentrations. Hence initial concentration is a useful variable to test the efficacy of a mutant to TNT. Mutants of *Arabidopsis* that possess greater resistance to TNT have been isolated and genetic and metabolic analyses on these are underway. These mutants can potentially have a greater TNT transformation efficiency than the wild-type.

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Toxicity of RDX on Germination of *Arabidopsis thaliana*

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Several ammunition manufacturing, packing, and testing sites have possible soil and groundwater contamination from energetic materials, such as RDX and TNT. Phytoremediation is an emerging technique that has the potential to remove energetic materials from soil and water, while being an inexpensive, self-sustaining, and environmentally friendly process. It is believed that RDX may be taken up by plants and stored as a parent compound. Currently, it is not understood what transformation pathway is used in the phytoremediation process or if any residue is returned to the soil or water. Before this technology can be applied, there must be a general understanding of how phytoremediation works. The goal of this research is to construct a genetic and biochemical knowledge base for the transformation pathway of RDX.

This study consists of evaluating the germination of *Arabidopsis thaliana* wild-type seeds in order to determine the toxicity level of RDX in solid media. The results of this work will be used to identify the concentration of RDX that should be used to screen mutant seeds. The concentration of seeds must also be determined by the assay. This study showed that future work should focus on using 1000 to 1500 mg/L RDX with 2000 seeds per plate. These conditions will be used to screen *A. thaliana* mutant seeds and select plants that perform better than the wild-type in the presence of high RDX concentrations. Selected mutants will be used to determine if there is a common gene that enhances remediation of RDX and/or limits the toxicity of RDX to the plant.

Introduction

The energetic materials RDX, TNT, HMX, and DNT have contaminated groundwater and surface soil at many Department of Defense training and testing sites. Phytoremediation can be used to continually reduce the level of energetic materials in the soil (1). Knowledge about the transformation pathways of RDX and other energetic materials would greatly increase our understanding of how phytoremediation occurs. With understanding of the transformation pathways, native plants could be selected by how well RDX is transformed, while mutant plants could be generated to enhance transformation. A well-defined pathway would also help identify what end products are formed by the remediation of RDX. In order to make the phytoremediation process more effective, a knowledge base of important genetic sequences and biochemical reaction pathways must be formed.

RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) is deposited on soil at ammunition manufacturing sites and testing sites. Once RDX has contaminated the soil, it can easily contaminate the groundwater, where it is particularly mobile (1). RDX is toxic to plants, animals, and humans, but can be taken up by some plants to reduce contamination of groundwater (2). RDX is thought to be incorporated into the biomass of the plant as a parent compound, since oxidation/reduction and conjugation occur less frequently than with other energetic materials (3).

The objective of our research is to use *Arabidopsis thaliana* mutants to determine if there are certain genes that promote RDX uptake and remediation, along with establishing a biochemical pathway in plants. *A. thaliana* is a useful model plant due to its small size, short life span and relatively simple genome (4). The transformation pathway of RDX in plants is still unknown, which makes it especially important to study this energetic material in depth. Since little is known about the transformation pathway of RDX, it is unclear if or how RDX is trans-formed, as well as what the end products of phytoremediation are. It is also not known what genetic mutations enhance RDX uptake and metabolism.

By exploiting the fact that RDX is toxic to plants, we can screen *A. thaliana* mutant seeds for plants that can survive exposure to high levels of RDX. Studying these mutants will then allow us to examine if there are recurrent genes that are present. Since the *A. thaliana* genome has been fully sequenced, it will be possible to identify the genes responsible for the metabolism of RDX. Metabolic analysis can then be used to determine exactly what step of the metabolism is altered by the mutation. Once the metabolic outcome and the genetic mutation are known, the role of the gene in the transformation pathway can be determined.

Before *A. thaliana* mutants can be screened and studied, the toxicity level for *A. thaliana* wild-type plants must be determined. The toxicity of RDX to some plants has been documented, but there is currently no information in the literature on the toxicity of RDX on *A. thaliana* (2, 4). Initial concentrations of RDX to study were estimated from previous phytoremediation work (5, 6, 7). Various concentrations of RDX were tested to evaluate the toxicity effects that RDX has on the germination of *A. thaliana* wild-type seeds in solid media. The time of germination was recorded for each concentration of RDX. The size of the plants was monitored for several weeks to determine how wild-type plants respond to growing in media containing RDX.

Materials and Methods

Half-strength Murashige and Skoog (MS) medium was used for all experiments. Medium consisted of 2.2 g of MS salt mixture, 3 mL of a 6% potassium phosphate monobasic (KH_2PO_4) solution, 1 mL of Gamborg's vitamin B_5 solution, 20 g of sucrose, and 0.5 g of 99% MES (2-morpholinoethanesulfonic acid) hydrate added to 1 L of water. The pH was adjusted to 5.8 with NaOH. Phytagel was then added to the media (2.0 g/L media) and the media was autoclaved.

RDX was obtained in acetone from AccuStandard and in acetonitrile from ChemService. The MS salt mixture was purchased from Gibco BRL Life Technologies. Potassium phosphate mono-basic was obtained in crystal form from Fisher Scientific. Gamborg's vitamin B_5 solution came from Sigma. Sucrose was

purchased from Midwest Scientific. 99% MES hydrate was obtained from Acros Organics. Phytagel was obtained from Sigma.

Seeds were sterilized by one of two methods. The first method consists of applying 20% bleach to seeds for 15 min and then rinsing with sterile water three times. The second method sterilizes seeds by exposing seeds to chlorine gas for 3 h. The chlorine gas is formed by combining 200 mL bleach with 20 mL concentrated HCl in a closed container.

Medium was autoclaved and then cooled in a 65°C water bath. RDX and acetone were added to the medium which was then poured into sterile petri dishes. The petri dishes were then cooled to let the media solidify. Then sterilized seeds were plated on the media. Lids were placed on the petri dishes, which were sealed with Parafilm to prevent evaporation and reduce the risk of contaminating the plates. Plates were then placed in a cold room at 4°C. After three days, the plates were then moved to the growth room to germinate, where they were kept at 22°C with constant light. Seeds were monitored to record the time of germination and the size of the plants over several weeks. Some plants were then transferred to clean media, with no acetone or RDX, or to soil to observe if they would be able to fully mature after exposure to high RDX concentrations.

Wild-type seeds were grown in solid media containing various concentrations of RDX in order to determine the toxicity of RDX on germination. Concentrations of RDX in the media ranged from 10 mg/L to 2000 mg/L. RDX was first obtained in a solution of acetonitrile (1000 µg/mL) from ChemService. Later, RDX was obtained in a more concentrated solution of acetone (50 mg/mL) from AccuStandard. Acetone concentrations were kept the same throughout a screening assay in order to compare the effect of the RDX only. Four replicates were used for each concentration of RDX studied. Two controls were used for the screening; one control consisted of media with no acetone or RDX added, while the other control consisted of media with acetone only. By using two controls, the toxic effects of RDX in acetone could be monitored by comparison with the first control and the toxic effects of RDX alone could be monitored by comparison with the second control. This allowed us to observe the effect of several concentrations of RDX on germination, by comparing those seeds to the controls. Various concentrations of seeds were also used to observe the effect that seed concentration had on RDX toxicity. Seed concentrations ranged from 75 to 2000 seeds per plate.

Results

RDX concentrations from 0 mg/L to 2000 mg/L were tested to determine the RDX level that will be used for mutant screening. Precipitation was first observed at 500 mg/L RDX and significant precipitation of RDX occurred at levels above 1000 mg/L RDX. Acetone concentrations remained at or below 40 mg/L. Seed concentrations ranged from 150 seeds per plate to 2000 seeds per plate. With higher concentrations of RDX, several toxic effects were observed, including stunted germination and plant growth. Although RDX stunted the germination of the seeds, all seeds eventually germinated at the RDX concentrations tested. This study determined that *A. thaliana* mutant seeds should be screened with an RDX concentration between 1000 and 1500 mg/L RDX with 2000 seeds per plate.

Several experiments with wild-type seeds were conducted to determine the concentration of RDX toxic to *A. thaliana* seed germination and the concentration of seeds to use to screen mutant seeds. An initial experiment with RDX in acetone compared plates with 50, 100, 150, 200, and 250 mg/L RDX. All of the plates had 100 seeds and 5 mL/L acetone. This experiment showed little toxic effect to seeds at any concentration, which demonstrated that the concentrations of RDX were too low and needed to be increased. The next experiment compared RDX concentrations of 500, 1000, 1500, and 2000 mg/L, with 40 mL/L acetone and 100 seeds per plate. The germination of the seeds at high RDX concentrations was severely delayed. Additional experiments were conducted between 1000 and 1500 mg/L RDX at various seed concentrations. The toxic effects on germination and plant growth of these RDX concentrations appeared to be fairly similar. Since the root length was severely stunted in these experiments, 24-day-old plants were transferred to media containing no RDX and to soil to determine if they would be able to develop to maturity. Developing to maturity is a requirement for selected mutants, since seeds need to be collected from the selected plants for future experiments. Plants that were transferred directly to soil had a hard time adjusting with their minimal root length. Plants transferred to media without RDX grew much more easily, which allowed for normal root growth. These plants could then be transferred to soil in order to fully develop. There was little difference between 1000 and 1500 mg/L RDX when observing germination and growth in RDX-containing media, but plants that were less stunted initially were able to develop much more rapidly when transferred to soil. Transplanting these plants to soil helped determine which RDX concentration and seed concentration to select for future work.

Discussion

The experiments demonstrated the toxic effects of high RDX concentrations, as well as showing the germination and growth of wild-type plants. Higher concentrations of RDX show more toxic effects on the germination and growth of the plants. Germination was delayed longer for seeds exposed to higher RDX levels. It should also be noted that the seeds that germinated first, matured first. Age of the plants was determined from the time the seeds were placed in the growth room.

RDX was originally purchased in acetonitrile (1000 µg/mL). One experiment was done to test concentrations of RDX from 0 mg/L to 40 mg/L. All of these plates had 40 ml/L of acetonitrile. Four replicates were used for each concentration. Out of the 20 plates tested, only two plates germinated. One plate was a control with 0 mg/L RDX, while the other plate had an RDX concentration of 30 mg/L. This experiment was inconclusive about the toxicity of RDX, but showed that acetonitrile was too toxic to be used as a solvent for screening studies. This experiment also demonstrated the need for two controls, one with no RDX and no solvent, and a second control with only solvent. Two controls are necessary in order to be able to observe the toxicity of the solvent, as well as the toxicity of RDX.

The results observed in this study can be summarized by three toxic effects. The first is that the concentration of RDX affected seed germination. With increasing concentrations of RDX, the toxic effects on germination also increased, which resulted in longer germination times. This study also showed that acetone was

particularly toxic to root length, and these toxic effects also increased with increasing concentrations of acetone. The seed concentration is also an important factor to consider. Decreasing the concentration of seeds per plate increased the toxic effects of both RDX and acetone.

Acknowledgements

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Phytoremediation of RDX: Using *Arabidopsis thaliana* to Determine the Genetics and Biochemistry of the Transformation Pathway

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Abstract

Many energetic materials, such as RDX and TNT, are possible surface soil and groundwater contaminants at Department of Defense testing and training sites. Phytoremediation is an inexpensive, self-sustaining, and environmentally friendly process that has the potential to remove residual energetic materials from surface soil and groundwater. It is believed that RDX is taken up by plants and accumulates as a parent compound. Currently, it is not understood what transformation pathway is used in the remediation process or if any transformation products are returned to the environment. Certain genes may be responsible for the phytoremediation of RDX. Understanding how phytoremediation works is an important step to being able to implement this technology. The goal of this research is to construct a genetic and biochemical knowledge base for the transformation pathway of RDX.

The study of important genes involved in the RDX transformation pathway involves screening *Arabidopsis thaliana* mutants to determine if there are common genes that enhance remediation of RDX and/or limit the toxicity of RDX to the plant. The selected plants must be able to withstand high concentrations of RDX (1400 mg/L) with no loss of growth to be a good candidate for remediation. Two-hundred eight super-pools of *Arabidopsis* mutants were screened to determine if any performed better than the wild-type plants in the presence of high RDX concentrations. Selected mutants will be genetically compared to determine if there are similarities between mutants that have an increased resistance to RDX toxicity as well as remediating RDX efficiently. Since the *Arabidopsis* genome has been sequenced, it will be possible to identify the genes responsible for the metabolism of RDX.

In order to develop a biochemical knowledge base about the RDX pathway in *Arabidopsis*, mass balances and metabolite analysis were performed. Several wild-type plants were grown in the presence of 15 mg/L, 30 mg/L and 35 mg/L of RDX.

Plant and media samples were taken at several time steps throughout the experiment to obtain growth curves and RDX uptake curves. Intracellular and extracellular samples from ^{14}C labeled experiments were taken periodically and analyzed with HPLC to identify RDX transformation products and their fate. This information will help build an understanding of the RDX pathway. Knowledge of other energetic transformation pathways will serve as a background for developing a breakdown pathway for RDX. The current status of the RDX pathway will be presented.

Keywords: phytoremediation, energetic materials, RDX, *Arabidopsis thaliana*

1. Introduction

Several ammunition manufacturing and testing sites have significant surface soil and groundwater contamination from energetic material, such as RDX and TNT (1). Contamination can also be found at ammunition manufacturing sites that are no longer in use (1, 2). These materials have been documented as toxic to a broad range of organisms (3-6). Development of an inexpensive and effective process to remove energetic materials from soil and groundwater could reduce the environmental and economic burden caused by this contamination. Significant research has been done on the remediation of energetic materials from soil and groundwater. Phytoremediation, as well as bioremediation, are potential methods to help reduce contamination (7, 8). Since significant contamination occurs in surface soil, phytoremediation may become an ideal treatment for energetic contamination (7, 9, 10).

Phytoremediation studies have shown that plants have the potential to remove energetic materials. Many plant species have been shown to transform TNT to non-toxic end products (7, 8, 11-16). A basic understanding of the TNT transformation pathway has also been developed (9, 10, 17). RDX can be taken up by plants, but little is known about the transformation pathway (2, 9, 10, 18-24). RDX has also been shown to accumulate in plant tissue as a parent compound. (2, 18, 20, 24). The phytophotolysis of RDX has been studied by Schnoor and co-workers and may be responsible for the first step in the degradation of RDX (21).

Phytoremediation is a slow process and may be limited by the toxicity of energetic materials to plants. The pathway and the fate of RDX by plants are unknown. Even the toxicity of RDX and its degradation products to plants is limited. The environmental fate of RDX and its transformation products, as well as their toxicity, must be known before phytoremediation will be the most successful. Knowledge about the transformation pathways of RDX and other energetic materials would greatly increase our understanding of how phytoremediation occurs. With understanding of the transformation pathways, native plants could be selected by how well RDX is transformed, while mutant plants could be generated to enhance transformation. A well-defined pathway would also help identify what end products are formed by the remediation of RDX. In order to make the phytoremediation process more effective, a knowledge base of important genetic sequences and biochemical reaction pathways must be formed.

The objective of our research is to use *Arabidopsis thaliana* mutants to determine if there are certain genes that promote RDX uptake and remediation, along

with establishing a biochemical pathway in plants. *Arabidopsis* is a useful model plant due to its small size, short life span and relatively simple genome (17). The transformation pathway of RDX in plants is still unknown, which makes it especially important to study this energetic material in depth. It is unclear if or how RDX is transformed, as well as what the end products of phytoremediation are. It is also not known what genetic mutations enhance RDX uptake and metabolism.

2. Experimental

2.1 Germination studies

Half-strength Murashige and Skoog (MS) medium was used for all experiments. Medium consisted of 2.2 g of MS salt mixture, 3 mL of a 6% potassium phosphate monobasic (KH_2PO_4) solution, 1 mL of Gamborg's vitamin B_5 solution, 20 g of sucrose, and 0.5 g of 99% MES (2-morpholinoethanesulfonic acid) hydrate added to 1 L of water. The pH was adjusted to 5.8 with NaOH. Phytagel was then added to the media (2.0 g/L media) and the media was autoclaved.

RDX was obtained in acetone from AccuStandard. The MS salt mixture was purchased from Gibco BRL Life Technologies. Potassium phosphate mono-basic was obtained in crystal form from Fisher Scientific. Gamborg's vitamin B_5 solution came from Sigma. Sucrose was purchased from Midwest Scientific. 99% MES hydrate was obtained from Acros Organics. Phytagel was obtained from Sigma.

Seeds were sterilized by one of two methods. The first method consists of applying 20% bleach to seeds for 15 minutes and then rinsing with sterile water three times. The second method sterilizes seeds by exposing seeds to chlorine gas for 3 hours. The chlorine gas is formed by combining 200 mL bleach with 20 mL concentrated HCl in a closed container.

Medium was autoclaved and then cooled in a 65°C water bath. RDX and acetone were added to the medium which was then poured into sterile petri dishes. The petri dishes were then cooled to let the media solidify. Then sterilized seeds were plated on the media. Lids were placed on the petri dishes, which were sealed with Parafilm to prevent evaporation and reduce the risk of contaminating the plates. Plates were then placed in a cold room at 4°C. After three days, the plates were then moved to the growth room to germinate, where they were kept at 22°C with constant light. Seeds were monitored to record the time of germination and the size of the plants over several weeks. Some plants were then transferred to clean media, with no acetone or RDX, or to soil to observe if they would be able to fully mature after exposure to high RDX concentrations.

Wild-type seeds were grown in solid media containing various concentrations of RDX in order to determine the toxicity of RDX on germination. Concentrations of RDX in the media ranged from 10 mg/L to 2000 mg/L. RDX was obtained in a solution of acetone (50 mg/mL) from AccuStandard. Acetone concentrations were kept the same throughout a screening assay in order to compare the effect of the RDX only. Four replicates were used for each concentration of RDX studied. Two controls were used for the screening; one control consisted of media with no acetone or RDX

added, while the other control consisted of media with acetone only. By using two controls, the toxic effects of RDX in acetone could be monitored by comparison with the first control and the toxic effects of RDX alone could be monitored by comparison with the second control. This allowed us to observe the effect of several concentrations of RDX on germination, by comparing those seeds to the controls. Various concentrations of seeds were also used to observe the effect that seed concentration had on RDX toxicity. Seed concentrations ranged from 75 to 2000 seeds per plate.

2.2 Growth and kinetic studies

Half-strength MS medium, adjusted to a pH of 5.8, was used. Seeds were sterilized by previously mentioned methods. *Arabidopsis* seeds were grown in 250 mL flasks at 25°C under constant illumination. Each flask contained 50 mL medium and 50 sterilized seeds. Flasks were covered in foam plugs and aluminum foil and then placed in a shaker set to 100 rpm. Seeds were cultured for seven days before 0 mg/L or 35 mg/L RDX were added to the cultures. Seedlings were sacrificed at 0, 7, 10, 14 and 21 days after RDX was added and the dried biomass was weighed. Extracellular samples were taken at 7, 10, 14 and 21 days and were analyzed with reverse-phased HPLC, with a polar mobile phase and a Nova-Pak C8 column.

3. Results and Discussion

3.1 Germination studies

RDX concentrations from 0 mg/L to 2000 mg/L were tested to determine the RDX level that will be used for mutant screening. Precipitation was first observed at 500 mg/L RDX and significant precipitation of RDX occurred at levels above 1000 mg/L RDX. Acetone concentrations remained at or below 40 mg/L. Seed concentrations ranged from 150 seeds per plate to 2000 seeds per plate. With higher concentrations of RDX, several toxic effects were observed, including stunted germination and plant growth. Although RDX stunted the germination of the seeds, all seeds eventually germinated at the RDX concentrations tested. This study determined that *Arabidopsis* mutant seeds should be screened with an RDX concentration between 1000 and 1500 mg/L RDX with 2000 seeds per plate.

Several experiments with wild-type seeds were conducted to determine the concentration of RDX toxic to *Arabidopsis* seed germination and the concentration of seeds to use to screen mutant seeds. An initial experiment with RDX in acetone compared plates with 50, 100, 150, 200, and 250 mg/L RDX. All of the plates had 100 seeds and 5 mL/L acetone. This experiment showed little toxic effect to seeds at any concentration, which demonstrated that the concentrations of RDX were too low and needed to be increased. The next experiment compared RDX concentrations of 500, 1000, 1500, and 2000 mg/L, with 40 mL/L acetone and 100 seeds per plate. The germination of the seeds at high RDX concentrations was severely delayed. Additional experiments were conducted between 1000 and 1500 mg/L RDX at various seed concentrations. The toxic effects on germination and plant growth of these RDX concentrations appeared to be fairly similar. Since the root length was severely stunted in these experiments, 24-day-old plants were transferred to media containing no RDX and to soil to determine if they would be able to develop to maturity. Developing to maturity is a requirement for selected mutants, since seeds

need to be collected from the selected plants for future experiments. Plants that were transferred directly to soil had a hard time adjusting with their minimal root length. Plants transferred to media without RDX grew much more easily, which allowed for normal root growth. These plants could then be transferred to soil in order to fully develop. There was little difference between 1000 and 1500 mg/L RDX when observing germination and growth in RDX-containing media, but plants that were less stunted initially were able to develop much more rapidly when transferred to soil. Transplanting these plants to soil helped determine which RDX concentration and seed concentration to select for future work.

The experiments demonstrated the toxic effects of high RDX concentrations, as well as showing the germination and growth of wild-type plants. Higher concentrations of RDX show more toxic effects on the germination and growth of the plants. Germination was delayed longer for seeds exposed to higher RDX levels. It should also be noted that the seeds that germinated first, matured first. Age of the plants was determined from the time the seeds were placed in the growth room.

The results observed in this study can be summarized by three toxic effects. The first is that the concentration of RDX affected seed germination. With increasing concentrations of RDX, the toxic effects on germination also increased, which resulted in longer germination times. This study also showed that acetone was particularly toxic to root length, and these toxic effects also increased with increasing concentrations of acetone. The seed concentration is also an important factor to consider. Decreasing the concentration of seeds per plate increased the toxic effects of both RDX and acetone.

3.2 Growth and kinetic studies

The results of the growth study can be used to observe RDX toxicity on *Arabidopsis* plants. No significant effect was observed for RDX toxicity at a concentration of 35 mg/L RDX. Either RDX was not toxic at this level or RDX was not toxic in the amount of time observed by the study. A growth curve for the study can be seen below in Figure 1. Additional studies are needed to determine if 35 mg/L would be toxic over a longer period of time.

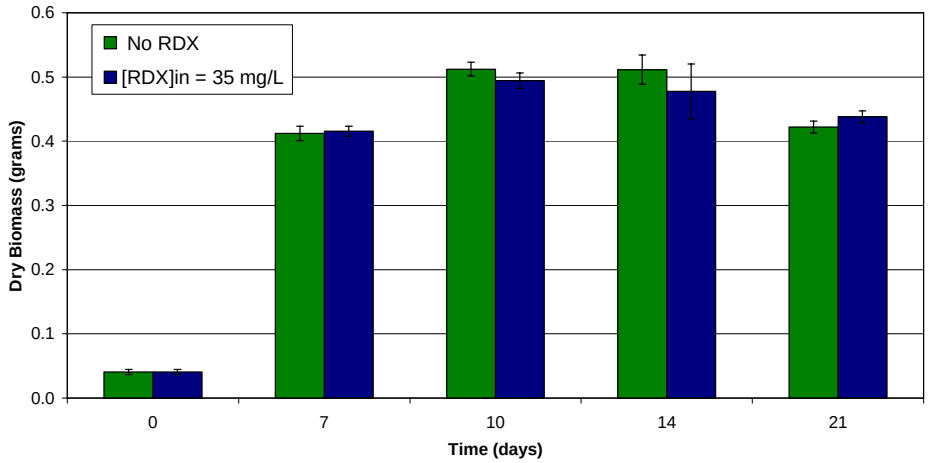

Figure 1: Effect of RDX on growth of *Arabidopsis* seedlings. RDX was added to one week old seedlings and the dry biomass was measured at several time steps. The system was maintained under sterile conditions. RDX does not appear to have an effect on the growth of the seedlings.

The results of the kinetics study can be used to observe the uptake of RDX by the plants. The RDX was taken up by the *Arabidopsis* plants. After 21 days, the plants reduced the RDX concentration from 35 mg/L to 10 mg/L (Figure 2). Initially, the RDX was removed rapidly from the media. After seven days, the uptake rate leveled out and the RDX was removed more slowly from the media. After 21 days, 70% of the initial RDX was removed from the system. Additional RDX may have been removed from the system if the experiment had proceeded past 21 days.

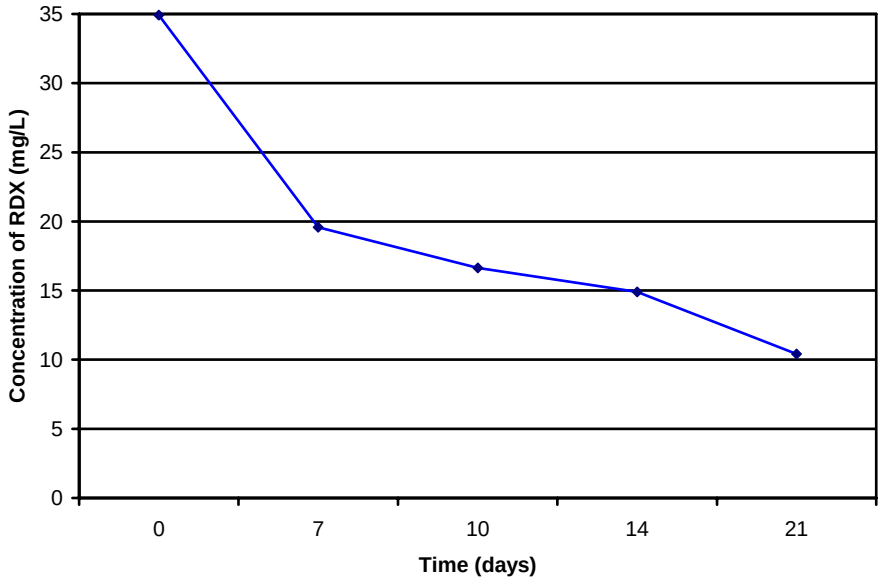

Figure 2: RDX removal from *Arabidopsis* seedlings. Extracellular RDX is shown to be taken up gradually by *Arabidopsis* seedlings, under sterile conditions. The plants were one week old when RDX was added to them. Concentrations were determined through reverse-phased HPLC, with a polar mobile phase and a Nova-Pak C8 column.

4. Conclusions

Arabidopsis exhibits toxicity to RDX, but high levels of RDX are needed to severally affect the plants. RDX was taken up *Arabidopsis*, but additional studies are needed to determine the fate and pathway of RDX in *Arabidopsis*. ^{14}C labeled experiments will allow the fate of RDX and its degradation products to be determined with more detail. These experiments will also help determine what degradation products are present with this plant system.

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Plant Transformation Pathways of Energetic Materials (RDX, TNT, DNTs)

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Summary

The energetic materials contaminating groundwater and soil are toxic to different organisms ranging from bacteria to mammals, including plants. Various plants have been shown the ability to remove and transform the energetic materials. Additionally, the fate of the energetic materials in plants after uptake and the corresponding phytotoxicity of the explosives are dependent on the plant species. Genetic and biochemical studies into the transformation pathways of the energetic materials as well as the development of transgenic plants has been undertaken to improve phytoremediation. Further research about post-harvesting fate and treatments is warranted.

Introduction

2,4,6-trinitrotoluene (TNT) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) are commonly found in surface soil and groundwater at ammunition production sites and military training sites. It is reported that 2000 facilities of the U.S. Department of Defense are contaminated with explosives in both soil and groundwater (Medina *et al*., 2003). Dinitrotoluenes (DNTs) such as 2,4-dinitrotoluene (2,4-DNT) and 2,6-dinitrotoluene (2,6-DNT) contaminated the sites as by-products of TNT. TNT and DNTs are classified as nitroaromatic explosives having aromatic ring structures while RDX belongs to nitramine explosives possessing N-nitro groups (Hannink *et al*., 2002). The explosives are transported to soil and groundwater after open detonation, seepage, and improper disposal at military and munition production sites. TNT and DNTs have higher octanol-water partition coefficients (K_{ow}) values than RDX suggesting that they will be strongly bound to organic matters in soils whilst RDX is very mobile due to its poor sorption. The explosives are not volatile due to their low vapor pressures. The physical and chemical properties of the explosives are shown in Table 1.

Several studies have reported abiotic treatments of explosives, such as incineration, carbon adsorption, alkaline hydrolysis, and catalytic and advanced oxidation (Garg *et al*., 1991; Rodgers and Bunce, 2001). Harmful by-products, requirement of further treatments, and transport of contaminated soils or groundwater have drawn attention to bioremediation. Phytoremediation is a promising technology using plants to clean up contaminated soil and groundwater *in situ* because of the low cost of maintenance and operation, and public acceptance (Schnoor *et al*., 1995). The fact that plants are able to accumulate metals to a high concentration inside their tissues has been well known (Salt *et al*., 1998). Phytoremediation research has been conducted on organic pollutants ranging from pesticides, i.e., atrazine (Burken and Schnoor, 1997) to industrial pollutants such as trichloroethylene, and polycyclic aromatic hydrocarbons (PAH) (Newman *et al*., 1997; Paquin *et al*., 2002; Vervaeke *et al*., 2003). The transformation products of xenobiotics by plants are less toxic than parent compounds. In addition, root exudates are reported to enhance microbial activity for degradation of xenobiotics (Miya and Firestone, 2001; Anderson *et al*., 1993). The

cost for soil remediation is 10-100 dollars per cubic meter while only 0.02 - 1 dollar per cubic meter is required for vegetative clean up of contaminated soils (Cunningham *et al*. 1995).

This short review summarizes the toxicity and transformation pathways of energetic materials (TNT, RDX, and DNTs) by plants.

Toxicity of the Explosives

Toxicity of TNT and RDX to various organisms has been studied. TNT and its degradation products have been reported to be mutagenic and toxic to several organisms. The survival of the midge (*Chironomus tentans)* decreased significantly after exposure to 200 mg/kg of TNT, 1,3,5,-trinitrobenzne (TNB), and 2,4-diamino-6-nitrotoluene (2,4-DANT); and the amphipod (*Hyalella azteca*) was more susceptible to TNT, TNB, and 2,4-DANT than the midge (Steevens *et al*., 2002). Gogal *et al*. (2002) reported that northern bobwhite quail exposed to TNT showed decreases in total red blood cell counts and plasma protein as dietary TNT intake increased, and they determined a low observed-adverse-effect level of 178 mg of TNT/kg of weight /day. Survival and growth of two freshwater invertebrates were not affected after a 10 day-exposure period at 1,000 mg of RDX/ kg of sediment (Steevens *et al*., 2002). The growth and survival of benthic invertebrates, *Neanthes arenaceodentata* and *Leptocheirus plumulosus* were not affected by exposure up to 1,000 μg/g RDX based on the dry weight of sediment (Lotufo *et al*., 2001). Inhibition to growth and reproduction of adult earthworms can occur at less than 95 mg/kg of RDX in artificial soil (Robidoux *et al*., 2000 and 2001), but acute toxicity was not observed up to 756 mg/kg based on dry soil for RDX.

Gong *et al*. (2002) investigated the influence of RDX on indigenous microbial activities. They measured soil dehydrogenase activity, potential nitrification activity, heterotrophic nitrogen fixation activity, substrate-induced respiration, and basal respiration for 12 weeks. Significant reduction (up to 30 % of control) of those parameters was observed in RDX spiked soil. In the case of luminescent marine bacterium (*Vibrio fischeri*), the EC_{50} value of RDX (115.5 mg/L) was above the solubility in water (42 mg/L for RDX) after incubation periods of 90 min (Drzyzga *et al*., 1995).

Both 2,4-DNT and 2,6-DNT were not mutagenic with Ames assay, but their hydroxylamine isomers proved to be mutagenic (Padda *et al*., 2003). Using the uptake response of H4IIE rat hepatoma cell cultures to neutral red, the NR_{50} values were 45 mg/L for 2,4-DNT, 50 mg/L for 2,6-DNT, and 7mg/L for TNT suggesting dinitrotoluenes were less cytotoxic than TNT (Mitchell and Burrows, 1995).

For phytotoxicity of the explosives, TNT was toxic to hybrid poplars at a concentration of 5 mg/L in hydroponic solution (Thompson and Schnoor, 1998) and at 50 mg/kg soil there were adverse effects to seed germination and seedling growth of cress and turnip (Gong *et al*., 1999). Alfalfa did not grow at 0.55 mM (100 mg/kg) of 2,4-DNT in soil (Dutta *et al*., 2003). Lettuce was the more sensitive than wheat, mustard, and lentil, indicating that phytotoxic effects of nitroaromatic explosives depended on plant species (Picka and Friedl, 2004). The highest non-observed adverse effect concentrations (NOAEC) for the growth of lettuce were 20 mg/kg for TNT, 2 mg/kg for 2,4-DNT, and 10 mg/kg for 2,6-DNT. The hydroponic toxicity of RDX to maize and wheat was estimated to be 21 mg/L RDX while soybean and sorghum did not show toxic effect up to 21 mg/L for 30 day exposures (Chen, 1993). RDX was not toxic to hybrid poplars up to 21 mg/L (Thompson 1997).

Mechanisms of Degradation of Xenobiotics by Plants

Prior to the introduction of xenobiotics to plant cells, they must be taken up by plants through roots. Several studies reviewed predictive relationships between the uptake rate of a compound and its physical-chemical properties (Briggs *et al*., 1982; Burken and Schnoor, 1998). Root uptake and translocation of the compounds are related to the logarithm of the octanol-water partition coefficient, log K_{ow}. Root concentration factor (RCF), defined as the ratio of the concentration sorbed to the roots divided by the concentration in the aqueous phase, is generally proportional to log K_{ow} values. Their relationship is proposed as the following equations:

$\log(RCF-3.0) = 0.65 \log K_{ow} - 1.57$ by Briggs *et al*. (1982)
$\log(RCF -0.82) = 0.77 \log K_{ow} -1.52$ by Burken and Schnoor (1998)

The transpiration stream concentration factor (TSCF) is calculated as the concentration in transpiration stream divided by the aqueous concentration. The values of TSCF for various chemicals show Gaussian distribution curves over the range of log K_{ow} values, indicating that hydrophilic compounds (log $K_{ow} < 1.8$) are not able to pass through lipid membranes of roots, while hydrophobic compounds (log $K_{ow} > 3.8$) tend to bind strongly to root tissues and can not then translocate from roots to shoots (Dietz and Schnoor, 2001). The relationship between TSCFs and log K_{ow} is predicted as follows:

$TSCF = 0.784 \exp\{-(\log K_{ow} - 1.78)^2/2.44\}$ by Briggs *et al*. (1982)
$TSCF = 0.756 \exp\{-(\log K_{ow} - 2.50)^2/2.58\}$ by Burken and Schnoor (1998)

Enzymatic transformation of xenobiotics by plants follows the green-liver model and involves three steps. At first, the foreign compounds taken up by plants are introduced and transformed by enzymes such as cytochrome P450, carboxylesterases, and peroxidase (Sandermann, 1994). Secondly, the transformed xenobiotics are conjugated with D-glucose, glutathione, or amino acids (Komba *et al*., 1995) by enzymes such as glutathione *S*-transferases, glucosyltransferases and malonyl-transferases, resulting in products either soluble or insoluble. The third step is storage and compartmentation. The soluble compounds are stored in vacuoles or as cell wall materials by further processing. Insoluble compounds are generally assumed to be stored in the cell wall (Schroder and Collins, 2002).

Uptake of the Energetic Materials by Plants

Nitroaromatic explosives showed different uptake and fate in plant systems than nitramine explosives. According to Thompson *et al*. (1998), 95 % of the TNT was removed from solution in less than 24 hours by hybrid poplars while 71 % of the RDX was removed from hydroponic solution in 7 days (Thompson, Ramer, and Schnoor, 1999). The uptake of both RDX and TNT in soil was slower than in the hydroponic systems because of the decrease of bioavailability of the compounds from soil. Bush beans took up less than 16 % of RDX in soil after 60 days while 60% was removed from solution after 7 days (Harvey *et al*., 1991).

Most of the radiolabel of RDX was located in the leaves unlike TNT where the radiolabel was found in roots, suggesting that RDX were translocated into leaves readily, but TNT was not. Over 60 % of the uptaken radioactivity of RDX was found in the aerial parts of hybrid poplars after 2 days. In contrast, 78 % of radioactivity of

^{14}C-TNT taken up by the poplars remained in roots after the same exposure time (Thompson, Ramer, and Schnoor, 1998).

In addition, an overall low recovery of RDX with no significant mineralization by plants suggested that final transformation products are volatile compounds (Just and Schnoor, 2000). Recently, it is reported that poplar nodule cultures mineralized RDX significantly under sterile conditions (Van Aken *et al*., 2004).

Regarding DNTs, knowledge of the uptake and transformation products by plants is limited compared to TNT and RDX. Best *et al*. (2001) applied wetland systems to remove explosives from groundwater at ammunition plants, resulting in an average 58 % and 61 % removal of 2,4-DNT and 2,6-DNT in a 115-day operation at the Volunteer Ammunition plant. Todd and Lange (1996) observed that 67 % of 2,4-DNT from soil was removed in a phytoremediation system using parrot feather. They showed that 4-amino-2-nitrotoluene (4A2NT) was found in the plant tissues after 90 hours of treatment prior to 2-amino-4-nitrotoluene (2A4NT) which was detected after 190 hours of exposure. However, other transformation products of the DNT's are unknown, as well as their fate in plants.

Transformation Pathways

2,4,6-trinitrotoluene (TNT)

Subramanian and Shanks (2003) proposed the TNT transformation pathway by plants based on experiments with periwinkle (*Catharanthus roseus*) and parrot feather (*Myriophylum aquaticum*) as shown in Figure 1.

Two monoamino compounds (2-amino-4,6-dinitrotoluene (2ADNT) and 4-amino-2,6-dinitrotoluene(4ADNT)) have been found as the primary reduction products by plants (Palazzo and Leggett 1986; Thompson *et al*. 1998; Bhadra *et al*., 1999a). Diaminotoluenes (2,4-diamino-6-nitrotoluene and 4,6-diamino-2-nitrotoluene) and azoxy compounds were observed under strong reducing conditions and by the condensation of hydroxylamines, respectively (Thompson *et al*., 1998; Sens *et al*., 1999; Pavlostathis *et al*., 1998).

As for oxidative transformation of TNT in plant systems, Bhadra *et al*. (1999b) isolated six oxidized metabolites such as 2-amino-4,6-dinitorbenzoic acid, 2,4-dinitro-6-hydroxy-benzyl alcohol, 2-*N*-acetoxyamino-4,6-dinitrobenzaldehyde, 2,4-dinitro-6-hydroxytoluene, and two binuclear metabolites from azoxytetranitro toluenes. In addition, they showed that oxidation of TNT by the plant could occur before the reductive transformation. This was based on results where monoamino compounds were added to plants and the oxidized metabolites of TNT were not produced. To date, oxidized metabolites have only been found in parrot feather (*Myriophylum aquaticum*) – they were not detected in *Catharanthus* or *Arabidopsis* (Subramanian, 2004).

2-hydroxylamino-4,6-dinitrotoluene (2HADNT) and 4- hydroxylamino-2,6-dinitrotoluene (4HADNT) were observed following reduction of nitro groups of TNT in non-axenic and aquatic plant systems (Pavlostathis *et al*., 1998; Wang *et al*., 2003). Measurement of hydroxylamines was difficult due to their instability. Wang and Hughes (1998) developed the efficient assay for hydroxylamines by derivatization with acetic anhydride. Recently, these hydroxylamines were shown to be present in axenic hairy roots of *Catharanthus* and axenic *Arabidopsis* seedlings (Subramanian, 2004; Subramanian *et al*., 2005). The hydroxylamines are considered the first transformation products resulting in other metabolites of TNT by reduction, oxidation, conjugation, and polymerization (Subramanian and Shanks, 2003; Wang *et al*. 2003).

The transformed products of TNT are further conjugated and sequestered in plant cells. Over 80 % of the TNT label was associated with plant biomass, suggesting the labeled carbon from TNT was sequestered in the plant tissues (Harvey *et al*., 1991). Thompson *et al*., (1998) showed that 75 % of the radioactivity of ^{14}C-TNT ended in the unextractable and bound residues in the poplar roots. Bhadra *et al*.(1999a) characterized the 4 conjugates of TNT metabolites with 6 carbon moiety by *C. roseus* and *M. aquaticum*. They found two of them have similar molecular structure to 2ADNT (labeled TNT-1 and 2A-1) and the rest of them are similar to 4ADNT (TNT-2 and 4A-2) indicating that the monoamines were precursors to the conjugates. Recent studies have elucidated these TNT conjugates. The conjugates of TNT metabolites by tobacco cell cultures are formed by conjugation of glucose on the hydroxylamine group of either 2HADNT or 4HADNT, and various diglycosides conjugates with gentiobioside or sophoroside forms were identified, including monoglycosides (Vila *et al*., 2005). Subramanian (2004 and 2005) showed the evidence for conjugation of monoamines and hydroxylamines with plant sugars by employing precursor-feeding studies.

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)

Studies on the transformation of RDX by plants are rarely done, while several microbial transformation pathways are proposed and established. After being taken up and translocated to leaf tissues, direct photolysis of RDX in the leaves is a feasible fate under natural sunlight. Just and Schnoor (2004) proposed the photodegradation pathway of RDX with presence of reed canary grass as shown in Figure 2. They identified ring cleavage products, such as nitrous oxide (NO_2) and 4-nitro-2,4-diazabutanal in leaves of reed canary grass under simulated sunlight, including nitrite (NO_2^-) and formaldehyde (CH_2O) in solution. Van Aken *et al*. (2004) proposed three processes for the degradation pathway of RDX by using poplar tissue cultures and leaf crude extracts as shown in Figure 2. At first, reduction products such as hexahydro-1-nitroso-1,3-dinitro-1,3,5-triazine (MNX) and hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX) were produced by intact plant cells regardless of light. In the second step, the reduced metabolites were further transformed to formaldehyde and methanol in both crude extracts and intact cultures under light. In the final step, light-independent mineralization of one-carbon metabolites by intact plant cultures, but not crude extracts, occurred. Some transformed products may be re-incorporated into plant cells. Formaldehyde may be conjugated by plant enzymes to form compounds like *S*-formyl-glutathione (Just and Schnoor, 2004). Small quantities of CO_2 produced by degradation of RDX by plants may be re-assimilated by photosynthesis (Van Aken *et al*., 2004).

Dinitrotoluenes (DNTs)

Unlike the situation with bacterial systems, little information is available on the transformation of DNTs by plants. Only one study mentioned vegetative transformation products. Monoamino isomers, 2A4NT and 4A2NT, were reported as reductive transformation products by plants (Todd and Anderson, 1996). The bacterial reduction of dinitrotoluenes can take place under aerobic and anaerobic conditions, resulting in the production of monoamines isomers (Sipegel and Welsch, 1997). Hydroxylaminotoluenes and dihydroxylaminotoluenes were produced anaerobically in *Clostridium acetobutylicum* cell cultures (Hughes *et al*., 1999). Further transformed products, aminohydroxylaminotoluenes and diaminotoluenes were observed in the cell extracts. *Hydrogenophaga palleronii* and *Burkholderia cepacia* produced

oxidative intermediates and mineralized DNTs into CO_2 by mono- or dioxygenases (Nishino *et al*., 1999). The bacteria converted 2,6-DNT into 3-methyl-4-nitrocatechol with release of nitrite, and then, 2-hydroxy-5-nitro-6-oxohepta-2,4,-dienoic acid, and 2-hydroxy-5-nitropenta-2,4,-dienoic acid (Nishino *et al*., 2000).

Transgenic Plants and Gene Expression

Significant activity in using genetic approaches for enhancing transformation or reducing phytotoxicity of energetic materials has emerged in the last six years. Genetically modified plants expressing bacterial genes have been developed for phytoremediation. Transgenic tobacco plants expressing nitroreductases of *Enterobacter cloacae* showed the enhanced ability to remove and tolerate TNT at high concentration (0.25 mM), which are toxic to their wild-type tobacco (Hannink *et al*., 2001). Another tobacco transgenic line expressing pentadrythritol tetranitrate reductase of the bacterium showed better germination and growth in the presence of TNT (0.05 mM) than wild-type plants (Rosser *et al*., 2001; French *et al*., 1999). In addition, these researchers have shown enhanced RDX removal in tobacco engineered with a XplA Cytochrome P450 from *Rhodococcus rhodochrous* (unpublished results). Clearly genetic modification with microbial redox enzymes has the potential to enable faster TNT and RDX transformation and reduced phytotoxicity.

Transcriptomic studies are providing clues to endogenous plant genes involved in transformation. Specific genes such as glutathione-*S*-transferases and cytochrome P450 in *Arabidopsis* were proposed to be involved in transformation of explosives by Ekman *et al*. (2003). They used serial analysis of gene expression (SAGE) for comparing 14-day-old *Arabidopsis* that were exposed to 15 mg/L of TNT after 24 hours to untreated plants. A glutathione-*S*-transferase was found to be highly induced up to 27 times. Among the highly induced genes are a cytochrome P450 (CYP81D11-A-TYPE), an ABC transporter which is known to expend ATP energy to transport hydrophobic molecules into or out of the cytoplasm, and a 12-oxophytodienoate reductase having high homology to nitroreductases of bacteria, *Enterobacter* sp.(Ekman *et al*., 2003). However, as noted previously, oxidative compounds were not found in *Arabidopsis* (Subramanian, 2004), thus the role of P450s in transformation pathways in *Arabidopsis* is unclear. In microarray experiments, *Arabidopsis* gene expression was monitored after long-term exposure (10 days) to different concentrations of TNT (Mentewab *et al*., 2005). In response to TNT amendment, 52 genes were upregulated and 47 genes were downregulated, and many of these genes have cell defense and cell detoxification function. Glutathione-S-transferases and cytochrome P450s were not found to be significantly upregulated in this study. Most of the genes differentially expressed were observed at the higher concentration of TNT amendment (10 μM) and genes expressed at 1 and 10 μM rarely overlapped. They confirmed the gene expressions of pathogenesis-related protein 1 precursor, DNA-binding protein, and ABC transporter-like protein by real-time PCR analysis.

The transcriptome studies provide clues to genes that may be involved in TNT transformation. Some of the genes whose expressions are upregulated may be the result of a generalized stress response and not genes encoding for enzymes involved in the TNT phytotransformation pathway or in a reduced phytotoxicity response. Reverse genetics approaches using the genes identified should enable further clarification of the transcriptome results. In a forward genetics approach, ten activation-tagged *Arabidopsis* mutant lines showing significantly better germination

rates than the wild-type on the TNT-amended media were isolated from 300,000 mutant seeds (Moon *et al*., 2004).

Selection of high-performing native plants, engineering plants with enhanced transformation capabilities, identifying the fate of transformation products in the plants, and designing the external variables to operate a more effective phytoremediation process are all dependent on a knowledge base of the genetic structure, enzymatic structure, and biochemical reaction pathways. The genetic approaches discussed here will enable the design of effective strategies for remediation of energetic materials in the future.

Conclusions and Future Directions

Plants can remove the contaminants in soil and groundwater, and transform them into less harmful compounds. Based on the information of transformation pathways and gene expressions, further studies on metabolic engineering and genetic modifications may make plants tolerant to higher concentrations of the xenobiotics by using less toxic metabolic pathway and faster rates of uptake. In addition, the explosives taken up by plants can be released from the plant tissues by action of water e.g. rain and river, and thus may be replenished to the environment as hazardous contaminants. Research of further treatment and the post-harvest fate of explosives is required. Information about phytoremediation of dinitrotoluenes is insufficient compared to TNT and RDX; thus, it is worthwhile for further investigation as well.

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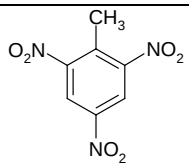
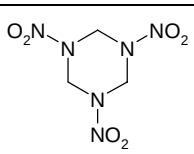
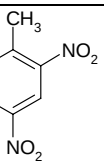
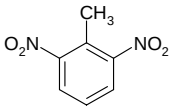
Table 1. Chemical and physical properties of TNT, RDX, and DNTs.

	TNT	RDX	2,4-DNT	2,6-DNT
Molecular Weight	227.15	222.26	182.14	182.14
Molecular Formula	$C_7H_5N_3O_6$	$C_3H_6N_6O_6$	$C_7H_6N_2O_4$	$C_7H_6N_2O_4$
Log K_{ow}	1.6-1.84	0.81-0.87	1.98	1.9-2.10
Solubility in water (mg/L)	100	42	270-273	910
Vapor Pressure (mmHg)	1.99×10^{-4}	$1.0\text{-}4.0 \times 10^{-9}$	1.47×10^{-4}	5.67×10^{-4}
Henry's Constant (atm-m^3/mole)	4.57×10^{-7}	1.2×10^{-5}	1.3×10^{-7}	9.26×10^{-8}
Molecular structures				

Data taken from Yinon and Zitrin, (1993); Talmage *et al*., (1999), HSDB (2000)

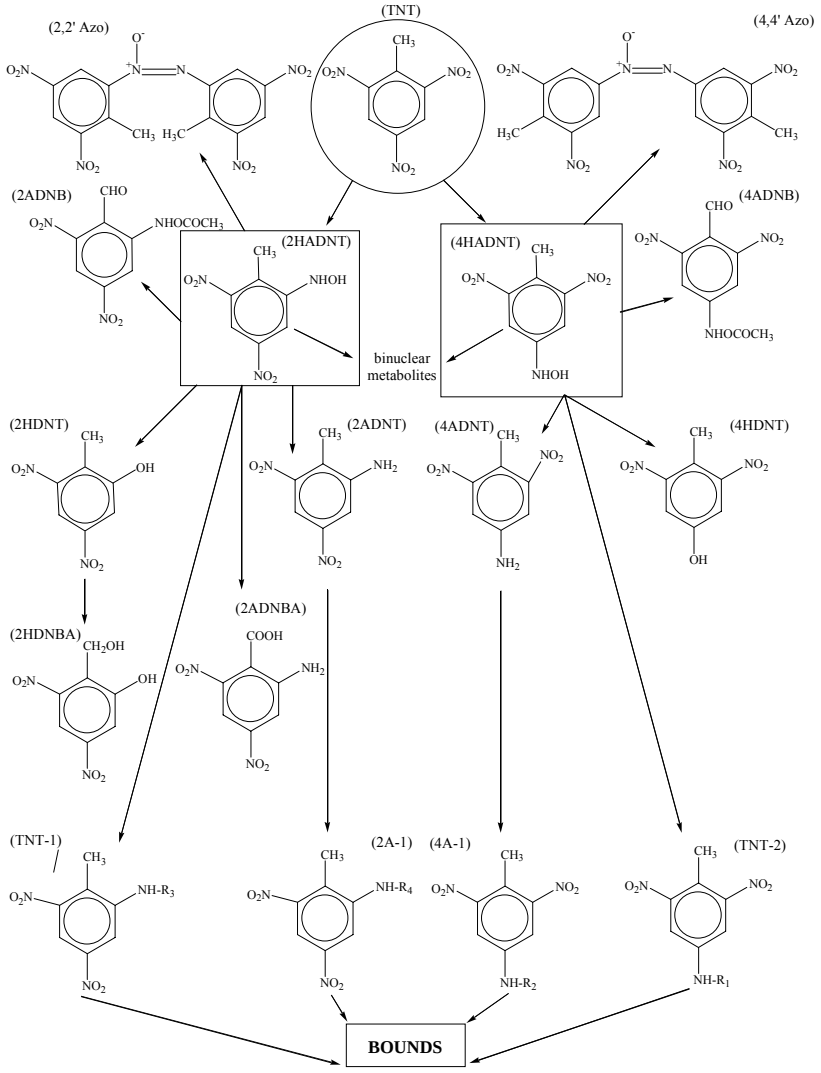


Figure 1. TNT transformation pathway by Subramanian and Shanks (2003). Abbreviations-TNT: 2,4,6-trinitrotoluene; 2ADNT: 2-amino-4,6-dinitrotoluene; 4ADNT: 4-amino-2,6-dinitrotoluene; 2HADNT:2-hydroxylamino-4,6-dinitrotoluene; 4HADNT: 4-hydroxylamino-2,6-dinitrotoluene; 4,4'Azo: 2,2',6,6'-tetranitro-4,4'-azoxytoluene; 2,2'Azo: 4,4',6,6'-tetranitro-2,2'-azoxytoluene; 2HDNT: 2-hydroxy-4,6-dinitrotoluene; 4HDNT: 4-hydroxy-2,6-dinitrotoluene; 2ADNB: 2-*N*-actamido-4,6-dinitrobenzaldehyde; 2ADNB: 4-*N*-actamido-2,6-didnitrobenzaldehyde; 2HDNBA: 2-hydroxy-4,6-dinitrobenzyl alcohol; and 2ADNBA: 2-amino-4,6-dinitrobenzoic acid. TNT-1, TNT-2, 2A-1 and 4A-1 represent conjugates with six carbon sugars (R1, R2, R3, and R4).

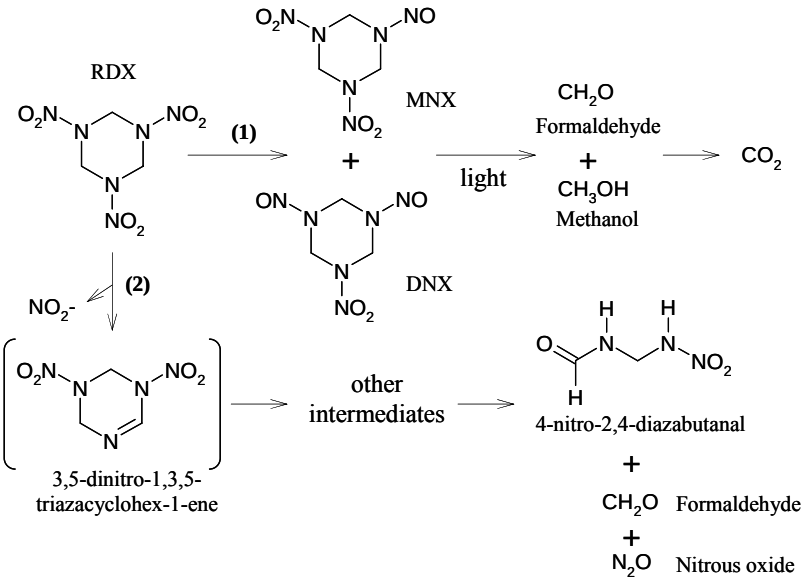


Figure 2. RDX degradation pathways proposed by (1) Van Aken *et al*. (2004) and (2) Just and Schnoor (2004). The bracketed compounds were not observed. Abbreviations-RDX: hexahydro-1,3,5-trinitro-1,3,5-triazine; MNX: hexahydro-1-nitroso-1,3-dinitro-1,3,5-triazine; and DNX: hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine.

TNT phytotransformation in native and genetically modified species: Significance of aromatic hydroxylamines in the metabolic pathway

by

Murali Subramanian

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ABSTRACT

The presence and role of hydroxylamines in TNT transformation were ascertained in two axenic (microbe-free) hydroponic plant systems through high TNT feeding experiments and the use of different analytical detection schemes. Hydroxylamines were formed within 10 hours of TNT addition, and usually disappeared within 20 hours, displaying a high turnover rate due to their instability and high reactivity. Hydroxylamines were shown to add biomolecules to their functional group and thereby directly form conjugates. The 4-isomer of the hydroxylamine was detected in very high levels (up to 30% of initial TNT added) in a transgenic tobacco system that had a bacterial nitroreductase inserted into its genome. It was shown that the bacterial enzyme was responsible for the formation of hydroxylamine, which disappeared rapidly from the media. These transgenic tobacco seedlings transformed TNT efficiently and were more resistant to high levels of TNT than the wild-type seedlings. A radiolabeled TNT mass balance was completed on Arabidopsis to delineate all branches of the TNT transformation pathway. It was observed that TNT ended up as intracellular-bounds, polymerized and bound to the plant biomass. The proportion of bounds reached greater than 80% by 168 hours representing complete TNT transformation. Other aspects of the Arabidopsis TNT transformation were also probed, and some specific characteristics of the pathway such as preference for 4-substituted metabolites were observed. Pseudo-first order rate constant estimates were obtained for various branches of the transformation pathway to reveal the rate-limiting steps of TNT transformation. Arabidopsis mutants were also analyzed to detect if their mutations had altered their metabolite profile. These mutants had demonstrated resistance to TNT toxicity in mutant library screening studies. The mutants were found to have greater resistance to TNT than wild-type seedlings, but did not appear to possess any specific advantages in the rate of TNT transformation. This seemed to indicate that the mutations did not target the TNT transformation biochemical pathway; rather they allowed these mutants to be more resistant to TNT. Based on all these experiments, a better understanding of TNT metabolism and the role of its transformation pathway in improving rates of phytoremediation have been obtained.

Chapter 1: Introduction

Pollution is an unfortunate by-product of development. From synthetic chemicals to energetic materials, every step of progress has seen the problem of pollution increase in magnitude. This problem reached massive proportions during the industrial revolution, and subsequently the science of cleaning up polluted wastes and preventing pollution received more attention. However, in spite of the advances in contaminant cleanup, pollution remains a serious problem in society. One of the chemicals contaminating the biosphere is the energetic material 2,4,6-trinitrotoluene (TNT) which is used in weapons and construction. Synthesized first in 1863 by the German scientist Joseph Wilbrand, commercial manufacture of TNT began by 1891 and by World War I it had replaced picric acid as the standard energetic. The widespread use of TNT in the 20th century has however had environmental ramifications. The manufacture, storage, transport and most importantly, testing of weapons has led to the contamination of over 40 land sites in the US alone (Spain, 2000). TNT contaminates these sites jointly with hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazine (HMX) with potentially adverse environmental, social and ecological consequences. These chemicals have known toxic and mutagenic effects on the local ecosystem. TNT has documented toxicity (Yinon, 1990) to humans (Nagel, Schmidt *et al.*, 1999), plants (Palazzo and Leggett, 1986), and microorganisms (Won *et al.*, 1976). The seriousness of energetic pollution of land sites is underscored in Massachusetts Munitions Range where RDX from the land has seeped into the ground water resources (EPA, 2004). Since TNT binds strongly to the soil, it has not, thus far, been detected in the ground water at this site although the possibility remains. Additionally, the re-vegetation of polluted sites can cause the entry of these energetic materials into the food chain (Palazzo and Leggett, 1986; Harvey *et al.*, 1990; Nagel, Scheffer *et al.*, 1999).

These legitimate concerns have spurred efforts to remediate these and other contaminated sites with a host of cleanup strategies. There exist, broadly, three branches of pollution abatement: physical, chemical and biological. While physical processes deal with operations such as thermal treatment and filtration, chemical processes usually involve a strong oxidizing or reducing agent to degrade the pollutant. Of recent origin are the

biological processes which use plants, bacteria, fungi and any biological slurry to decontaminate wastes. Phytoremediation of TNT, the subject of this dissertation, falls under this category.

Phytoremediation, the application of plants and plant-based systems to clean up polluted sites, is derived from *phyton* (Greek for plant), and *remedium* (Latin for remedy). Phytoremediation, as a concept, was first recognized in 1966 when the uptake of fluorine from air by plants was observed (Jacobson *et al.*, 1966); subsequently, phytoremediation was shown to have a role in water purification (Felgner and Meissner, 1968). However, phytoremediation as a technology is still a specialized niche area with only a handful of real-world applications. This is changing, with more traditional environmental companies investing in phytotechnologies as an alternative to physical and microbiological cleanup. Field proof of principle for the phytoremediation of land and groundwater contaminated with selenium, trichloroethylene (TCE), methyl tertiary butyl ether (MTBE), perchloroethylene (PCE) and carbon tetrachloride (CCl_4) has been demonstrated (McCutcheon and Schnoor, 2003).

Currently, the annual market for phytoremediation stands at the order of 10^7 billion US dollars, compared to the pharmaceutical industry at 10^9 billion US dollars (McCutcheon and Schnoor, 2003). Since phytoremediation has minimal capital investment requirements, low raw material requirements and is not manual-labor intensive, it is capable of delivering the most cost-effective cleanup of a contaminated site. In addition to these economic advantages, phytoremediation is also an eco-friendly, sustainable technology that utilizes solar energy as its main driving force. In light of these factors, phytoremediation has a high public acceptance (Table 3.1). In contrast, the currently used cleanup technology for energetic material-contaminated soil is incineration (Ahmad and Hughes, 2000). Not only does this require excavation and transport of the soil, but it also produces secondary pollutants in the form of smoke. This makes incineration an expensive, unsustainable process. There exist newly developed processes for the cleanup of contaminated soils, such as UV-radiation and pump and treat methods with carbon filters. However, these technologies have high material and labor costs associated with them, and hence are expensive (Ahmad and Hughes, 2000).

Biological removal of contaminants is a process with immense potential. Composting, wherein the soil is exposed to mixed populations of native bacteria, fungi and rhizoidal microorganisms, is a technology that has shown promise. Bioremediation, the microbiological analogue of phytoremediation, has also been demonstrated for a variety of pollutants and media. Biological schemes of remediation, however, suffer two main disadvantages compared to phytoremediation. Composting requires mixing and aeration of the soil and bioremediation requires the use of lagoons or reactors to obtain effective cleanup. In contrast, phytoremediation systems do not require these additional steps since the plants grow into the contaminated soil, which helps maintain low cleanup costs. The second, and more important advantage of phytoremediation is that microbes being heterotrophs require external substrates for their growth and maintenance, while plants are self-sustaining autotrophs capable of producing their own nutrition using photosynthesis. Hence, plant-based remediation schemes entail lower maintenance requirements and costs compared to microbial remediation.

While phytoremediation has the potential to overcome many of the drawbacks associated with other processes, it suffers from its own liabilities, chief amongst them being a slow rate of remediation (Table 3.1). In addition, high concentrations of the contaminants can prevent or stunt growth of the plants. Even if plants can survive these elevated concentrations, they can take months to a few years for complete remediation. For phytoremediation to be a viable option in the cleanup of such sites, its long process time and limitations due to toxicity of the contaminant need to be overcome. These are not issues of engineering or implementation, but drawbacks with the process itself. Hence, overcoming slow rates and high toxicity requires basic research in addition to experiments at the implementation level.

1.1: Dissertation Format

This dissertation deals specifically with the analysis of plant metabolic pathways in the transformation of TNT. Metabolic pathway analysis is a powerful tool that can be of considerable use in understanding and improving TNT transformation. It involves determining information on the metabolites (reaction products), the concentration profiles of

these metabolites, sequence of reactions, rates of each branch of the pathway and final fate of the xenobiotic. Once this information has been compiled, the genes and enzymes involved in each step of the pathway can also be determined. In addition, toxicity pathways and mechanisms of individual reactions can be ascertained. Such a database for TNT transformation that includes details on metabolites, kinetics, enzymes and genes can be of considerable utility. With this knowledge it is possible to suggest potential pathway modifications that will result in improved TNT removal efficiencies, lowered formation of toxic intermediates and enhanced formation of preferred products. This will help make phytoremediation of TNT a faster, more robust process and hasten its application in the field. The following sections lay out the details of each chapter, and make a case for the role of pathway studies in improvement of phytoremediation rates.

Studies in Chapters 3 and 4 demonstrate the presence and role of hydroxylamines (2-hydroxylamine-4,6-dinitrotoluene, 2HADNT and 4-hydroxylamine-2,6-dinitrotoluene, 4HADNT) in the TNT transformation pathway. These are difficult to detect metabolites formed early in the TNT transformation pathway. Chapter 3 proves its presence in two axenic (microbe-free) systems using high concentration TNT feeding experiments, while Chapter 4 delineates the various reactions the unstable hydroxylamines can undergo. These studies also revealed other openings in the pathway that could be exploited for improving the efficiency TNT transformation. For example, curbing the formation of a certain group of metabolites (the monoamines) and encouraging the formation of other products (such as conjugates or azoxies) may speed up the rate of TNT removal. The advantage of this technique is that genetic modifications do not need to be made to the system; rather amending the environment may produce the desired effect. Pathway information can therefore help suggest conditions conducive to faster TNT removal.

The knowledge of hydroxylamines obtained from Chapters 3 and 4 was utilized in Chapter 5 in the analysis of transgenic tobacco seedlings. These seedlings had a bacterial enzyme (nitroreductase) inserted in them which allowed for much faster TNT uptake. Metabolic profiling of these transgenic seedlings upon TNT exposure revealed high amounts of the potentially toxic 4HADNT in the media and seedlings. However, rapid removal of this metabolite prevented its toxic effects from killing the plants, and the transgenic seedlings, in

fact, displayed better health and greater TNT resistance than the wild-type plants. Pathway studies were thus able to accurately determine the effect of the bacterial enzyme expressed in the plant, and determine the specific advantages conferred. Results from this chapter have been prepared for publication in the journal Plant Physiology (ASPB Publication). While transgenic plants have the greatest power to improve TNT removal efficiencies, abundant caution is necessary in their application. The release of transgenics in nature is not fully understood and achieving public and regulatory acceptance will be a huge challenge. Genetic drift between species is possible; studies examining the effect of over-expressing TNT-transformation related enzymes (such as nitroreductases and Cytochrome P450 monooxygenases) on the health of plants are essential.

Chapter 6 presents a complete analysis of TNT uptake characteristics in Arabidopsis (mustard), the preferred system for plant molecular biologists. A complete mass balance on all the branches of the TNT transformation pathway was completed using ^{14}C radiolabeled TNT. The differences in transformation characteristics in this system when compared to those reported in literature were highlighted. Results from Chapters 3, 4 and 6 have been compiled and prepared for publication in Environmental Science and Technology (ACS publications). Chapter 7 used the basic understanding of TNT transformation from Chapter 6 to analyze Arabidopsis mutants. These mutants were screened from libraries of randomly generated mutants and demonstrated enhanced TNT resistance capabilities. The mutants were tested against a wide range of TNT concentrations to determine those with most resistance. Genetic information on the mutations was obtained and the metabolic and genetic information correlated to determine the general nature of the mutations. Further strategies to refine these studies have been outlined in Chapter 8.

While field studies on TNT phytoremediation are very important, a necessary improvement in the rates of phytoremediation will require metabolic pathway studies. In addition, these studies can help delineate modified transformation steps in genetically altered plants, which is necessary for the regulatory approval. These factors have been addressed in this dissertation, with specific emphasis on the role of hydroxylamines and the characterization of transgenic and mutant pathways.

Table 1.1: Advantages and Limitations of Phytoremediation (EPA, 1998)

Advantages	Limitations
In-situ	Limited to shallow soils, streams and groundwater
Passive	High concentrations of hazardous materials can be toxic to plants
Solar	Mass transfer limitations
Costs 10 to 20% of mechanical treatments	Slower than mechanical treatments
Transfer is faster than natural attenuation	Only effective for moderately hydrophobic contaminants
High public acceptance	Toxicity and bioavailability of degradation products is not known.
Fewer air and water emissions	Contaminants may be mobilized into the groundwater
Generate less secondary wastes	Potential for contaminants to enter food chain through animal consumption
Soils remain in place and are usable following treatment	Unfamiliar to many regulators

Chapter 2: Review of Literature

Sections of this chapter have been published in "Role of Plants in the Transformation of Explosives", by M. Subramanian and J. V. Shanks, in the book "Phytoremediation: Transformation and Control of Contaminants", edited by Steven C. McCutcheon and Jerry L. Schnoor, Wiley Publications, 2003.

2.1: Overview of Plant-Assisted Remediation

The removal of organic compounds and metals from soil and water by plants is a well-established procedure, although much remains unknown about the biochemistry and genetics involved. Excellent general reviews on bioremediation of foreign-compounds (Lenke *et al.*, 2000; Nishino *et al.*, 2000; Esteve-Nunez *et al.*, 2001), and phytoremediation of organics and metals (Salt, 1998; Burken *et al.*, 2000; Macek *et al.*, 2000; Meagher, 2000; Trapp and Karlson, 2001) have been published in recent years. This review will focus mainly on the salient and relevant aspects of phytoremediation of 2,4,6-trinitrotoluene (TNT).

Plants possess an array of mechanisms to protect themselves from xenobiotics, ranging from altering the physical conditions of the soil to taking up and immobilizing the compounds (Figure 2.1). These mechanisms eventually reduce the bioavailability of these toxic pollutants that assists in remediating the site or water body. Phytoremediation, the process of remediating sites using plants, encompasses all these types of pollution-abatement mechanisms. Plants, by their mere presence in the ecosystem, alter the water balance, the redox potential and pH of the soil, and stimulate microbial activity in the soil (Burken *et al.*, 2000; Trapp and Karlson, 2001). By increasing the transpiration of water from the soil, plants reduce the possibility of explosives reaching groundwater sources, and increase protection against erosion (Trapp and Karlson, 2001). Plants also affect the physio-chemical parameters of the soil by lowering the pH of the soil (Schnoor *et al.*, 1995) and increasing the oxygen flux in the soil pores (pores emptied of their water by transpiration) (Trapp and Karlson, 2001). The other mechanisms that are usually referred to as falling within the purview of phytoremediation include phytoextraction, phytodegradation, phytovolatization, phytostabilization and rhizodegradation (Figure 2.1), (Burken *et al.*, 2000). Phytoextraction refers to the mere uptake and concentration of contaminant such as metals, usually in a

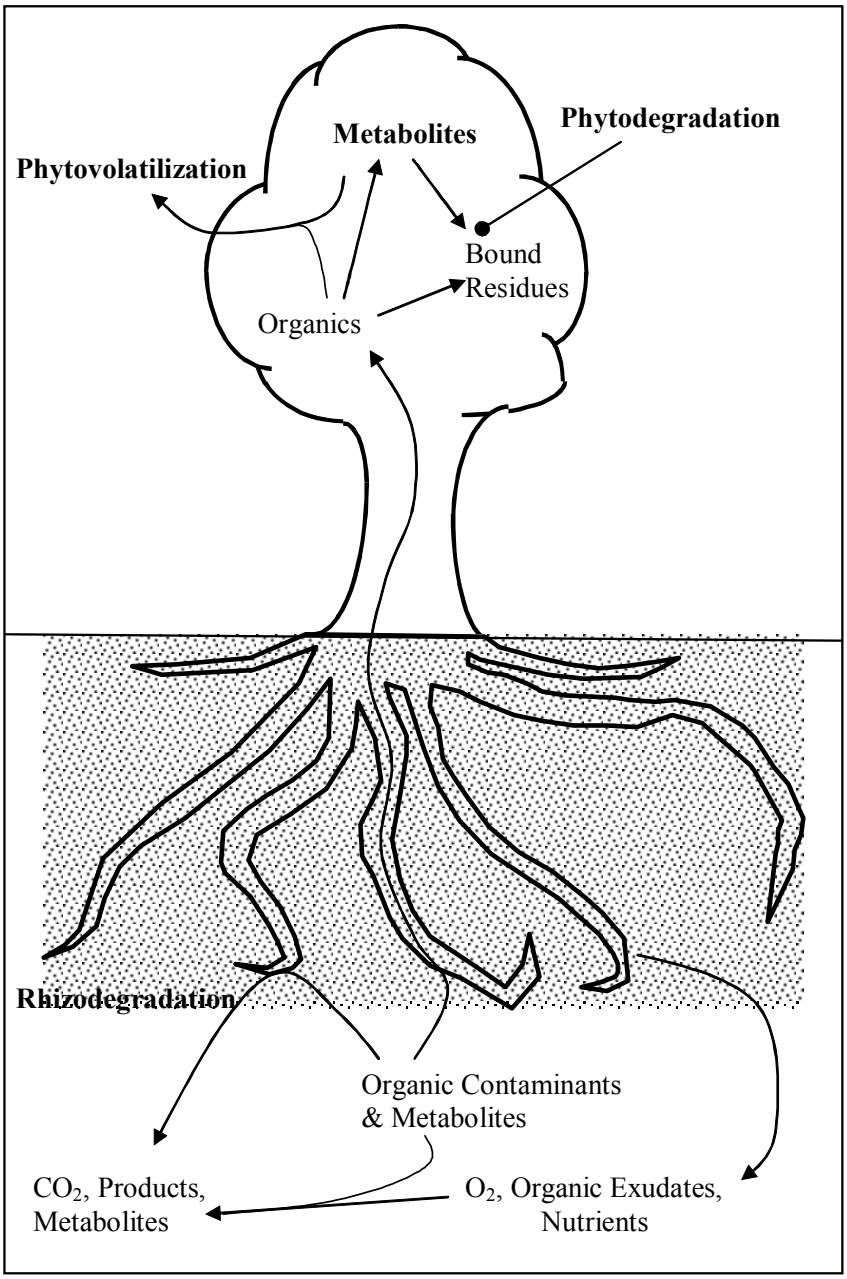


Figure 2.1: Plant-Assisted Remediation: Phytoremediation mechanisms in a terrestrial, deep-rooted plant. Rhizodegradation, phytovolatilization, and phytodegradation pathways are shown and are the potential degradation and removal mechanism for organic compounds. Figure adapted from (Burken *et al.*, 2000).

recoverable form (Salt, 1998). Ultimate disposal of the plant by harvesting and incineration is a conceivable solution to the pollution problem at the site. Phytodegradation, the process of uptake and transformation of the contaminant by the plant enzymes, is observed in TNT metabolism experiments on a lab scale (Salt, 1998). This is the most common form of phytoremediation for organics, and the most promising. Phytovolatization is the ability of plants to volatize the compound down to its gaseous form and has been observed for trichloroethylene (TCE) and some metals (Salt, 1998; Burken *et al.*, 2000). This is a highly desirable end-point for nitroaromatic removal. Phytostabilization is the immobilization of the compounds in the soil, or the stabilization of the soil to prevent its erosion (Trapp and Karlson, 2001). Rhizodegradation refers to the action of the microbes living in the rhizosphere, the microbe-rich zone in contact with the roots. The supply of nutrients like phosphate or nitrogen and availability of a habitat on the plant roots provide for a symbiotic relationship between plants and microbes, which stimulate the growth of microbes along the plant roots (Trapp and Karlson, 2001). These microbes may partially or fully degrade the pollutants in the soil matrix. While, a desirable end-point for organics removal from the soil would be mineralization, i.e. degradation of the pollutant-derived carbon to CO_2, this is rarely achieved and if observed, is in very small percentages (Burken *et al.*, 2000; Nishino *et al.*, 2000).

All pollutants, however, are not uniformly taken up and transformed by plants. One of the key parameters in determining the physical uptake of the pollutants from the ground into the plant is the octanol-water coefficient (log K_{ow}), which is a measure of the lipophilicity of the compound (Briggs *et al.*, 1982). Compounds with high polarity (low lipophilicity) cannot pass biological membranes, while compounds with low polarity tend to adhere too strongly to the roots; hence, there exists an optimum polarity region for uptake of contaminants by plants (Trapp and Karlson, 2001). Compounds with octanol-water coefficient within the range of 0.5 to 3.0 can be transported into the plant. TNT has a log K_{ow} of 1.60 which lies within the optimal octanol-water coefficient for plant uptake (between 1.5 and 2.0) (Briggs *et al.*, 1982). While these physical and chemical characteristics influence the ultimate response of the plant to the compound, the biology of the process is also of great interest.

2.2: Motivation for Understanding Plant Metabolism of Xenobiotics

The final application of phytoremediation on contaminated sites depends on disparate factors such as toxicity of the pollutant to the plant, speed of remediation, use of native plants, regulatory approval and local acceptance. However, to reach a stage where it is conceivable to use plants on the field requires prior knowledge of the metabolism of the contaminants by the plant. Understanding plant transformation pathways at three different levels – metabolites, enzymes and genes – will prove to be important for several reasons (Figure 2.2). The final fates of the pollutant and its transformation metabolites are crucial for regulatory reasons and as a measure of the effectiveness of the process. Some studies indicate that plant growth may be severely inhibited by explosives even at low concentration (Palazzo and Leggett, 1986), while many other studies report an absence of conspicuous deleterious effect of explosives on plants (Salt, 1998; Bhadra, Spanggord *et al.*, 1999). Plant health after prolonged exposure to the pollutants, and effectiveness of plants in pollution control over continued periods are further factors that can be better understood after carrying out basic studies in transformation pathways and analyzing the products formed. The maximum concentration of pollutants a given plant species can effectively remove is another parameter that needs attention, since it imposes an upper-limit on the applicability of phytoremediation. The final fate of the contaminant is extremely important in the design of successful systems. It is important to be able to assess when the process of phytotransformation is complete, the residual toxicity of the plants used in the decontamination process, and final bioavailability of the contaminant. This will determine the time scales involved in the process, and whether the plants need to be harvested and incinerated, or if the resultant biomass lacks any measurable toxicity. All these parameters need to be deduced in prior metabolite-scale experiments.

Understanding the types of enzymes and corresponding genes involved is important, since they suggest not only a mechanism for transformation, but perhaps also a way of improving efficiency by generating genetically modified plants, with the sole purpose of transforming pollutants (French *et al.*, 1999; Doty *et al.*, 2000). This would have several inherent advantages, one being more efficient pollutant removal since the key enzyme(s)

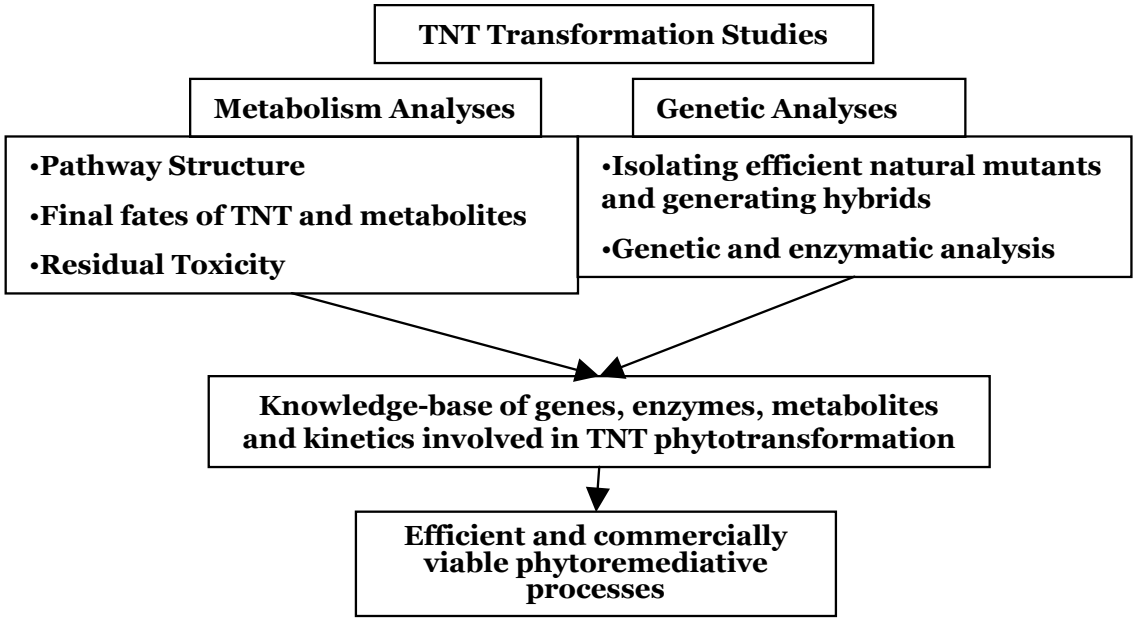


Figure 2.2: Role of Pathway Studies: While field-scale studies are very significant, basic metabolism-scale studies are important too as they help isolate natural mutants and generate efficient hybrids. In addition, the toxicity and end-points of phytoremediation are fully understood.

involved in flux-limitation(s) in the pathway can be over-expressed. Ideally, this would result in a faster rate of pollutant removal, lower biomass requirements and well-characterized fate of the pollutants. Another potential advantage would be phytotoxicity management. Some of the TNT transformation products in the natural environment, both from soil bacteria and from plants, are phytotoxic (detrimental to plant growth) (Tadros *et al.*, 2000). Metabolic engineering of the pathways to decrease levels of these compounds may be possible, resulting in plants that may tolerate higher initial levels of the pollutants. In addition, new routes of degradation through the application of foreign genes may be emphasized, resulting in less-toxic metabolites than those produced by the traditional pathway.

2.3: Experimental Systems Used in TNT Transformation Studies

Axenic or microbe-free cultures of plants are used extensively in phytotransformation studies in the lab. When whole plants transplanted from soil are used in phytotransformation studies, there are associated bacteria present too which have their own capacity to transform TNT. Hence, isolating the specific role of plants in TNT transformation is not easily achieved in these systems. Using sterile tissue cultures in the lab help isolate the unique role of plants in the transformation of contaminants. Since TNT is a widespread pollutant found in many contaminated land and water systems, it has been a subject of widespread research. Studies concentrating on remediating TNT using plants have systems from terrestrial plants to aquatic species to tissue-cultured plants in the lab. All these systems have shown the ability of plants to take up and transform TNT to varying extents. Of all these systems, axenic cultured hairy roots of *C. roseus* (Figure 2.3) and native *M. aquaticum* plants (Figure 2.4) have been extensively analyzed for the metabolites produced and the TNT transformation pathway. These systems offer their own unique set of advantages and drawbacks. *C. roseus* hairy root cultures, in particular, offer a number of significant advantages over using whole plants in TNT-transformation pathway studies. They are genetically stable and the results obtained are highly reproducible (Shanks and Morgan, 1999). They are experimentally versatile and large amounts of biomass can be generated in a controlled setting, as they can be grown in shake-flasks and bioreactors (Shanks and Morgan, 1999). However, in the

Figure 2.3: ***C. roseus*** **roots**

Figure 2.4: ***M. aquaticum*** **Plants**

Figure 2.5: Arabidopsis Seedlings: Two week old axenic *Arabidopsis* seedlings grown from 50 sterile seeds at a temperature of 25 Celsius and 100 rpm in a 250 ml flask with 50 ml of MS media, showing the development of roots and leaves.

context of phytoremediation, the key advantage in using them would be to obtain a definite verification of the capacity of plants to transform explosives, since the cultures are fully free of microbial contamination. Therefore, all results obtained using *C. roseus* are indicative of the potential of plant roots to metabolize TNT, and do not include any microbial or symbiotic relationships. Another significant advantage in using root cultures to carry out pathway studies would be the simplified plant physiology involved, relative to using whole plants. Phenomena like transport of substrates into the vascular bundle and leaves and photosynthesis could be conveniently excluded, while greater emphasis would be placed on the biological mechanisms roots adopt to counter nitroaromatics. This simplified model system helps in better elucidating pathway information from the observations. In addition, since studies indicate that TNT transformation occurs predominantly in the root zone, *C. roseus* roots are ideally suited for such studies.

Axenic cultures of the aquatic plant species *M. aquaticum* have been grown (Hughes *et al.*, 1997), but this system is problematic as the axenic plants are not as robust as their native counterparts. The important advantage of native *M. aquaticum* is its documented TNT removal abilities and its importance in the field, as it has been used in various field studies for wetland systems (Best *et al.*, 1997; Best *et al.*, 1999). The whole plant may give different results than a specific organ culture, namely difference between tissues in isolation versus an intact plant. Any similarities observed between results among *M. aquaticum* and *C. roseus* have interesting implications for plant selection criteria, as they are two widely different plant species. Similarities in TNT transformation point to the possibility that several different plant species may utilize similar metabolic schemes.

Axenically grown Arabidopsis (Figure 2.5) is currently being extensively used by researchers in TNT metabolism because of the variety of powerful genetic tools available. Arabidopsis belongs to the mustard (Brassicaceae) family, which also includes cabbage and radish. Seedlings of Arabidopsis can be grown under sterile conditions in the lab, starting from seeds, ensuring reproducibility of results. With the sequence of the Arabidopsis genome completed, emphasis is being placed on determining the genes implicated in TNT transformation. Genetic analysis of Arabidopsis mutants resistant to TNT may lead to knowledge of the enzymes involved in detoxifying the explosive, and this knowledge can be

used in ex-situ enzymatic systems, or the construction of hybrids or transgenics for remediation. However, for genetic and transcriptional studies to yield useful information, they need to be supplemented with metabolic and pathway information. A recent paper on TNT transcription studies to determine genes involved in TNT transformation draws heavily on metabolite and pathway studies to speculate on the genetics of TNT metabolism (Ekman *et al.*, 2003). In addition, metabolite and pathway studies on transgenic tobacco helped isolate the effect of the inserted gene on TNT transformation (Chapter 5).

2.4: "Green Liver Model" of TNT Transformation

Most research to date on phytoremediation of explosives has concentrated on elucidating the ability of plants to transform TNT. Several small-scale studies show that plants possess an inherent capacity to remove TNT and other nitroaromatics from soil and water (Palazzo and Leggett, 1986; Gorge *et al.*, 1994; Best *et al.*, 1997; Hughes *et al.*, 1997; Burken and Schnoor, 1998; Pavlostathis *et al.*, 1998; Rivera *et al.*, 1998; Salt, 1998; Scheidemann *et al.*, 1998; Thompson *et al.*, 1998; Best *et al.*, 1999; Bhadra, Spanggord *et al.*, 1999; Sens *et al.*, 1999). While TNT can be taken-up and metabolized, it is not broken down to its constituent elemental forms, neither is it broken down to CO_2 and nitrogen compounds. Instead, the TNT is detoxified and assimilated within the plant biomass, thereby reducing its bioavailablity. The process that governs the uptake and transformation of TNT has been termed the "Green Liver Model" (Sandermann, 1994) (Figure 2.6). According to this model plants, unlike microorganisms, cannot use xenobiotics as substrates for growth and hence they have to metabolize them using detoxification pathways, similar to the way the human liver metabolizes xenobiotics. This involves an initial activation step, a conjugation step and final polymerization and sequestration step. Together, these steps reduce the toxicity of the contaminant and reduce its bioavailability in the soil or water. Reduction, oxidation or hydroxylation of the xenobiotic is usually the first step in activating the inert molecule and preparing it for conjugation by plant molecules. In the case of TNT, the three nitro groups present on the ring confer it with an electrophilic nature, a characteristic shared by most nitroaromatics (Esteve-Nunez *et al.*, 2001). A strong positive field exists over the benzene

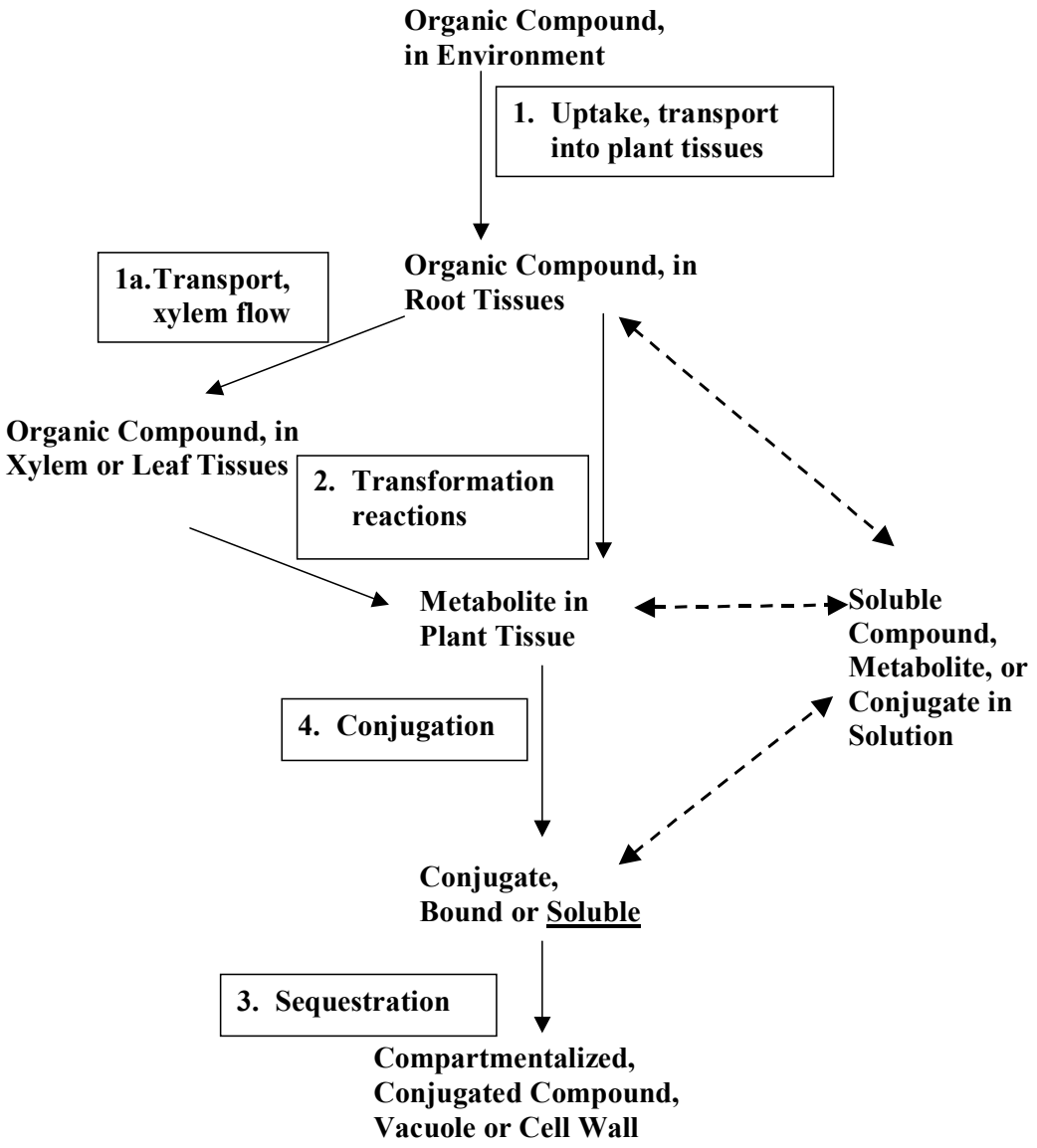


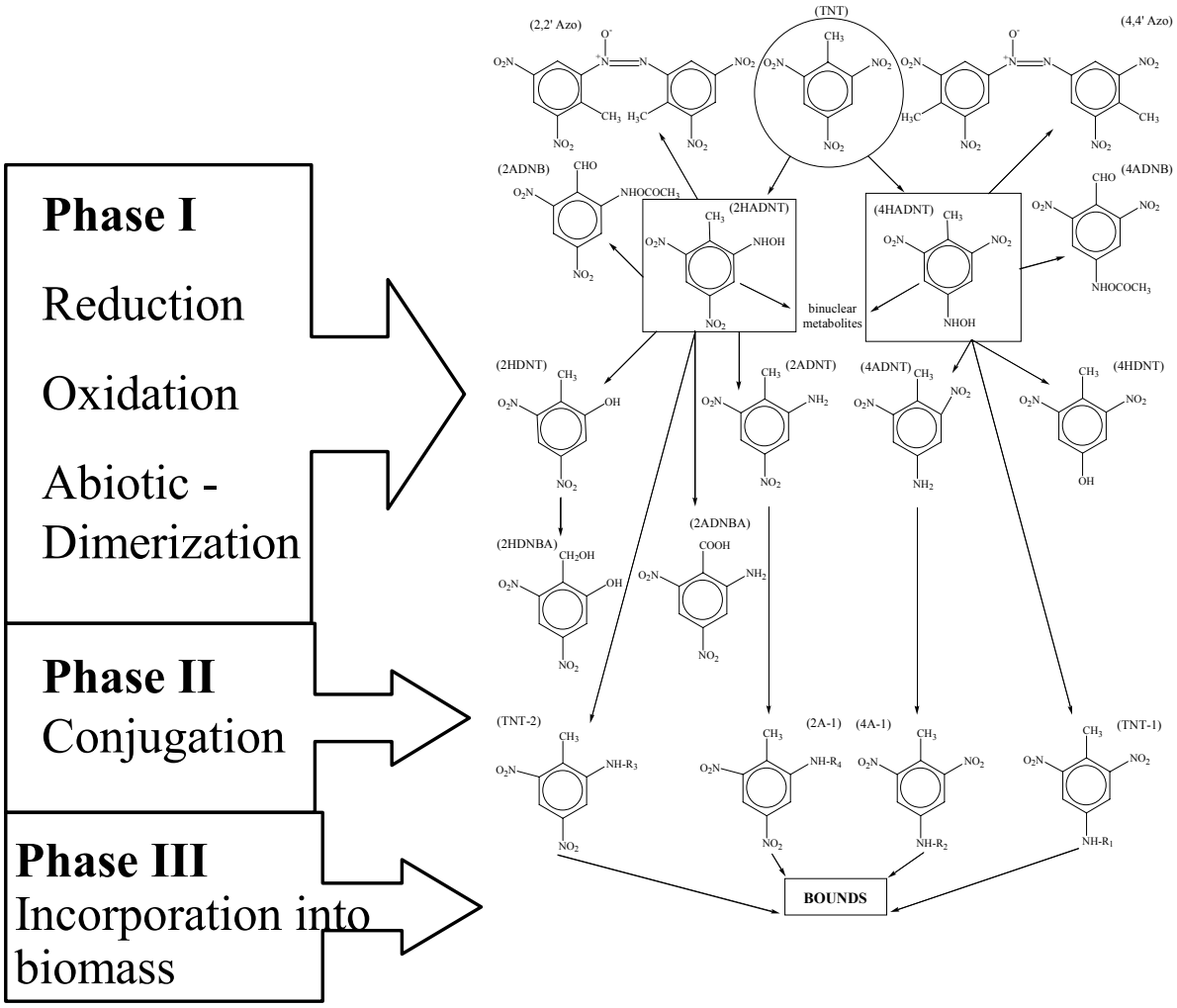
Figure 2.6: Outline of Green Liver Model: Schematic of the green liver model for metabolism of xenobiotics in plants. Dotted lines indicate diffusion/transport possible in aquatic plants. Figure adapted from (Burken *et al.*, 2000)

rings, which attracts reducing agents to the compound, and this makes reduction the most likely form of initial biotransformation reaction. This is almost universally true for both plants and microbes, evidenced by presence of mono and dinitroaminotoluenes in most TNT transformation studies (Burken *et al.*, 2000). Conjugation, the second step, is a phenomenon exclusive to plants. It entails the attachment of a plant molecule, like glucose or glutathione, to the metabolite and a consequent decline in toxicity of the metabolite (Bhadra, Wayment *et al.*, 1999). The conjugated products are finally bound inextricably to the plant biomass through, it is believed, a covalent linkage between the conjugates and the plant lignin. These transformation steps also increase the polar nature of the compound and hence their water-solubility (Coleman *et al.*, 1997). As TNT is continually transformed, the metabolites formed are increasingly polar in nature. Increasing polarity of the metabolites implies a reduced ability to partition in the cell membranes, and thereby reducing their presence in cells (Coleman *et al.*, 1997). Reduced exposure of the cells and tissues towards the metabolites results in lower toxicity effects. It also necessitates a different scheme for long-term storage of the metabolites, usually as lignin-like bounds, termed sequestration. In addition to an increase in polarity of the compound, and its role in final storage, transformation of the xenobiotic also reduces its toxicity. While the initial step of reduction may increase or decrease toxicity of the metabolite, the formation of conjugates and their storage always lowers toxicity levels (Coleman *et al.*, 1997). Figure 2.7 gives a detailed overview of the various sections of the Green Liver model, in conjunction with the metabolites formed and the chemistry involved. The various branches of the Green Liver Model are dealt with in detail in the following sections.

2.4.1: Reduction

Reduction is the most commonly observed scheme of transformation for nitroaromatics, as evidenced by the number of studies reporting the existence of amine products formed from the reduction of nitro groups. 2-amino-4,6-dinitrotoluene (2ADNT) and 4-amino-2,6-dinitrotoluene (4ADNT), the proposed primary TNT reduction products, are two of the most common transformation products and have been ubiquitously identified in TNT-removal studies, although in very low concentrations (Palazzo and Leggett, 1986;

Figure 2.7: Schematic Green Liver Model: A proposed TNT transformation pathway describes the uptake and transformation of TNT to various metabolites and conjugates (adapted from Subramanian and Shanks, in "Phytoremediation", edited by Steve McCutcheon and Jerry Schnoor, p389-408). TNT is completely removed from the system within 120 hours. A combination of HPLC-PDA, MS and NMR were used to identify the structure of the metabolites formed. This pathway, based on the "green liver model", shows the initial reduction of TNT to the hydroxylamines- 2-hydroxylamine-4,6-dinitrotoluene and its isomer the 4-hydroxylamine-2,6,-dinitrotoluene. The hydroxylamines are subsequently completely reduced to the monoamines- 2-amino-4,6-dinitrotoluene and 4-amino-2,6,-dinitrotoluene. Alternatively, the hydroxylamines can also be oxidized or isomerized to other metabolite branches. These reduced, oxidized and isomerized metabolites are then subjected to a plant conjugation mechanism, wherein plant biomolecules like sugars are attached to their functional groups. These transformation steps serve to reduce the toxicity of the parent TNT and polarize the compound. The conjugated metabolites are then polymerized by the plant enzymes and attached to the plant biomass, often irreversibly. Since these compounds have a final fate of being "bound" to the plant, they are not immediately bioavailable in the ecosystem.


Phase I
Reduction
Oxidation
Abiotic - Dimerization
Phase II
Conjugation
Phase III
Incorporation into biomass
(TNT)
(2,2' Azo)
(4,4' Azo)
(2ADNB)
(2HADNT)
(4HADNT)
(4ADNB)
binuclear metabolites
(2HDNT)
(2ADNT)
(4ADNT)
(4HDNT)
(2HDNBA)
(2ADNBA)
(TNT-2)
(2A-1)
(4A-1)
(TNT-1)
BOUNDS

Harvey *et al.*, 1990; Best *et al.*, 1997; Hughes *et al.*, 1997; Larson, 1997; Vanderford *et al.*, 1997; Burken and Schnoor, 1998; Pavlostathis *et al.*, 1998; Rivera *et al.*, 1998; Thompson *et al.*, 1998; Bhadra, Spanggord *et al.*, 1999; Bhadra, Wayment *et al.*, 1999; Nagel, Scheffer *et al.*, 1999; Sens *et al.*, 1999; Wayment *et al.*, 1999). Bhadra, Wayment (1999) show the formation of 2ADNT and 4ADNT in quantities less than 5% of the initial TNT added during the complete course of the experiment in *C. roseus* roots. In some cases, 2,4-diamino-6-nitrotoluene and its corresponding 4,6-diamino isomer have been reported, although infrequently since they require quite strong reducing conditions (Thompson *et al.*, 1998; Sens *et al.*, 1999). Rivera (1998) have proposed the reduction of TNT all the way to triaminotoulene and subsequent ring cleavage, although it has pointed out by Burken (2000) that this is unlikely given the strong reducing conditions required. In addition to aminated toulenes, azoxy compounds such as 2,2'-azoxy-tetranitrotoluenes have also been observed in trace amounts, indicating the condensation of hydroxylamines to form them (Pavlostathis *et al.*, 1998; Wang *et al.*, 2003). Two unidentified binuclear metabolites have also been observed in studies with *M. aquaticum* in fairly significant quantities- 5.6% molar percent of initial TNT (Bhadra, Spanggord *et al.*, 1999).

Nitroreductase, an NADPH dependent enzyme requiring anaerobic conditions, may be one of the enzymes involved in the reduction of the nitro group (Hatzios and Penner, 1982). Since aerobic conditions prevail in these systems, activity of the nitroreductases may have been low and hence low concentrations of monoamines result. Other studies with Ferrodoxin NADP-oxidoreductase from spinach leaves seem to indicate their ability to carry out nitro to amino reductions efficiently (Shah and Campbell, 1997), although this seems to require anaerobic conditions too. The corresponding hydroxylamine is an observed intermediate in these studies (Shah and Campbell, 1997). Recent studies, including work presented in this dissertation indicate that hydroxylamines could be the first metabolites to be formed during TNT transformation (Chapter 3) as precursors to monoamines. Their instability and their rapid degradation to either azoxy compounds or monoamines are likely the reason for the early formation of monoamines in transformation studies, usually within 10 hours of exposure of the plant to TNT (Hughes *et al.*, 1997; Vanderford *et al.*, 1997; Bhadra,

Spanggord *et al.*, 1999; Bhadra, Wayment *et al.*, 1999). Further details on the presence and role of hydroxylamines are given in the next section of this chapter and in Chapters 3 and 4.

While reduced compounds are never formed in significant quantities in transformation studies, they undoubtedly represent the most common defense adopted by the plant towards the xenobiotic. It is speculated that all of the flux from the transformation of the parent contaminant passes, in most species, through at least one reductive step. Reduction serves to reduce the electrophilicity of TNT and prepare it for further, potentially toxicity-reducing reactions. Reduction, however, may not be a desirable goal of phytoremediation studies, since reduced products tend to be almost as toxic, or in cases more toxic, than the parent contaminant. In addition, reduced products are rarely subject to ring-cleavage in microorganisms, an essential step for complete degradation of xenobiotics to carbon dioxide. For these reasons, transgenics with pathways that direct flux away from reduced metabolites will be of special interest. It has also been envisaged that a two-stage process- an initial reduction step, followed by an oxidative step can effectively treat xenobiotics (Lenke *et al.*, 2000), although no practical implementation of such a scheme exists. However, this is not to underestimate the efficacy of reduction as a metabolic process. Transgenic plants over-expressing nitroreductase enzymes have been developed and have shown to be much more resistant to TNT than the wild-types (French *et al.*, 1999), evidence that the reductive process plays a key role in contaminant transformation.

2.4.2: Oxidation

While oxidative metabolism is a significant feature of herbicide detoxification by plants (Hatzios and Penner, 1982), its role in TNT and nitroaromatic transformation is more limited. While it is plausible that oxidative metabolites are formed, given the number of unidentified polar compounds formed in various TNT studies, positive confirmation for their presence exists only in one plant-based study (Bhadra, Spanggord *et al.*, 1999). Oxidation can only follow the initial reduction of TNT to hydroxylamines or monoamines. Six oxidized metabolites, contributing nearly a significant 30 percent of the transformative pathway, were identified in experiments with *M. aquaticum* (Bhadra, Spanggord *et al.*, 1999). Oxidation of the methyl groups and hydroxylation of the aromatic group after removal of a nitro group are

some of the reactions that may be involved. In addition, carboxylation, hydroxylation, addition of aldehydes and addition of an acetoxyamino group were observed, which seem to suggest the interplay of many different enzymes in TNT transformation (Bhadra, Spanggord *et al.*, 1999). Studies on microbial remediation of TNT have also confirmed the formation of oxidative metabolites, usually after an initial reduction step (Nagel, Schmidt *et al.*, 1999; Nishino *et al.*, 2000). Nagel (1999) extracted the oxidized TNT metabolites 2,4,6-trinitrobenzoic acid and 2-amino-4,6-dinitrobenzoic acid from soil at low levels; however, no microcosm studies were performed. These oxidized metabolites in the are soil due to both natural biotic and abiotic degradation of TNT. Hence, it is unclear which bacterial system, if any, can oxidize TNT.

Although oxidative metabolism of TNT in plants has been observed just once, they are significant in the context of TNT transformation pathways. Metabolites from these pathways may exert lower toxicity levels than their reduced counterparts, and hence present a more attractive end-point solution for phytoremediation. In addition, eventual ring cleavage of oxidative metabolites may lead to mineralization of the parent contaminant, a highly desirable goal. One study on over-expression of a cytochrome P450 reported the removal of TCE 640 times as fast as wild-type controls (Doty *et al.*, 2000), proving the importance of oxidative reactions in xenobiotic metabolism. For these reasons, the enzymes involved in TNT-oxidation are of extreme interest to current researchers.

Cytochrome P450 oxygenases are probably the enzyme group involved in the formation of oxidative metabolites. These are a diverse group of enzymes involved in detoxification of many herbicides by oxidizing their lipophylic substrates, increasing their polarity and allowing further conjugation, and storage in vacuoles or cell wall (Schuler, 1996; Schalk *et al.*, 1997). These NADPH or NADH requiring enzymes split dioxygens into two monooxygens, one oxygen atom being incorporated into the compound, and the other becoming a part of a water molecule (Hatzios and Penner, 1982). These heme-dependent mixed-function-oxygenases have been implicated in the oxidation of many xenobiotics and herbicides (Bolwell *et al.*, 1994; Barrett, 1995; Schuler, 1996; Durst *et al.*, 1997; Khatisashvili *et al.*, 1997; Schalk *et al.*, 1997; Doty *et al.*, 2000). Cytochrome-P450 oxygenases catalyzing addition of hydroxyl groups and oxidizing methyl groups have been

isolated and characterized in various plants (Schuler, 1996). In addition, in higher plants these enzymes have been implicated in the metabolism of lipids, terpenoids, phenyl-propanoids, hormones, pigments, herbicides and other xenobiotics (Schuler, 1996; Schalk *et al.*, 1997). Cytochrome P450s are also inducible by a wide range of conditions and molecules ranging from light and temperature to xenobiotics, alcohols and acids (Durst *et al.*, 1997). As the role of cytochrome P450s in explosive metabolism is better characterized, TNT metabolism will be better understood.

The complete absence of identified oxidative products in *C. roseus* could be due to one of several reasons. Studies with hairy roots have shown that their characteristic of growing in clumps could significantly reduce the concentration of oxygen available to them (Yu *et al.*, 1997). Experiments have shown oxygen deprivation of the roots in shake flask cultures is a natural phenomenon, and this manifests as deficient growth of the roots (Yu *et al.*, 1997). Cytochrome P450 monooxygenases need dioxygen, both as a substrate and probably as an inducer as well (Schuler, 1996), and hence the observed low oxygen levels could result in their non-activation. Other reasons for the absence of oxidative products could include the fact that oxidative products could have a high reactivity and hence a short lifetime. There may also be significant differences in the enzymes implicated while using whole plants (*M. aquaticum*) as opposed to using roots (*C. roseus*). All these criteria can be manipulated under experimental conditions, and hence the hypotheses tested.

2.4.3: Conjugation and Sequestration

After the initial activation steps of reduction, oxidation or hydroxylation, the metabolites usually bond with a plant organic molecule, like sugar, fatty acid, amino acid, thiol, or a gluthatione (Hatzios and Penner, 1982), to form a conjugate. The molecule is added onto one of the functional groups of the plant, usually on one of the more reactive centers. Conjugate formation is an effective tool in protecting against the xenobiotic, since it usually reduces the toxicity of the metabolite (Coleman *et al.*, 1997).

In studies with *C. roseus* and *M. aquaticum*, four unique conjugates were observed that had sugar molecules bonded to the amino group on the ring (Bhadra, Spanggord *et al.*, 1999; Bhadra, Wayment *et al.*, 1999; Wayment *et al.*, 1999). The structures of these

conjugates have not been determined, but it has been found that two of them bear similarity to 2ADNT (labeled TNT-1 and 2A-1) and the remaining two are similar to 4ADNT (labeled TNT-2 and 4A-2), which is reason to believe that the monoamines are the substrates for conjugative enzymes. However, monoamine precursor-feeding studies seem to indicate the possibility of direct addition of plant sugars to the hydroxylamine group; hence, both monoamines and hydroxylamines seem to be capable of being conjugated. Confirmation for the conjugation of hydroxylamines is given in Chapter 4. The types of conjugates formed differ depending on the parent contaminant, plant species, conditions of transformation and other factors. This suggests a wide array of plant enzymes catalyzing the conjugative reactions. Further analysis of conjugate formation is essential from health perspectives, since digestive enzymes of some animal species appear capable of cleaving the bond between the plant molecule and the metabolite, thereby releasing the potentially toxic metabolite in the living systems of the animal (Coleman *et al.*, 1997).

Since plants, unlike animals, cannot release these conjugates via excretory processes they need a storage scheme for them, usually in the form of non-extractable bound residues, which are then irreversibly incorporated in the plant biomass (Hatzios and Penner, 1982; Hatzios, 1991; Coleman *et al.*, 1997; Bhadra, Spanggord *et al.*, 1999; Bhadra, Wayment *et al.*, 1999; Sens *et al.*, 1999; Wayment *et al.*, 1999; Burken *et al.*, 2000). Studies have found that nearly 20 percent of the total pollutant added passes through the conjugative pathway, and greater than 30 percent ends up as bounds (Khan, 1988; Hughes *et al.*, 1997; Vanderford *et al.*, 1997; Sens *et al.*, 1999). One study found that a significant portion of the bounds was found in the lignin fraction of the biomass (27%) and lesser amounts were found in the pectin, hemicellulose, and protein fractions of the biomass (Sens *et al.*, 1999). Therefore, the conjugates appear to polymerize and covalently bond to the lignin, or else be isolated in the vacuole, to form the end products of TNT metabolism. While these bounds are not released into the environment, they may still have residual toxic effects on the local fauna, and hence the nature and toxicity of bounds is an important practical consideration for phytoremediation studies.

2.5: Analysis of TNT Metabolism

Plant and microbial metabolism of TNT is understood within certain limits; hence, a comparison of the two proves instructive. There exist some significant similarities between plant and microbial response to nitroaromatic compounds, underscored mainly by the fact that both of them cannot mineralize the nitroaromatics, but use a process of co-metabolism to deal with them (Burken *et al.*, 2000; Nishino *et al.*, 2000). Co-metabolism implies that the nitroaromatics are not used as the primary nutrient sources, but may be used as a secondary nutrient source. Hence, they may not be broken down to the smallest organic molecules, but are transformed because of the organism's metabolic processes. In addition, while both plants and microbes have an array of transformation reactions to choose from, the first step is usually one of reduction of the nitro-groups. While this is in part due to the electrophilic nature of the target compound (Rieger and Knackmuss, 1994), it also implies similar enzymes acting on the foreign compound in both plants and microbes, and this fact has been exploited in developing transgenic plants to over-express a particular bacterial enzyme (French *et al.*, 1999; Doty *et al.*, 2000).

However, in spite of these similarities between plant and microbial remediation, there are key differences between the two systems, which make fundamental research on genes, enzymes and mechanisms in plants important. Microbes have various enzymatic systems, which can transform the nitroaromatics into different end-points, depending on the nature of the compound, the microbe and the conditions of transformation (Nishino *et al.*, 2000). Complete degradation of TNT to basic molecules like carbon-dioxide and water, however, is rarely observed, and even if observed is usually of a low percentage (Nishino *et al.*, 2000). The final fates of these metabolites are usually bound to the biomass in a less toxic form. Ammonia maybe released as a by-product of the transformation processes (Nishino *et al.*, 2000). Plants, while operating a similar system have much fewer mechanisms to detoxify nitroaromatics, since they are heterotrophs (Burken *et al.*, 2000). Photosynthesis provides the carbon sources and energy required for the survival of plants, and hence transformation of the contaminants is strictly co-metabolic (Burken *et al.*, 2000). In addition, conjugation and sequestration of the amended nitroaromatic within the vacuole of the cell appears to be a

unique plant and mammalian defensive mechanism. Figures 2.8 and 2.9 give the final metabolite ratio found in *C. roseus* and *M. aquaticum* upon complete TNT removal.

Analysis of the unknown concentration distribution of TNT transformation products, in *C. roseus* reveals a higher percentage of unknowns (unidentified metabolites) in the intracellular-extractable portion as compared to the extracellular portion (Figure 2.10) (Hughes *et al.*, 1997; Vanderford *et al.*, 1997; Bhadra, Spanggord *et al.*, 1999; Bhadra, Wayment *et al.*, 1999; Wayment *et al.*, 1999). Initially, the unknown extracellular concentration is around 15 percent, but this falls to less that 10 percent at the end of the experiment. In contrast, the unaccounted intracellular-extractable fraction increases from around 17 percent to 36 percent (Bhadra, Spanggord *et al.*, 1999; Bhadra, Wayment *et al.*, 1999; Wayment *et al.*, 1999). This seems to indicate that the unidentified product in the extracellular phase is getting continuously further transformed into products in the intracellular-extractable phase. The unaccounted portion of the carbon could be unidentified conjugates, other reduced or oxidized metabolites, or conjugates formed by bonding of the methyl group to plant organic molecules. The methyl group could be modified, e.g. oxidized, before conjugation. A very dynamic state exists in the roots and its surrounding media, with the concentration of all the metabolites changing constantly.

2.6: TNT Transformation Pathway: Knowledge and Structure

Based on the literature surveyed, a working model of the TNT transformation pathway is proposed and illustrated in Figures 2.11 and 2.12. Both figures represent the same pathway mechanism; Figure 2.12 is expanded from Figure 2.11 and shows more detail. This working model is based on a composite of experiments with *C. roseus* and *M. aquaticum*. The abbreviations for the various metabolites present in the pathway have been listed in the Figures 2.13 and 2.14, along with their IUPAC names. Studies on *C. roseus* hairy roots yielded reduced mono-amino products and conjugates, but no oxidized products studies on *M. aquaticum* (parrot feather) resulted in the production of a wide array of oxidized, reduced, conjugated and binuclear products (Bhadra, Spanggord *et al.*, 1999; Bhadra, Wayment *et al.*, 1999; Wayment *et al.*, 1999). The TNT transformation pathway is comprised of these distinct reduced, oxidized, conjugated and azoxy products. Reduction metabolites

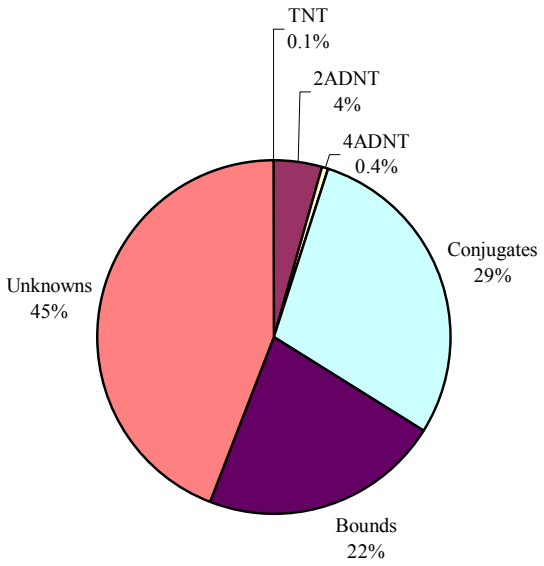


Figure 2.8: *C. roseus* Molar Balance: *Catharanthus roseus* molar carbon balance 75 hours after amendment with TNT. Figure adapted from (Bhadra, Wayment *et al.*, 1999).

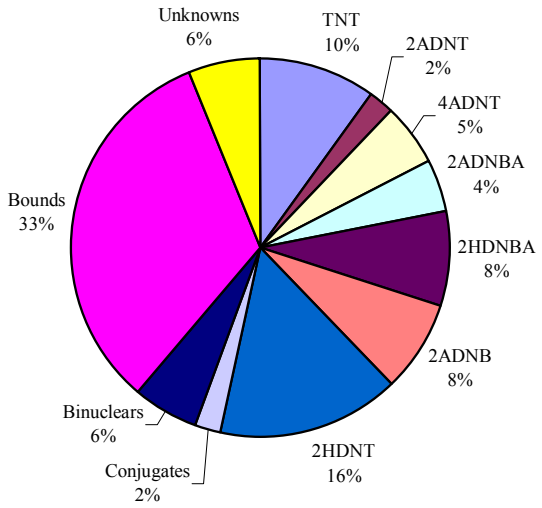


Figure 2.9*: M. aquaticum* Molar Balance: *Myriophyllum aquaticum* molar carbon balance estimate 12 days after amendment with TNT. Figure adapted from (Bhadra, Spanggord *et al.*, 1999).

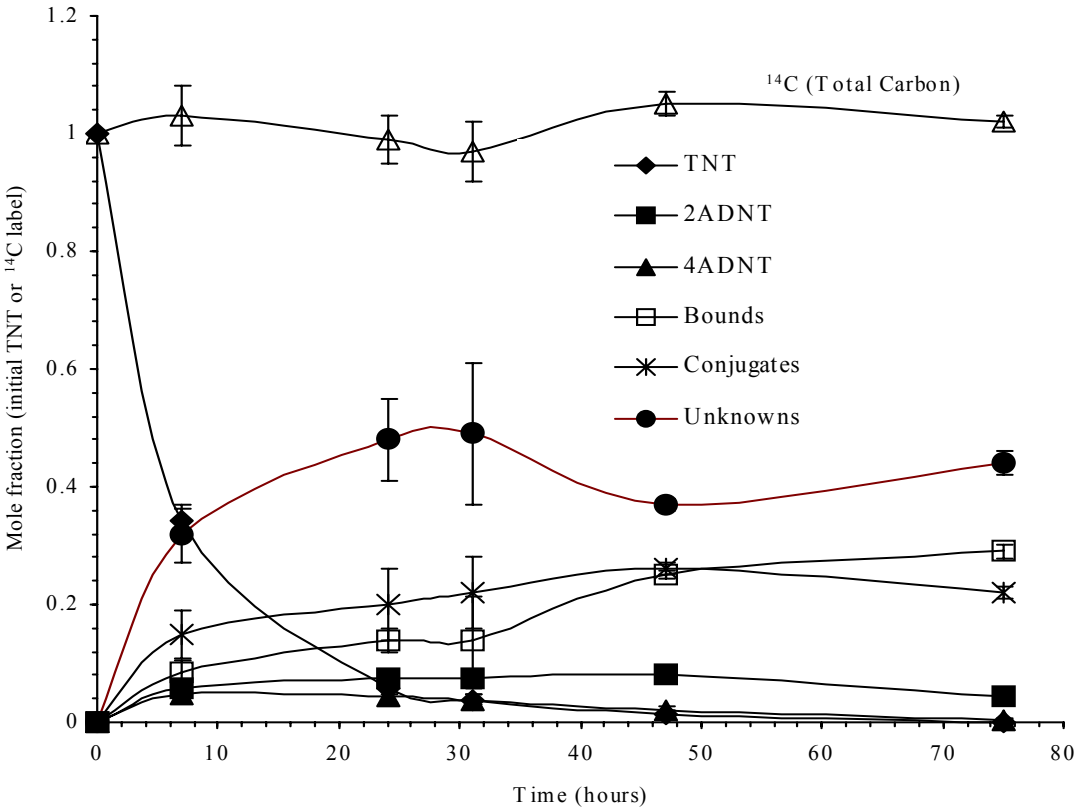


Figure 2.10: TNT Mass Balance: Transient mole fractions of TNT, ADNTs, Conjugates, and Bounds, in *Catharanthus roseus*, upon addition of TNT, and total ^{14}C fraction. Data taken from (Bhadra, Wayment *et al.*, 1999).

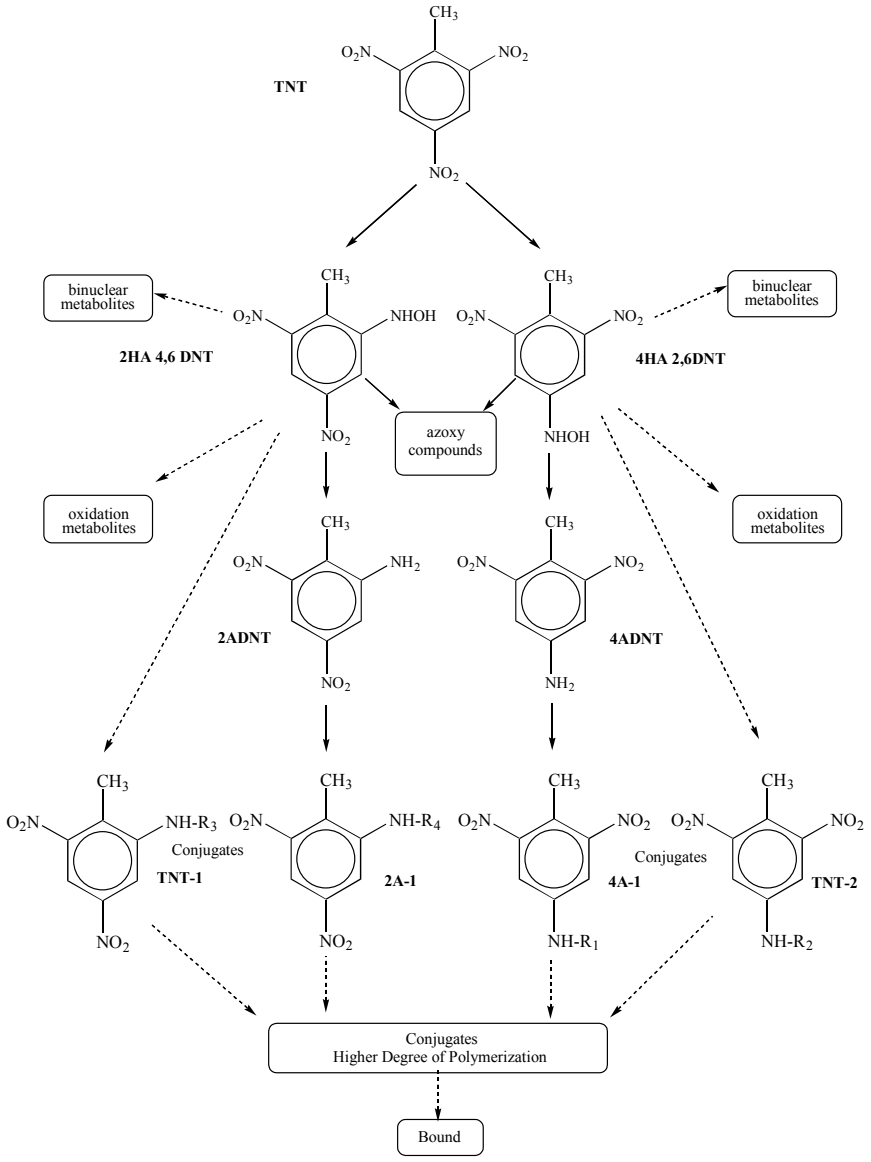


Figure 2.11: TNT Transformation Pathway: Hypothetical TNT transformation pathway in plants. Figure adapted from (Burken *et al.*, 2000).

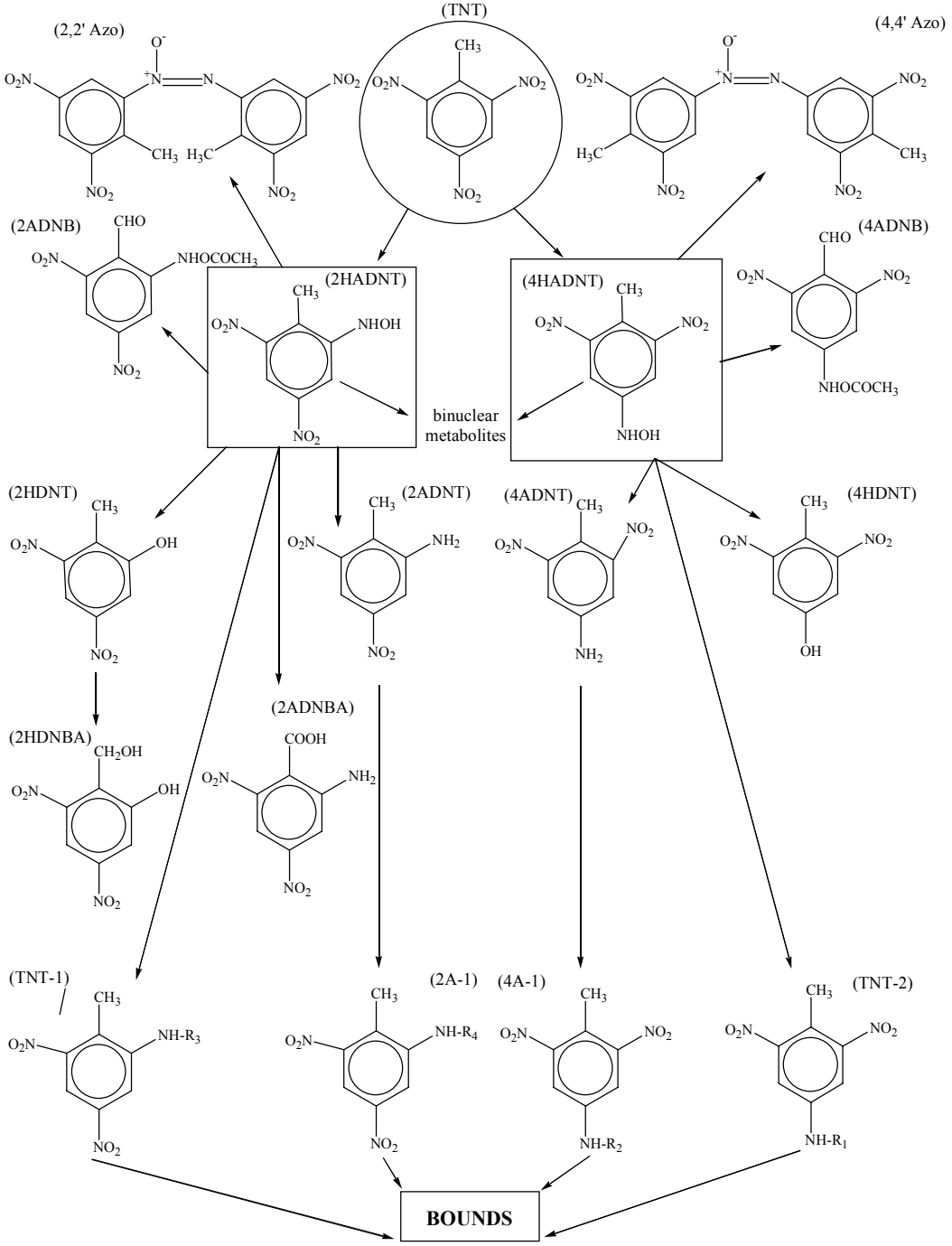


Figure 2.12: TNT Transformation Pathway: Hypothetical TNT transformative pathway in plants, encompassing all known metabolites produced. Arrows are indicative of the general directions of the pathway, and do not necessarily represent a single step reaction.

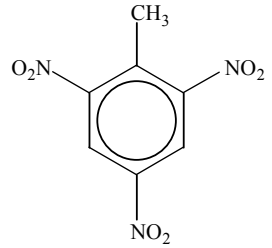
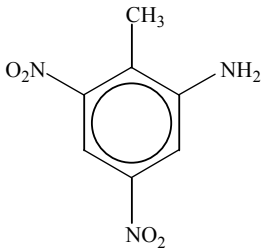
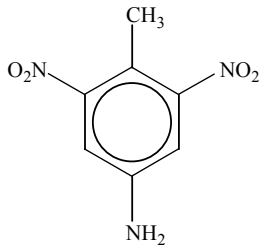
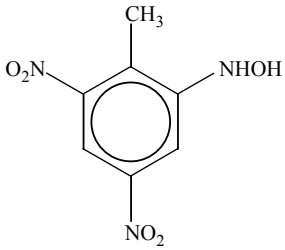
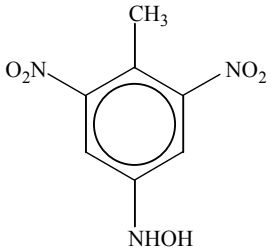
Abbreviation	Compound	Structure
TNT	2,4,6-Trinitrotoluene	
2ADNT	2-amino-4,6-dinitrotoluene	
4ADNT	4-amino-2,6-dinitrotoluene	
2HADNT	2-hydroxylamino-4,6-dinitrotoluene	
4HADNT	4-hydroxylamino-2,6-dinitrotoluene	

Figure 2.13: Reduced Metabolites: List of reduced metabolites formed in *Catharanthus roseus* and *Myriophyllum aquaticum* upon amendment with TNT. Figure adapted from (Burken *et al.*, 2000).

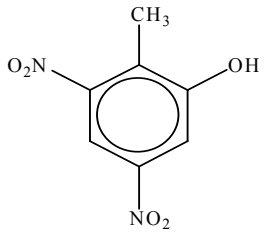
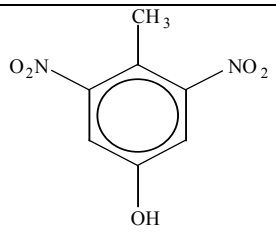
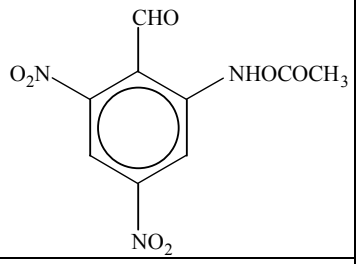
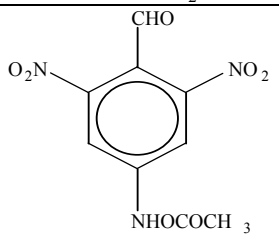
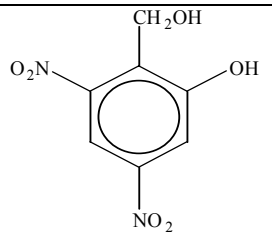
Abbreviation	Compound	Structure
2HDNT	2-hydroxy-4,6-dinitrotoluene	
4HDNT	4-hydroxy-2,6-dinitrotoluene	
2ADNB	2-N-acetamido-4,6-dinitrobenzaldehyde	
4ADNB	4-N-acetamido-2,6-dinitrobenzalydehyde	
2HDNBA	2-hydroxy-4,6-dinitrobenzyl alcohol	

Figure 2.14: Oxidized Metabolites: List of oxidized metabolites formed in *Catharanthus roseus* and *Myriophyllum aquaticum* upon amendment with TNT. Figure adapted from (Burken *et al.*, 2000).

are observed in several aquatic and terrestrial species (Burken *et al.*, 2000). Oxidative products have only been reported for *M. aquaticum* and not in any other plant-based study, although they have been identified in microbial systems (Nagel, Scheffer *et al.*, 1999; Nagel, Schmidt *et al.*, 1999). With a significant percentage of transformation metabolites remaining unidentified in most studies, it is conceivable that various other mechanisms are involved in TNT transformation (Hughes *et al.*, 1997; Vanderford *et al.*, 1997; Thompson *et al.*, 1998; Sens *et al.*, 1999)

The importance of this proposed pathway, however, lies in the fact that it provides a fairly complete picture of TNT-transformation, and hence is a good first step in understanding the biochemistry behind the transformation process. Moreover, with a nearly complete mass balance in *M. aquaticum*, a substantially complete TNT-fate balance can be modeled from the information presented in the pathway. Determination of the key branches in the pathway and pathway modification to increase the rate of removal of TNT are some of the plausible offshoots from the information.

2.7: Role of Hydroxylamines in TNT Transformation

Two of the probable metabolites shown in the pathway are 2HADNT and 4HADNT. These hydroxylamines are formed upon the sequential reduction of nitro groups, via an intermediary nitroso group (Ahmad and Hughes, 2000); these reductions can be one electron or two electron transfers depending on the type of enzyme (Figure 2.15) (Bryant *et al.*, 1991; Corbett and Corbett, 1995; Ahmad and Hughes, 2000). Hydroxylamines and nitroso compounds derived from TNT are electrophilic, unlike the individual nitro and amino groups (Corbett and Corbett, 1995). This makes them candidates for a number of transformation reactions, including further reduction, oxidation, conjugation, polymerization and azoxy formation (Ahmad and Hughes, 2000). Since hydroxylamines are probably the first metabolites formed in TNT transformation, their identification and quantification in phytoremediation systems are important. In addition, due to their high reactivity, it is conceivable that the pathway flux can be redirected from the hydroxylamines to other preferred metabolite schemes. Further details on the importance, presence and role of

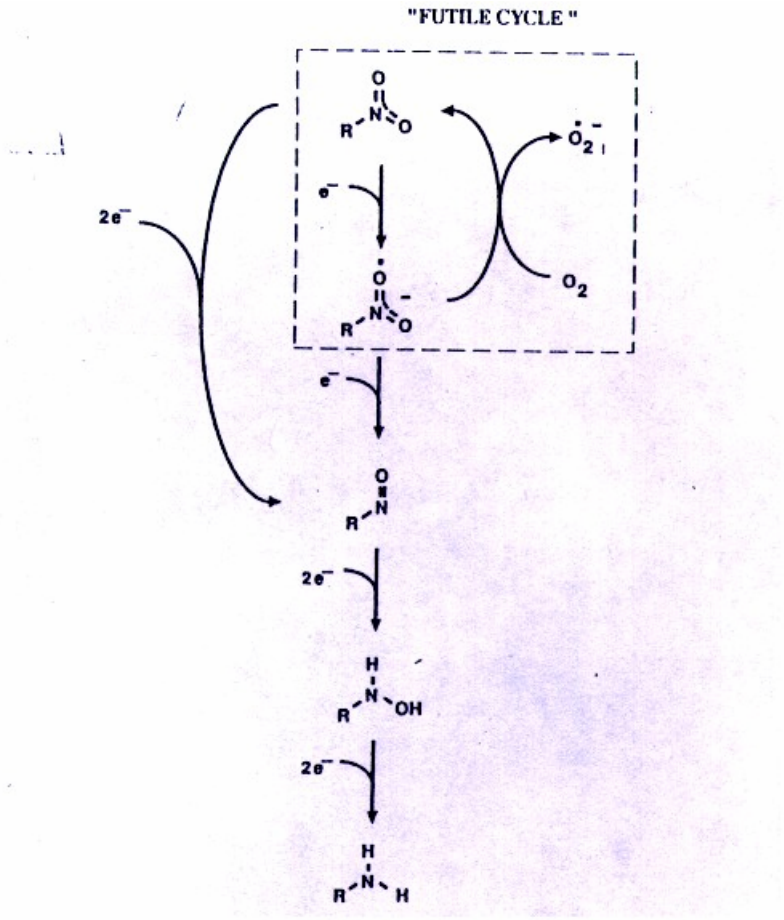


Figure 2.15: Nitroreductase Mechanism: Nitro-reduction mechanism in Type I and Type II nitroreductases, showing the formation of hydroxylamines. The futile cycle is established in the presence of oxygen for Type II (oxygen sensitive) nitroreductases. Figure adapted from (Bryant *et al.*, 1991)

hydroxylamines in TNT transformation completed are given in Chapters 3 and 4.

While hydroxylamines have not been reliably identified in axenic phytoremediation systems, a couple of non-axenic plant systems and a few bacterial remediation studies have shown their presence. Although TNT was identified in two plant systems (Pavlostathis *et al.*, 1998; Wang *et al.*, 2003), both these systems were non-axenic and aquatic. Pavlostathis (1998) show the formation of 4HADNT at low concentrations during the first 60 hours of after TNT addition. TNT, added in initial levels of 50 μM disappeared from the media within 70 hours. Wang (2003) offer positive UV-visible spectral comparison and NMR confirmation for the presence of both 2HADNT and 4HADNT. When TNT was added at an initial concentration of 25 mg/L, these metabolites were shown to be present in the system from the second day to the end of the experiment (15 days), but were never observed in amounts greater than 10% of initial TNT added. The plants took 15 days to remove all of the TNT from the media (Wang *et al.*, 2003).

Most of the microbial systems that showed the presence of hydroxylamines were anaerobic (Fiorella and Spain, 1997; Hughes, Wang, Bhadra *et al.*, 1998; Hughes, Wang, Yesland *et al.*, 1998; Hughes *et al.*, 1999; Naumov *et al.*, 1999; Ahmad and Hughes, 2000) but one recent study reports the formation of 2HADNT in an aerobic system too (Vasilyeva *et al.*, 2000). Naumov (1999) showed the complete transformation of TNT, in almost molar quantities of TNT to 2 and 4HADNT in the bacteria *Lactobacilli*. Hughes (1998) have reported the formation of Bamberger intermediates via hydroxylamines during TNT biotransformation by anaerobic *Clostridium*. Vasilyeva (2000) show the conversion of over 50% of TNT to hydroxylamines within 15 hours by the bacterium *Pseudomonas aeruginosa* under aerobic conditions. 12% of the TNT was converted to 2ADNT, while 65% was transformed to 2,2'-Azoxy (Vasilyeva *et al.*, 2000). The bacterial strain displayed a preference for the 2-substituted metabolites of TNT transformation. Although hydroxylamines have not been convincingly identified in axenic, microbe-free plant systems, this may be attributed to their instability in aqueous, aerobic media and high chemical reactivity. It is probable that hydroxylamines are formed in most plant systems, but have very short lives and hence are not detected. Studies detailing the detection and role of

hydroxylamines in TNT transformation studies in axenic plant systems are given in Chapters 3 and 4.

2.8. Role of Nitroreductases in TNT Transformation

Nitroreductases, enzymes responsible for the reduction of the nitro group in TNT, are found in both prokaryotic and eukaryotic systems. Lately, a number of bacterial nitroreductases have been isolated and characterized, in an effort to understand their precise role in TNT metabolism (Bryant *et al.*, 1991; Fiorella and Spain, 1997; Huang *et al.*, 2000; Pak *et al.*, 2000; Esteve-Nunez *et al.*, 2001; Oh *et al.*, 2001). There exist two types of nitroreductase enzymes Type I and Type II reductases (Shah and Spain, 1996). Type I reductases are oxygen insensitive nitroreductases and use a two electron mechanism to reduce the nitro group of the xenobiotic to nitroso and hydroxylamine groups (Bryant *et al.*, 1991); these enzymes are not affected by the presence of oxygen. Type II reductases use a one-electron scheme to reduce the nitro group to form a superoxide of the nitroso group. However, in the presence of oxygen, the superoxide loses an oxygen atom to form the parent nitro group, and hence setting up a reverse flux and thereby preventing the complete reduction of the nitro group (Bryant *et al.*, 1991). Therefore, these nitroreductases are oxygen sensitive; this has been partially validated for the *E. coli* nitroreductases (Peterson *et al.*, 1979; Bryant *et al.*, 1991). Figure 2.15 shows figuratively the associated chemistry of these two reductases. Apart from a single mammalian reductase (quinone oxidoreductase) and several classical nitroreductases from enteric bacteria, all the other reductases are oxygen sensitive (Peterson *et al.*, 1979, Bryant *et al.*, 1991). This seems to imply that reduction of trinitrotoluene may proceed inefficiently in plant and mammalian systems in the presence of oxygen, which may explain the low levels of monoamines seen in all TNT phytotransformation studies. Obtaining detailed information for plant nitroreductases will prove significant in determining the ambient conditions necessary for TNT reduction and oxidation in plants (Wolfe *et al.*, 1994).

Nitroreductase enzymes isolated and characterized in bacteria include carbon monoxide dehydrogenase, nitroreductase from *Pseudomonas*, nitroreductase from *Enterobacterium*, xenobiotics reductase B, cytochrome P450 reductase, xanthine

dehydrogenase and quinone reductase (Peterson *et al.*, 1979; Bryant *et al.*, 1991; Shah and Spain, 1996; Huang *et al.*, 2000; Pak *et al.*, 2000; Esteve-Nunez *et al.*, 2001; Oh *et al.*, 2001). These enzymes were capable of reducing the nitro group to hydroxylamines, and in some cases, all the way to amines. In many anaerobic systems probed, the nitroreductases reduced the nitro group to hydroxylamines, and subsequent Bamberger rearrangement or azoxy formation followed (Hughes, Wang, Yesland *et al.*, 1998). Nitroso compounds were never convincingly identified in these systems, probably due to their great instability (Ahmad and Hughes, 2002). Some studies report the formation of triaminotoluene (TAT), a completely reduced product and it is speculated that ring breakage could occur from TAT. However, it has been argued that reduction to TAT requires a highly reductive atmosphere, beyond what is normally present in biological systems (Burken *et al.*, 2000; Lenke *et al.*, 2000).

Nitroreductases from the spinach enzymes have shown potential in offering an alternative degradative mechanism for TNT. Ferrodoxin NADP Oxidoreductase from spinach leaves is capable of eliminating a nitrite group from 2,4,6-trinitrophenylmethylnitramine (tetryl), under anaerobic conditions (Shah and Spain, 1996). However, the same enzyme when used on nitrobenzene resulted in the partial reduction of the nitro group to hydroxylamine (Shah and Campbell, 1997). Removal of functional groups or ring cleavage in TNT is extremely rare. Consequently the role of this reductase in eliminating nitro groups from TNT bears further research.

2.9: Toxicity of TNT and its Metabolites

Understanding and being able to quantify nitroaromatic toxicity to plants and animals is essential in determining the value and success in any phytoremediation project. Since phytoremediation typically does not break down the contaminants to their basic molecular constituents and neither does it mineralize them, it is important to be able to measure the toxicity of the final products of the phytotransformative process. While the toxicity of individual nitroaromatics to various parts of the ecosystem have extensively studied, comparative toxicities of TNT and its metabolites under common standards has not received much attention (Won *et al.*, 1976; Harvey *et al.*, 1990; Yinon, 1990; Tan *et al.*, 1992; Honeycutt *et al.*, 1996; Berthe-Corti *et al.*, 1998; Dodard *et al.*, 1999; Nagel, Scheffer *et al.*,

1999; Naumov *et al.*, 1999; Schafer and Achazi, 1999; Padda *et al.*, 2000; Reddy *et al.*, 2000; Tadros *et al.*, 2000). This section will summarize some of the current finding and trends in toxicity of TNT and its metabolites to plants and animals.

2.9.1: Mechanisms of Toxicity

In deciphering the toxicity of TNT to various test species, it is necessary to understand the metabolism of TNT by these species. It has been speculated that the toxicity and mutagenicity of TNT are due to its breakdown products, and not TNT itself (Fu, 1990; Naumov *et al.*, 1999; Esteve-Nunez *et al.*, 2001). Nitroso and hydroxylamines analogues, the first and also the most reactive metabolites formed in TNT metabolism, probably cause toxic, carcinogenic, and mutagenic effects on the organism (Fu, 1990; Naumov *et al.*, 1999; Esteve-Nunez *et al.*, 2001) activity by binding to the -SH groups and therefore inhibiting proteins and enzymes (Fu, 1990; Naumov *et al.*, 1999). In addition, hydroxylamines can form adducts on DNA (Fu, 1990; Naumov *et al.*, 1999), and catalyze the oxidation of oxyhemoglobin to ferrihemoglobin and render it useless (Esteve-Nunez *et al.*, 2001). These hemotoxic symptoms deplete the ability of blood to transport oxygen. Hydroxylamines also have been shown to inhibit lactic acid enzyme activity much more than TNT (Naumov *et al.*, 1999). Other manifestations of toxic, carcinogenic and mutagenic effects of nitroaromatics include neoplastic transformations of human diploid fibroblasts, induction of DNA strand breaks and repair, induction of sister chromatid exchanges and induction of chromosome aberrations (Fu, 1990). Formation of tumors, inhibition of growth, sterility and death are some of the commonly observed effects in TNT toxicity experiments.

TNT toxicity and mutagenicity have been studied in a wide range of species; however, there are no studies on TNT carcinogenicity. Since monoamines are known to be carcinogenic (Harvey *et al.*, 1990), it is important to determine the carcinogenicities of the other metabolites too. Mutagenicity of nitroaromatics has been studied using the Ames assay on *Salmonella typhimurium* or the mutatox assay on *Vibrio fischeri*. The Ames assay, a well-validated and often-used test, uses *Salmonella* strains (usually TA98 and TA100) that lack histidine biosynthesis capacity due to a mutation; hence, these strains need histidine in the media to survive (Honeycutt *et al.*, 1996). A reverse mutation, caused by a mutagenic

compound like TNT, can restore the organism's histidine synthesis pathway. Hence, the number of colonies that grow on histidine free media due to the addition of TNT or its metabolites is a measure of their mutagenicity of the compound (Honeycutt *et al.*, 1996). There also exists a nitroreductase-deficient strain of *Salmonella* (TA98NR), which can be used in understanding the role of nitroreductases in TNT metabolism and mutagenicity (Fu, 1990; Rafii *et al.*, 1994). The mutatox assay consists of causing forward mutations in a dark *Virbrio fischeri* strain, causing it to regain luminescence (Honeycutt *et al.*, 1996). Hence, the bioluminescence of the bacterium is a measure of the mutagenicity of the chemical. In contrast to the well-defined systems for mutagenic studies, toxicity analyses have used a wide range of test species, from bacterium to rats. Use of multiple species better represents the ecosystem as a whole, although basic formalization of toxicity versus species would help in analyzing and comparing the toxicity data from various species.

2.9.2: TNT Toxicity and Mutagenicity Analyses

The results of the various toxicity and mutagenic tests performed are tabulated in Tables 2.1, 2.2 and 2.3. While Table 2.1 has the toxicity and mutagenicity information for TNT, Tables 2.2 and 2.3 have the same for its metabolites. Many useful observations are borne out of comparing data from these tables. Monohydroxylamines are always more toxic than the corresponding monoamines, and the monoamines are, in turn more toxic than the diamines (Tan *et al.*, 1992; Honeycutt *et al.*, 1996; Schafer and Achazi, 1999; Padda *et al.*, 2000; Tadros *et al.*, 2000). Dihydroxylamines are the most toxic and mutagenic metabolites, showing greater mutagenicity than TNT (Padda *et al.*, 2000). The toxicities and mutagenicities seem to correlate directly with reactivities, as the highly unstable hydroxylamines are more toxic and mutagenic than the much more stable monoamines. The only exception appears to be TNT, the stable parent molecule; TNT is more toxic and mutagenic than its metabolites in most studies (Tan *et al.*, 1992; Honeycutt *et al.*, 1996; Padda *et al.*, 2000; Tadros *et al.*, 2000). Another observation well worth noting is the influence of the position of the hydroxylamine or monoamine group on toxicity. The 2-substituted position compounds were found to be more toxic and mutagenic than the 4-substituted position (Tan *et al.*, 1992; Padda *et al.*, 2000) with one exception (Honeycutt *et*

Table 2.1: TNT Toxicity and Mutagenicity: Toxicity and mutagenicity of TNT on various plant and microbial organisms. LD_{50} (or LC_{50}), IC_{50} and EC_{50} are the concentrations required to cause 50% reduction in number of living organisms, functional activity or luminescence.

Test Organism	Toxicity	Mutagenicity (Ames Assay)	Reference
Enchyraeus crypticus	LD_{50} =570 mg/kg		(Schafer and Achazi, 1999)
Folsomia candida	LD_{50} =185 mg/kg		(Schafer and Achazi, 1999)
Tidepool codepods *Tigriopus californicus*	LD_{50} =5 mg/L		(Won *et al.*, 1976)
Oyster larvae *Crassostrea gigas*	LD_{50} =3-5 mg/L		(Won *et al.*, 1976)
Vibrio fischeri	IC_{50} = 3.4 uM		(Dodard *et al.*, 1999)
Salmonella typhimurium	EC_{50}= 2.5 uM		(Dodard *et al.*, 1999)
Selenastrum capricornutum	EC_{50} = 0.48 mg/L		(Dodard *et al.*, 1999)
H4HE- Rat hepatoma cells	LC_{50}= 4 ug/ml media		(Honeycutt *et al.*, 1996)
Peromyscus leucopus (white-footed mouse)	LD_{50} =Greater than 600 mg/kg/day; unattained		(Johnson *et al.*, 2000)
Sigmodon hispidus Hispid cotton rat	LD_{50} =607 to 767 mg/kg; unattained		(Reddy *et al.*, 2000)
Salmonella typhimurium, TA100		16,200 revertants/mg	(Padda *et al.*, 2000)
Salmonella typhimurium TA100		6600 revertants/mg	(Tan *et al.*, 1992)
Salmonella typhimurium TA100		14,000 revertants/mg	(Won *et al.*, 1976)
Salmonella typhimurium TA100		0 revertants/mg	(Honeycutt *et al.*, 1996)

Table 2.2: TNT Toxicity: Toxicity of TNT metabolites on plant and microbial species. LD_{50} (or LC_{50}), IC_{50} and EC_{50} are the concentrations required to cause 50% reduction in number of living organisms, functional activity or luminescence.

Metabolite	Test Organism	Toxicity	Reference
2ADNT	H4HE- Rat hepatoma cells	LC_{50}= 18 μg/ml media	(Honeycutt *et al.*, 1996)
4ADNT	H4HE- Rat hepatoma cells	LC_{50}= 66 μg/ml media	(Honeycutt *et al.*, 1996)
4HADNT	H4HE- Rat hepatoma cells	LC_{50}= 6 μg/ml media	(Honeycutt *et al.*, 1996)
2,4DANT	H4HE- Rat hepatoma cells	LC_{50} > 250 μg/ml media	(Honeycutt *et al.*, 1996)
2HADNT	*Selenastrum capricornutum*	EC_{50} = 2.9 mg/L	(Tadros *et al.*, 2000)
24DHANT	*Selenastrum capricornutum*	EC_{50} = 2.4 mg/L	(Tadros *et al.*, 2000)
26DA4NT	*Selenastrum capricornutum*	EC_{50} = 6.0 mg/L	(Tadros *et al.*, 2000)
2,2' Azo	H4HE- Rat hepatoma cells	LC_{50}= 4 μg/ml media	(Honeycutt *et al.*, 1996)
4,4'Azo	H4HE- Rat hepatoma cells	LC_{50}= 13 μg/ml media	(Honeycutt *et al.*, 1996)
TAT*	*Enchyraeus crypticus*	Greater than 1000 mg/kg unattained	(Schafer and Achazi, 1999)
TAT*	*Folsomia candida*	Greater than 1000 mg/kg unattained	(Schafer and Achazi, 1999)

* TAT is triaminotoluene

Table 2.3: Metabolite Mutagenicity: Mutagenicity of TNT metabolites on plant and microbial species. LD_{50} (or LC_{50}), IC_{50} and EC_{50} are the concentrations required to cause 50% reduction in number of living organisms, functional activity or luminescence.

Metabolite	Test Organism	Mutagenicity	Reference
2HADNT	*Salmonella typhimurium*, TA100	10,200 revertants/mg	(Padda *et al.*, 2000)
4HADNT	*Salmonella typhimurium*, TA100	4330 revertants/mg	(Padda *et al.*, 2000)
2ADNT	*Salmonella typhimurium* TA100	2300 revertants/mg	(Tan *et al.*, 1992)
4ADNT	*Salmonella typhimurium* TA100	500 revertants/mg	(Tan *et al.*, 1992)
2ADNT	*Salmonella typhimurium* TA100	218 revertants/mg	(Honeycutt *et al.*, 1996)
4ADNT	*Salmonella typhimurium* TA100	402 revertants/mg	(Honeycutt *et al.*, 1996)
2,4DA6NT	*Salmonella typhimurium* TA100	0 revertants/mg	(Tan *et al.*, 1992)
2,6DA4NT	*Salmonella typhimurium* TA100	750 revertants/mg	(Tan *et al.*, 1992)
2,4DHANT	*Salmonella typhimurium*, TA100	127,000 revertants/mg	(Padda *et al.*, 2000)
2,4DANT	*Salmonella typhimurium* TA100	0 revertants/mg	(Honeycutt *et al.*, 1996)
2,2' Azo	*Salmonella typhimurium* TA100	0 revertants/mg	(Honeycutt *et al.*, 1996)
4,4'Azo	*Salmonella typhimurium* TA100	1460 revertants/mg	(Honeycutt *et al.*, 1996)

al., 1996). Hence, 2HADNTs and 2ADNTs were more toxic and mutagenic than the corresponding 4HADNTs and 4ADNTs, respectively. While the reasons for the above observations are unclear, it may be advantageous to divert the TNT metabolic flux through the 4-substituted pathway, in metabolically engineered mutants, to help reduce the overall toxicity effect.

While it has been speculated that hydroxylamines and nitroso compounds are responsible for TNT toxicity, experimental evidence seems to indicate TNT has a greater toxicity than the hydroxylamines and its other metabolites (Tan *et al.*, 1992; Honeycutt *et al.*, 1996; Padda *et al.*, 2000; Tadros *et al.*, 2000). This can be explained, in part, by the fact that TNT breaks down into numerous metabolites and the toxicity of TNT is the total of toxicities of all these metabolites. Hence, hydroxylamines and monoamines in isolation have a lower toxicity than TNT. Understanding TNT toxicity must involve understanding its metabolism in the host species. The 'Green Liver Model' for plants, the normal detoxification pathways via the liver for animals, and the enzymatic metabolic pathways of TNT by unicellular organisms may be invoked to further understand toxicity. One study found RDX and HMX to be less mutagenic than TNT (Tan *et al.*, 1992), although plants transform TNT with greater ease (Bhadra *et al.*, 2001).

The toxicity of the final products of phytoremediation such as the conjugates and bound residues is of crucial importance from a practical view, since the local flora and fauna are ultimately exposed to these compounds. Generally, phytoremediation succeeds in reducing the toxicity of the system, although the final products have a residual toxicity. While most results find that TNT is toxic even at low concentrations, and its metabolites are generally less toxic than the parent compound, there are many exceptions to this trend. Mechanistic information about TNT toxicity is essential in understanding and developing means of reducing toxicity. Oxidative pathways are shown to exist for TNT phytoremediation by *Myriophyllum aquaticum* (Bhadra, Spanggord *et al.*, 1999), and testing the toxicity and mutagenicity of oxidative metabolites is essential in discovering if oxidative pathways have reduced toxicity levels.

2.10: Genetics of TNT Phytotransformation

Recent approaches to phytoremediation have focused on creating recombinant plants that express bacterial and mammalian genes to transform a certain pollutant (French *et al.*, 1999; Doty *et al.*, 2000; Meagher, 2000; Hannink *et al.*, 2001). These genetically modified plants show a greater tolerance to the pollutants, and metabolize them faster than the wild-type plants. In addition, the number of unknown products formed is reduced, since most of the flux passes through the recombinant pathways. Hannink (2001) showed the increased resistance to TNT of transgenics overexpressing a bacterial nitroreductase; 14-day-old transgenic tobacco seedlings removed TNT at concentration of 0.2 mM (45.6 mg/L) within 24 hours, whereas similarly grown 14-day-old wild-type seedlings did not remove any of the TNT from the system (Hannink *et al.*, 2001). Doty (2000) developed tobacco seedlings expressing the mammalian cytochrome P450 2E1, which showed a 640 fold increase in the metabolism of TCE. Ethylene dibromide (EDB) was also efficiently transformed by these transgenic tobacco seedlings (Doty *et al.*, 2000). These studies show the potential for transgenic plants to reveal insights about the enzymatics of TNT transformation, and in addition may find potential applications in the field.

Understanding the genetics of TNT transformation is another actively researched area. With the complete elucidation of the *Arabidopsis* genome, there exist powerful genetic tools to determine the genes and enzymes implicated in TNT transformation. This is accomplished by measuring DNA, RNA and metabolites levels and correlating the two. A recent paper detailing measurements of the levels of RNA in *Arabidopsis* seedlings exposed to TNT revealed the overexpression of several enzymes suspected in TNT phytotransformation (Ekman *et al.*, 2003). 15 mg/L of TNT was added to 14-day-old seedlings for a period of 24 hours, following which the tissue was frozen in liquid nitrogen and a SAGE transcription analysis was performed to measure RNA levels in the tissue. A glutathione-S-transferase was overexpressed 27-fold over uninduced *Arabidopsis* seedlings (Ekman *et al.*, 2003); this enzyme has been postulated to be involved in many xenobiotic and herbicide resistance studies. A Cytochrome P450 (CYP81D11-A-TYPE) and a possible nitroreductase (12-Oxyphytodienoate reductase, OPR-1) showed 14 and 10 fold increases in RNA levels, respectively (Ekman *et al.*, 2003). Both these families of enzymes have long

been speculated to be involved in TNT transformation. OPR-1 has an amino acid sequence with a high degree of homology to a nitroreductases from an *Enterobacter*. An ABC (ATP Binding Cassette) transporter was also induced 21-fold in the tissue (Ekman *et al.*, 2003); these transporters expend ATP energy to transport hydrophobic molecules into or out of the cytoplasm, often over a concentration gradient (Locher and Borths, 2004). While RNA expression information provides valuable information regarding the potential enzymes involved in TNT detoxification, complementary genetic and metabolic information is essential to make valid conclusions. Elucidating the specific roles of the implicated enzymes in the TNT transformation pathway by obtaining mutants over or under-expressing the genes coding for these enzymes will help to conclusively prove their role. Work on using TNT transformation studies and metabolite analyses on *Arabidopsis* mutants has been presented in Chapter 7.

2.11: In Conclusion

TNT metabolism has been understood a great deal in plants. A hypothetical pathway has been proposed for the same, and new studies are revealing information about the genes and enzymes involved. Field studies, from mesocosm levels, to actual field applications are also being actively conducted. Books and databases of information for TNT and xenobiotic remediation are being developed. Breeding of native hybrids which are robust in the field but possess enhanced TNT removal characteristics is a potential key to fast and efficient TNT removal in contaminated sites. Further advances in understanding the metabolism, genetics, enzymatics and kinetics of TNT transformation in plants is continuing in an effort to apply these elegant, green principles on a large scale.

Chapter 3: Presence of Hydroxylamines in the TNT Transformative Pathway

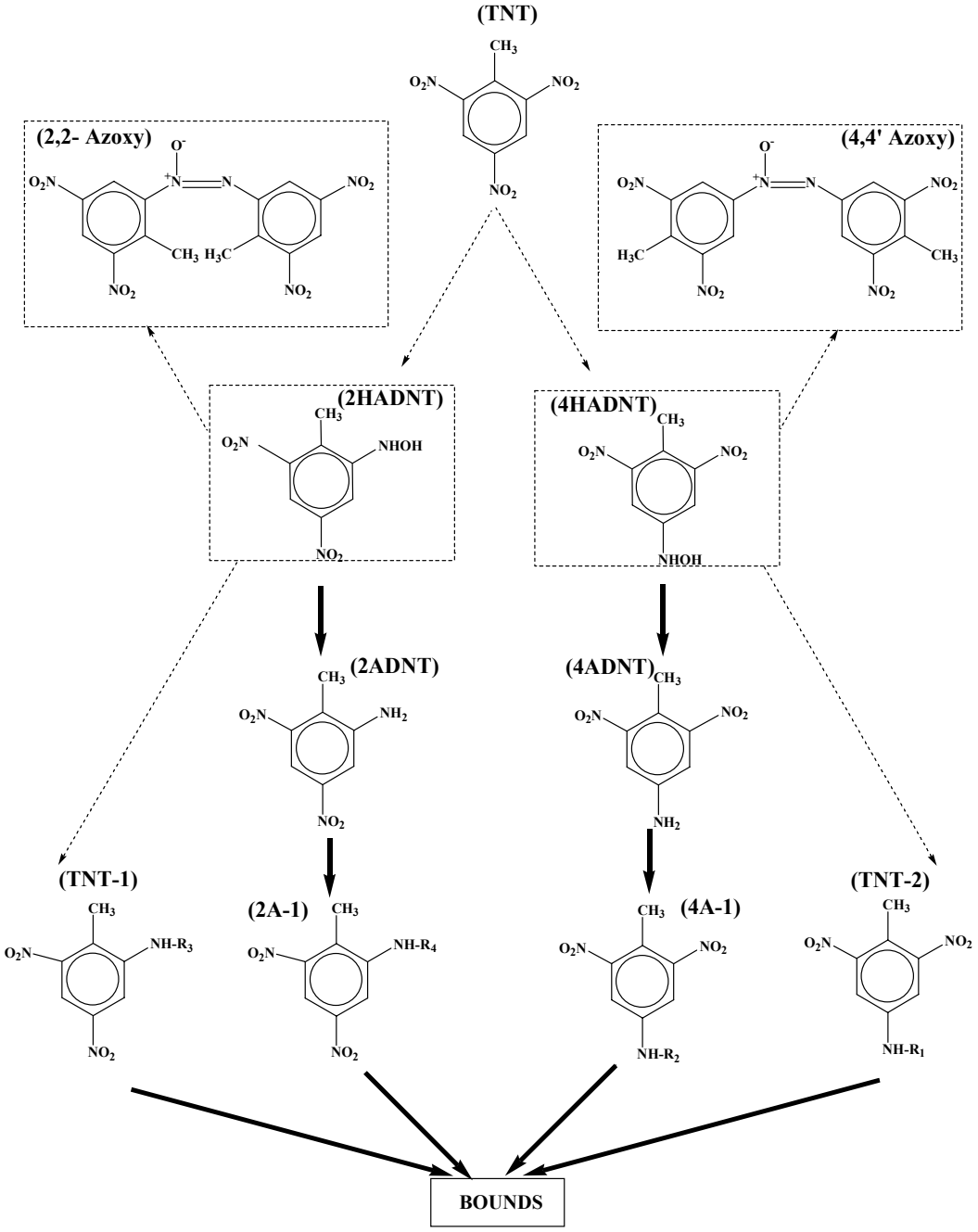
Sections of Chapter 3, 4 and 6 have been combined and prepared for publication in the journal Environmental Science and Technology (ACS Publications).

3.1: Objectives

Although TNT is taken up and transformed efficiently in a number of aquatic, terrestrial and tissue cultured plant systems, the metabolic pathways involved have not been completely delineated. A Green Liver model that describes the uptake and transformation of TNT by plants, wherein various reductive and oxidative steps are succeeded by the addition of plant biomolecules to the xenobiotic has been proposed. A final process of polymerization and sequestration inactivates the compound within the plant biomass. This model, which essentially predicts a reduction in the bioavailability of the xenobiotic, has been described in detail in the previous chapter (Chapter 2, Section 2.4). In experiments presented in this chapter, we will prove the formation of hydroxylamines as the primary metabolites in TNT transformation in the Green Liver model in two axenic (microbe-free) plant systems, using different analytical techniques. We will show that these hydroxylamines are formed ahead of the monoamines during TNT transformation and their inherent instability in aqueous, aerobic systems is responsible for their non-identification in all previous axenic plant systems exposed to TNT.

As shown in the TNT transformation pathway (Figure 3.1), plants transform TNT by reducing its nitro-groups to amine-groups, resulting in the formation of the monoamines 2-amino-4,6-dinitrotoluene (2ADNT) and 4-amino-2,6-dinitrotoluene (4ADNT). This is hypothesized to be accomplished via the partially reduced hydroxylamine intermediates, 2-hydroxylamine-4,6-dinitrotoluene (2HADNT) and 4-hydroxylamine-2,6-dinitrotoluene (4HADNT) (Burken *et al.*, 2000; Subramanian and Shanks, 2003). These hydroxylamines are also thought to be capable of direct conjugation, oxidation or bimolecular nucleation (Corbett and Corbett, 1995). The monoamines and hydroxylamines are subjected to further conjugative reactions, wherein a sugar or another plant biomolecule is added to the amino group. All these conjugates polymerize and irreversibly bind to the plant biomass representing

Figure 3.1: TNT Transformation Pathway: Proposed TNT transformation pathway in axenic plants as determined by xenobiotic feeding experiments in *C. roseus* and Arabidopsis. High level TNT feeding studies in *C. roseus* and Arabidopsis (described in this chapter) proved the presence of hydroxylamines and azoxies (shown in boxes) while the dotted arrows show novel sections of the pathway (confirmed with metabolite feeding studies presented in the next chapter). The bold arrows represent sections of the pathway confirmed by previously published studies (Burken *et al.*, 2000). All these steps, in total, represent the "Green Liver" model of TNT transformation by plants.


(TNT)
(2,2- Azoxy)
(4,4' Azoxy)
(2HADNT)
(4HADNT)
(2ADNT)
(4ADNT)
(TNT-1)
(2A-1)
(4A-1)
(TNT-2)
BOUNDS

the final fate (bounds) of the xenobiotic. Monoamines have ubiquitously been postulated to be the first stable metabolites identified in the transformation of TNT (Burken *et al.*, 2000; Subramanian and Shanks, 2003). In one representative study, the formation of 2ADNT and 4ADNT in quantities less than 5% of the initial TNT added during the complete course of the experiment in *C. roseus* roots was shown (Bhadra, Wayment *et al.*, 1999). Most studies show monoamines formed in amounts lesser than 10 to 15% of the initial TNT added. Since these monoamines are less toxic than TNT (Honeycutt *et al.*, 1996; Schafer and Achazi, 1999; Padda *et al.*, 2000; Tadros *et al.*, 2000), phytoremediation is assumed to lower the toxicity of the system.

However, in spite of the theoretical postulation of hydroxylamine formation, experimental evidence for the presence and role of hydroxylamines in the pathway is not readily available. Of the entire research detailing TNT transformation in plants, only two studies, to the best of our knowledge, report the formation of hydroxylamines as precursors to the monoamines (Pavlostathis *et al.*, 1998; Wang *et al.*, 2003). Pavlostathis (1998) shows the formation of 4HADNT in Eurasian Watermilfoil (*Myriophyllum spicaticum*) at low concentrations during the first 60 hours after TNT addition. TNT, added in initial levels of 50 μM disappeared from the media within 70 hours. Wang (2003) offers positive UV-visible spectral comparison and NMR confirmation for the presence of both 2HADNT and 4HADNT in Parrotfeather (*Myriophyllum aquaticum*). When TNT was added at an initial concentration of 25 mg/L to *M. aquaticum*, these metabolites were shown to be present in the system from the second day to the end of the experiment (15 days), but were never observed in totals of greater than 10% of initial TNT added. The plants took 15 days to remove all the TNT from the media. Both studies, however, used aquatic plants wherein complete axenic conditions were not achievable. Since these systems were not free of microbial presence, the role of plants in the formation of hydroxylamines is unconfirmed. Many bacterial systems, mainly anaerobic, have shown the formation of hydroxylamines (Esteve-Nunez *et al.*, 2001); it is possible that these bacterial enzymes were responsible for the presence of hydroxylamines in these aforementioned studies. Results presented here will overcome that deficiency and conclusively prove the formation of hydroxylamines, by plant enzymes, as the first metabolites to be formed in TNT phytotransformation.

As observed, although hydroxylamines have been postulated to be the primary metabolites formed during TNT transformation, and the gateway to many oxidized, reduced, conjugated and binuclear products, they have not been observed in any axenic TNT transformation study in plants. This is probably due to multiple reasons, the chief among them being the instability of hydroxylamines in an aqueous, aerobic system. Most phytoremediation studies are performed in a hydroponic aqueous system, and hence the hydroxylamines disappear before they can be detected. In addition, hydroxylamines are inherently very reactive, and can form oxidized, reduced, conjugated and binuclear metabolites, and hence this further reduces its lifetime in the system (Corbett and Corbett, 1995). Bacterial systems offset many of these factors, and hence hydroxylamines are more readily identified in those systems (Hughes, Wang, Bhadra *et al.*, 1998; Hughes, Wang, Yesland *et al.*, 1998; Naumov *et al.*, 1999; Esteve-Nunez *et al.*, 2001). Anaerobic bacterial systems provide conditions where hydroxylamines are more stable, and the higher rate of TNT reduction gives rise to a higher concentration of hydroxylamines in the system.

In stability studies on hydroxylamines described in this chapter, the degradation of 2HADNT and 4HADNT in methanol and water were monitored. The studies were performed to estimate the residence times of the hydroxylamines in a hydroponic experimental system, and to develop an analytical detection scheme that accounted for their (in)stabilities. The two plant species tested for hydroxylamine presence were the hairy roots *Catharanthus roseus* and the fully sequenced mustard *Arabidopsis thaliana*. *C. roseus* was chosen as a system because previous studies have elucidated the pathway involved in TNT transformation (Burken *et al.*, 2000). *C. roseus*, as mentioned in Section 2.3 of Chapter 2, is a robust plant system with high genetic stability, capable of producing reproducible results and hence has been used extensively in phytotransformation studies. Much of the research presented in this thesis uses these hairy roots as a test system. In previously published studies with *C. roseus*, initial TNT concentrations up to 60 mg/L failed to show the formation of hydroxylamines (Bhadra, Wayment *et al.*, 1999; Subramanian and Shanks, 2003). Hence, higher concentrations from 80 to 110 mg/L of initial TNT for 50 ml of media were used. A dual effect was expected from this experiment: the high concentration of TNT would raise levels of all metabolites in the system, including the hydroxylamines. In addition, the high TNT concentration would

exert toxic effects on the system, and slow the metabolism of the plant as a whole. This would reduce the rate of hydroxylamine degradation to other metabolites. It was hoped that the combination of these two factors could help in the detection of hydroxylamines.

Arabidopsis was chosen as the other test system because it is a versatile, fully sequenced plant and can be grown under sterile conditions in the lab. Since the genome of this plant is fully sequenced, it is a powerful tool in understanding TNT transformation genetics and metabolism. Many of the studies conducted in this thesis use Arabidopsis seedlings to determine the metabolic and genetic characteristics governing TNT transformation. The analytical methods employed play a key role in detecting the hydroxylamines and azoxies. Many different schemes were attempted, and the results from the most commonly employed EPA8330 method (using a C8 column) and a new, more suitable method utilizing a C18 column under non-polar conditions are presented.

3.2: Materials and Methods

3.2.1: Plants

Two different plant species were used in the studies described here. The first, hairy roots of *Catharanthus roseus*, which were grown as described in Hughes (1997), propagate solely as roots. *C. roseus* roots were grown in 50 ml of half strength Gamborg B5 media in 250 ml flasks, and shaken at 100 rpm in the dark. 14 days old, exponential phase roots were used in the TNT feeding experiments.

The other plant system tested, *Arabidopsis thaliana*, was grown axenically from seeds. 50 seeds of the plant were surface sterilized with 20% bleach for 15 minutes and subsequently rinsed three times with sterile water. They were next transferred to 50 ml of Arabidopsis growth media (4.2 mg/L of MS media salts, 20 g sucrose, 1 ml Gamborg B-5 vitamins, MES buffer, potassium phosphate in 1 L of nanopurified water at a pH of 5.7) in a 250-ml erlenmeyer flask and shaken at 100 rpm under light. Seedlings 14 days old were used in the TNT and metabolite feeding experiments. These seedlings consisted of both roots and leaves.

3.2.2: Chemicals

Solid TNT for feeding experiments was purchased from ChemServices (West Chester, PA), while liquid HPLC standards of TNT, 2ADNT, 4ADNT, 2HADNT, 4HADNT and 4,4'-azoxy were purchased from AccuStandards (New Haven, CT). All solvents, including 2-propanol, methanol, ethanol, ethyl ether and acetonitrile were purchased from Fisher Scientific.

3.2.3: Analytical Methods

Reverse-phase HPLC was used for the separation and identification of metabolites from the phytotransformation studies. A Waters system with a 717 autosampler equipped with a PDA detector was used for these studies. Two types of columns were used, a NovaPak C8 and a NovaPak C18 column. A mobile phase of 82% water and 18% 2-propanol were used with the C8 column to isolate and identify TNT, 2ADNT, 4ADNT and the previously identified conjugates of TNT-1, TNT-2, 2A-1 and 4A-1 (Bhadra, Wayment *et al.*, 1999). For the C18 column, conditions as described in Wang (2003), with a gradient mobile phase of 60% water and 40% acetonitrile ramped to 40% water and 60% acetonitrile, at a flow rate of 0.6 ml, was used. From minute 0 to 5, 75:25 of water: acetonitrile was used, from minute 5 to 12, 70:30 of water: acetonitrile was used, from minute 12 to 20, 40:60 of water: acetonitrile was used, and from minute 20 to 25, 75:25 of water: acetonitrile was used. This column was used to identify the metabolites 2HADNT, 4HADNT, 2,2'-azoxy and 4,4'-azoxy. Identification and quantification was done on the basis of retention time and spectral matches of samples to standards. Secondary confirmation for hydroxylamine presence was accomplished using Electron Impact Mass Spectroscopy. The sample was collected via HPLC separation, twice extracted into ethyl ether, and concentrated. Electron Impact ionization experiments were performed on a Finnigan TSQ700 triple quadrupole mass spectrometer Finnigan MAT (San Jose, CA) fitted with a Finnigan EI/CI ion source. Samples were introduced to the mass spectrometer using the solids probe. The probe was heated gradually from 100 to 400 degrees. The instrument was used as a single quadrupole and scanned from 50 to 1000 daltons. The spectrum obtained was compared to a previously reported spectrum (Fiorella and Spain, 1997)

3.2.4: Stability Analysis

In order to determine the stability of hydroxylamines in aqueous and organic solvents, a stability analysis was performed. An acidic pH of 1 and a near neutral pH of 6 were used for water and methanol to compare stabilities at different conditions. Samples were run periodically through the HPLC, with a C8 column, to measure concentration of hydroxylamines remaining.

3.2.5: High Concentration TNT Studies

In *C. roseus*, three different concentration levels of TNT- 0.33 mM (75 mg/L), 0.37 mM (85 mg/L) and 0.47 mM (108 mg/L) were added to separate flasks, and the flasks shaken at 100 rpm in the dark at 26°C. This experiment was performed with single samples. Filter sterilized TNT solution was used in all the studies. Samples were taken periodically while maintaining the sterility of the system and analyzed with a C8 column. For Arabidopsis seedlings, TNT was added to 14-day-old hydroponic seedling cultures and media samples taken periodically until 120 hours. An initial concentration of 100 mg/L (0.44 mM) of TNT, in triplicate, was used for the experiment. The flasks were constantly shaken at 100 rpm, and were placed under light.

3.3: Results and Discussion

3.3.1: Stability Analyses of Hydroxylamines

The stability studies were performed to estimate the residence time and turnover rate of hydroxylamines in both aqueous and organic systems. Since all phytoremediation systems are mainly aqueous, the results in aqueous systems are of special interest. Figure 3.2A and B show the stability plot for 2HADNT and 4HADNT in methanol, at a near neutral and acidic pH; while Figures 3.3A and B show similar data for aqueous systems. As seen in Figures 3.2A and B, the HADNTs have good stability in methanol, with little or no degradation in 30 hours. Variation of pH seemed to have no significant effect on the stabilities. However, in aqueous media, the HADNTs have pronounced lower stabilities, with 80% of the 2HADNT

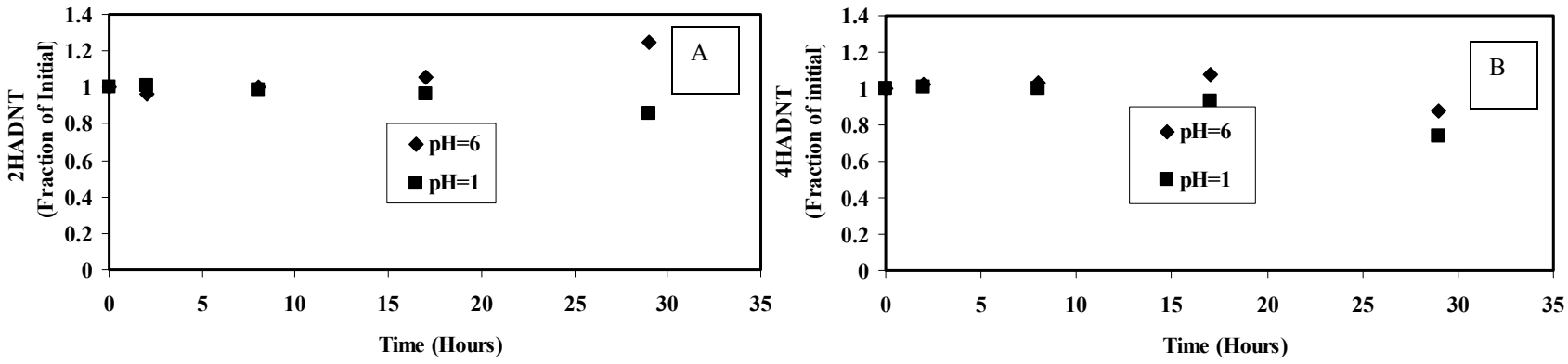


Figure 3.2: Stability of Hydroxylamines in Methanol: Stability of 2HADNT (A) and 4HADNT (B), dissolved in methanol, starting with an initial concentration of 5 mg/L as measured by reverse-phased HPLC. 2HADNT and 4HADNT are seen to be highly stable in methanol, with little or no degradation in 36 hours. The apparent increase in HADNT levels at the pH of 6 is due to evaporation of the solvent methanol. No significant difference in stability levels were seen at both pH levels. While only singles were used in this experiment, typical errors bars are of the range 10%.

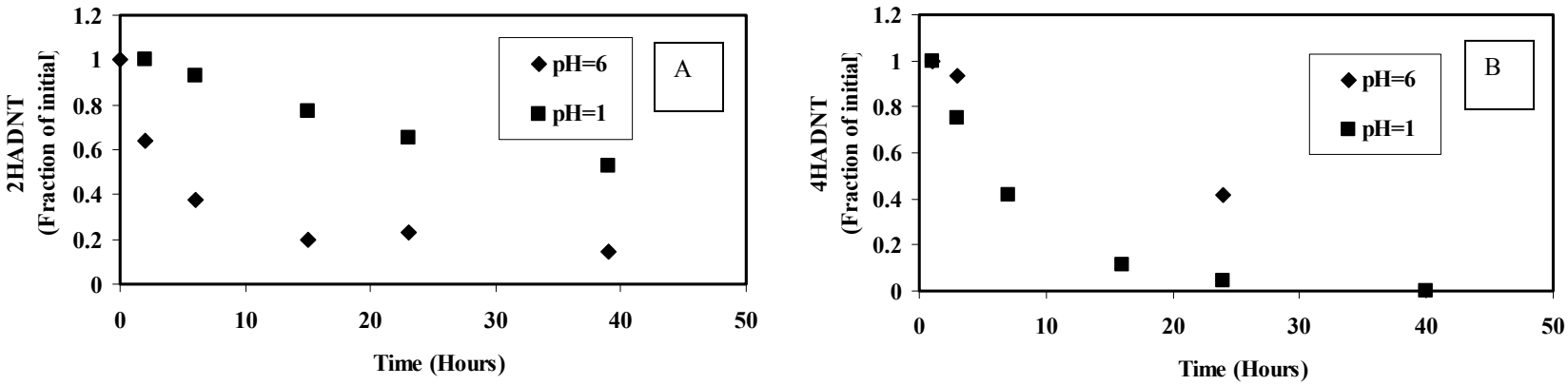


Figure 3.3: Stability of Hydroxylamines in Water: Stability of 2HADNT (A) and 4HADNT (B) in water, starting with an initial concentration of 5 mg/L. Stability studies were performed at a near neutral and acidic pH to determine more favorable conditions for measuring hydroxylamines. Since most phytoremediation studies are in aqueous conditions under neutral pH conditions, results from that study are the most important. More than 80% degradation of 2HADNT was observed within 40 hours, at the pH of 6. Complete degradation of 4HADNT was observed within 40 hours at the pH of 6. This makes detection of these metabolites in a phytoremediation system very challenging. While only singles were used in this experiment, typical error bars have been of the magnitude of 10%.

and 60% of 4HANDT disappearing in 40 hours at a pH 6 (Figures 3.3A and B). At a lower pH of 1, 60% of 2HADNT disappeared in 40 hours, and all of the 4HADNT disappeared in 40 hours. Hence, the stabilities of HADNTs in aqueous media are quite poor, with significant degradations occurring within the first few hours. While these results appear to contrast with those previously published (Wang *et al.*, 2000), which showed better stability for hydroxylamines under aqueous conditions, the one important difference is starting concentration of hydroxylamines. While Wang (2000) used an initial concentration of 50 mg/L, we used concentrations of 5 mg/L in an effort to mimic our in-vivo systems. Hence, at such low concentrations, even small amounts of degradation are reflected as large percentage drops in the amount remaining (zero-order degradation, independent of starting concentration). Low concentrations of hydroxylamines could also entail higher amounts of dissolved oxygen in the system, which can further reduce their stability. This is an additional probable cause for the difference in observed stability levels.

3.3.2: Hydroxylamine Detection in *C. roseus* and Arabidopsis

When high levels of TNT were added to *C. roseus* and Arabidopsis, hydroxylamines (2HADNT and 4HADNT) were detected for short periods as primary metabolites in both the systems. Primary identification for hydroxylamine presence was obtained from matching their retention time and full absorbance spectra with standards (Figure 3.4). Secondary confirmation for hydroxylamine presence was done through Electron Impact Mass Spectroscopy. Mass fragments from the sample were compared with standards. The hydroxylamines showed major mass fragments of 212, 197, 165 and 155 (Figure 3.5). Since hydroxylamines are highly unstable in aqueous media, their detection was found to be a very strong function of sample handling and applied analytical chemistry. Samples run immediately, in a C18 column, under a strong non-polar mobile phase, were more likely to reveal the formation of the hydroxylamines. Samples stored at -20 ºC for many hours were likely to result in the degradation of the hydroxylamines. Samples run through a C8 column were also likely to have the hydroxylamines degraded during their separation run. Hence, the C18 column was used for hydroxylamine detection, with minimal storage time. Samples that

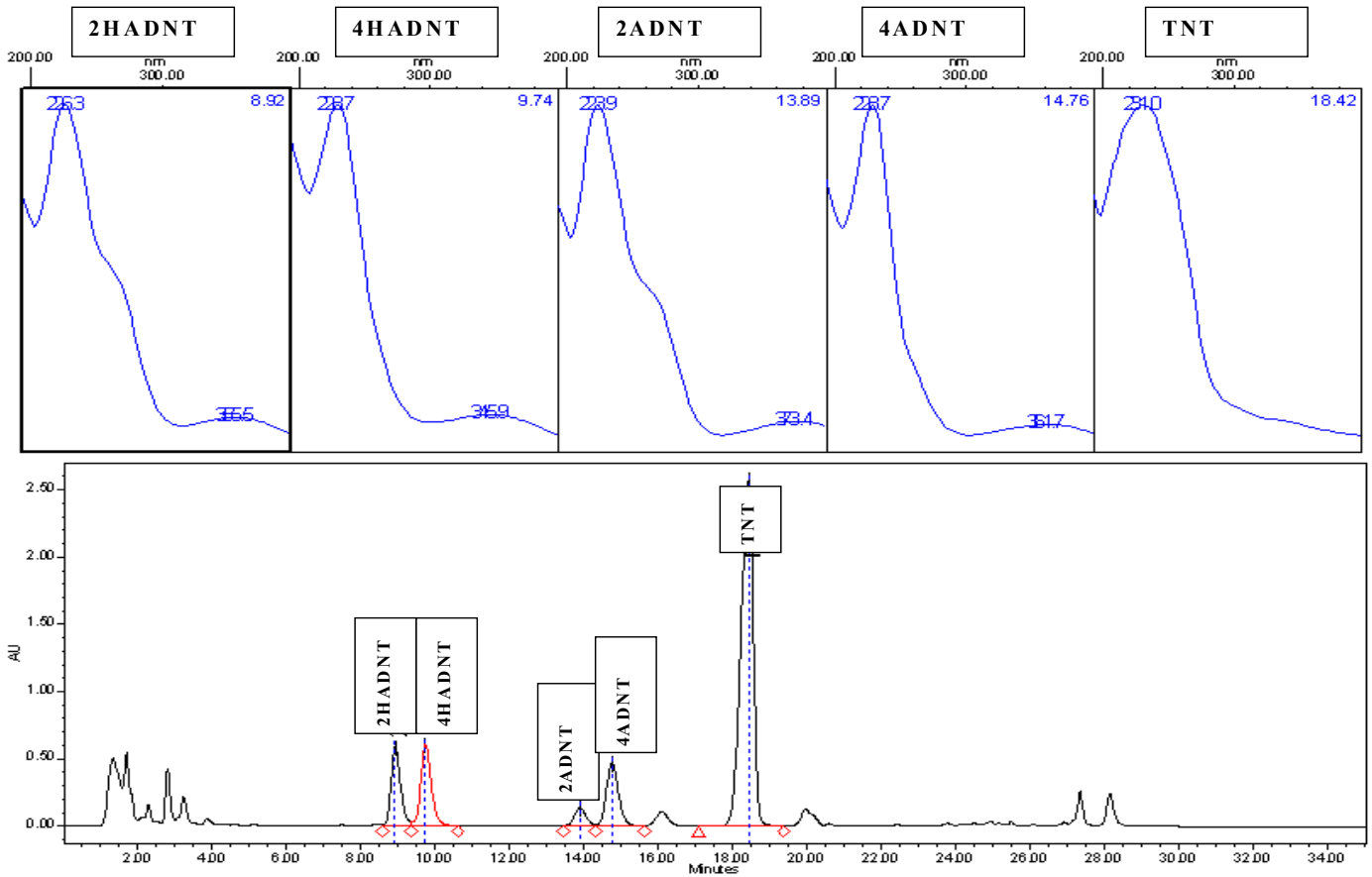


Figure 3.4: Metabolite Spectra and Chromatogram: Trace of a sample run showing the elution of TNT, 2HADNT, 4HADNT, 2ADNT and 4ADNT on a Nova-Pak C18 column with 2-propanol and water in the mobile phase. The PDA spectra for the metabolites are also shown, normalized along the y-axis for absorbance and x-axis for wavelength. The similarity between the 2ADNT and 2HADNT, and 4ADNT and 4HADNT spectra is observed. This makes distinguishing between the ADNTs and HADNTs a challenge, and hence their identification is dependent on the difference in retention times.

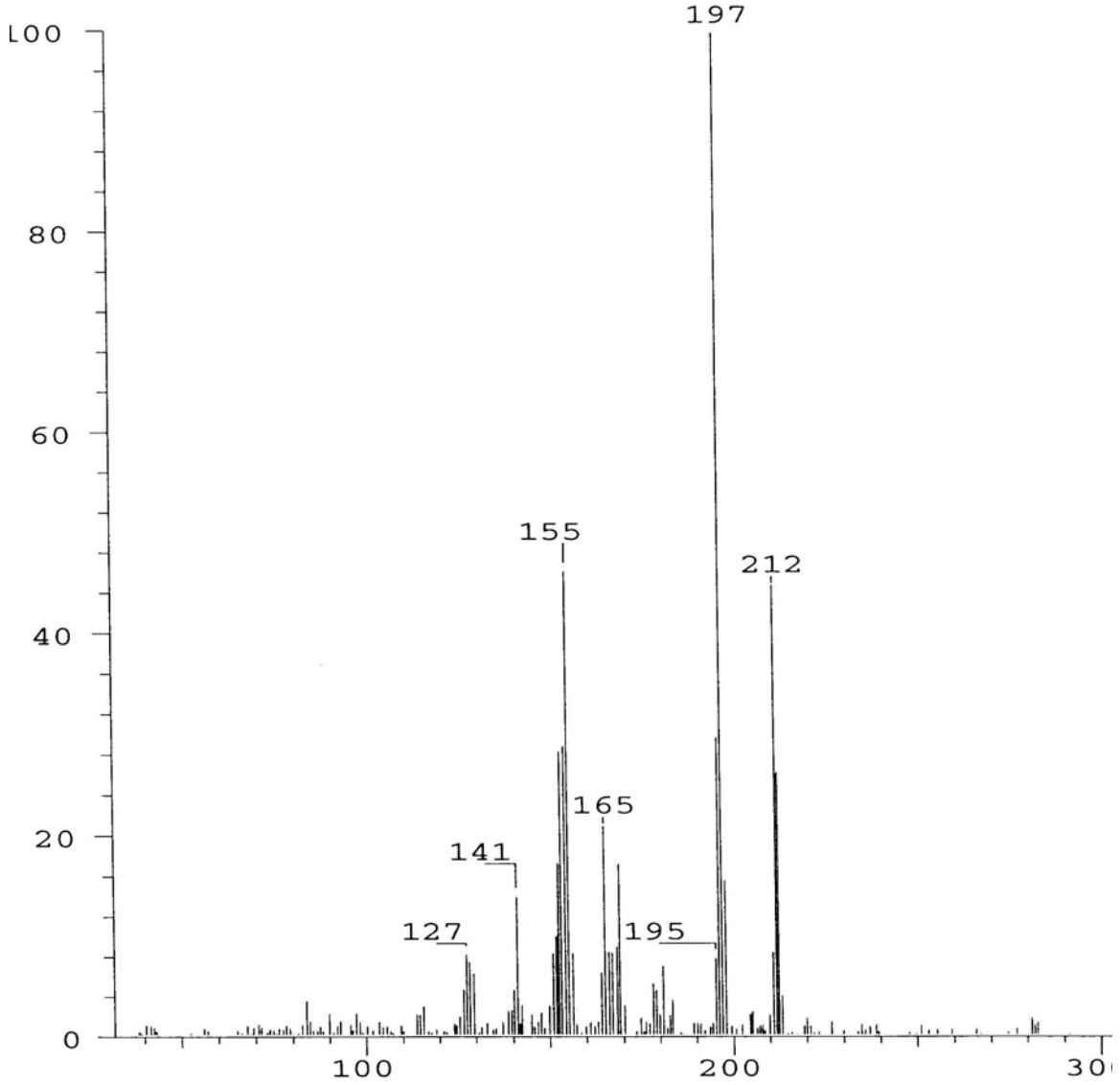


Figure 3.5: EI Mass Spectra of Hydroxylamines: Electron impact spectra of hydroxylamine samples as determined by a Finnigan TSQ700 triple quadrupole mass spectrometer. The x-axis represents (mass/charge), while the y-axis represents the relative intensity. The two main peaks are observed at 212 and 197; the first peak represents the molecular weight of the HADNT, while the second peak represents the largest ion. The EI spectrum is constant for any compound; this spectrum was compared to a standard from literature (Fiorella and Spain, 1997) to ensure that it belonged to HADNT.

were not run immediately were stored at -80 ºC until they could be analyzed; at this temperature the hydroxylamines tend to degrade slowly.

In *C. roseus*, when 80 to 110 mg/L of TNT were added to the roots, 2HADNT was detected in levels up to 3 mg/L, but disappeared within 20 hours of TNT addition. The temporal profile of TNT removal is shown in Figure 3.6, while the transient profile of 2HADNT is shown in Figure 3.7. The other hydroxylamine, 4HADNT was detected at only one time step in the 80 and 100 mg/L systems, at a maximum concentration of 1.7 mg/L (7.8 μM) 4.5 hours after TNT amendment (Table 3.1). The detection of these metabolites was possible because of the high initial levels of TNT since previous experiments with lower levels of TNT amendment had failed to reveal the formation of these metabolites (Hughes *et al.*, 1997; Vanderford *et al.*, 1997). In Arabidopsis, 2HADNT and 4HADNT were observed for the first 12 to 18 hours after 100 mg/L of TNT was added to the seedlings (Figure 3.8). 4HADNT (28% of initial TNT) was observed in greater levels than 2HADNT (22% of initial TNT). In addition to hydroxylamines, monoamines and conjugates were also observed during the course of the experiment. The transient profiles of these metabolites are discussed in detail in Chapter 6.

3.4: Discussion: Stability and Presence of Hydroxylamines in TNT Transformation

While an advanced understanding of the TNT metabolic pathway in plants exists (Subramanian and Shanks, 2003), the initial TNT transformation steps were never completely confirmed in axenic plant systems. Most of the phytotransformation studies published postulated monoamines to be the primary metabolites formed during TNT transformation (Burken *et al.*, 2000; Subramanian and Shanks, 2003). Wang (2003) in a recent paper show the formation of hydroxylamines as primary metabolites; they speculate, and we concur, that the reason for non-observance of hydroxylamines are artifacts of the analytical chemistry used. Essentially, the frequently used EPA 8330 scheme of explosive and their metabolite detection does not reliably work for either hydroxylamine or azoxy detection. In addition, hydroxylamines are highly unstable in aqueous, aerobic media (Wang and Hughes, 1998; Wang *et al.*, 2000) and turned over extremely rapidly (Subramanian and Shanks, 2003). The stability studies on hydroxylamines presented here show their poor

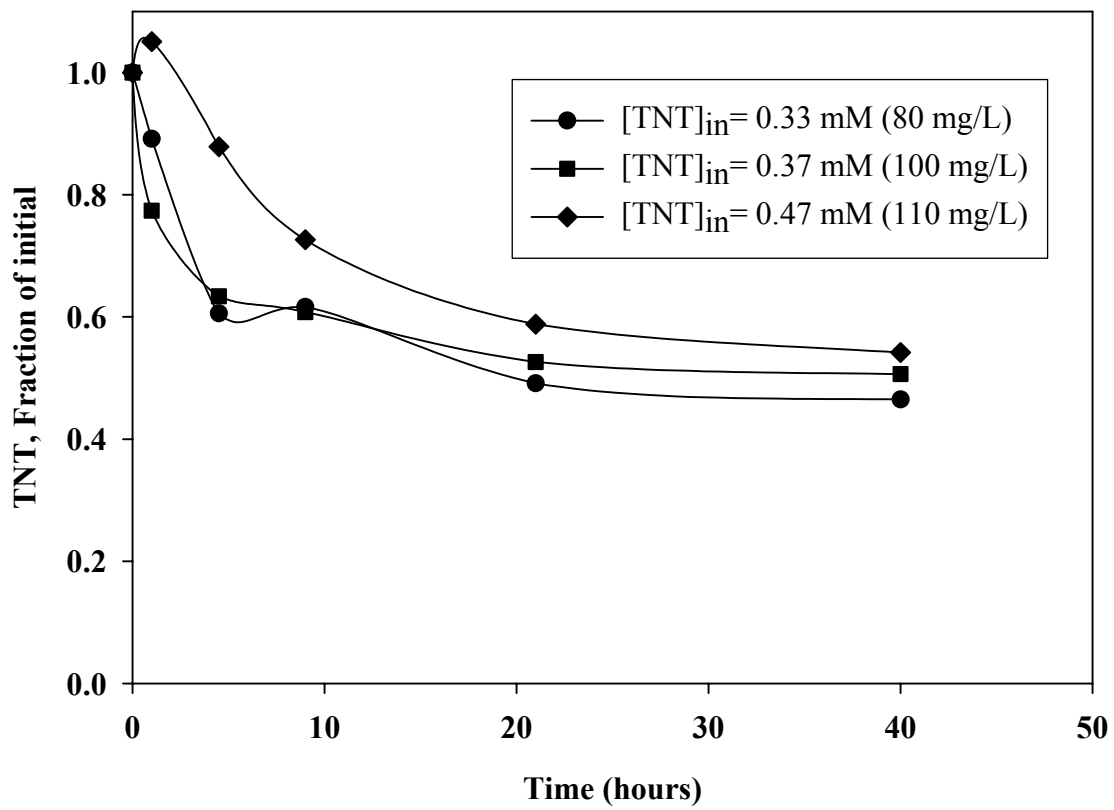


Figure 3.6: TNT Profile in *C. roseus*: Transient concentration profile of extracellular TNT in *C. roseus* hairy roots in high concentration TNT feeding studies. The higher levels of TNT used ensured higher concentrations of all metabolites, including hydroxylamines. The *C. roseus* seedlings were inefficient in their transformation of TNT, as evidenced by the slow rate of removal. Toxic effects including stunted growth and browning of root tips were observed.

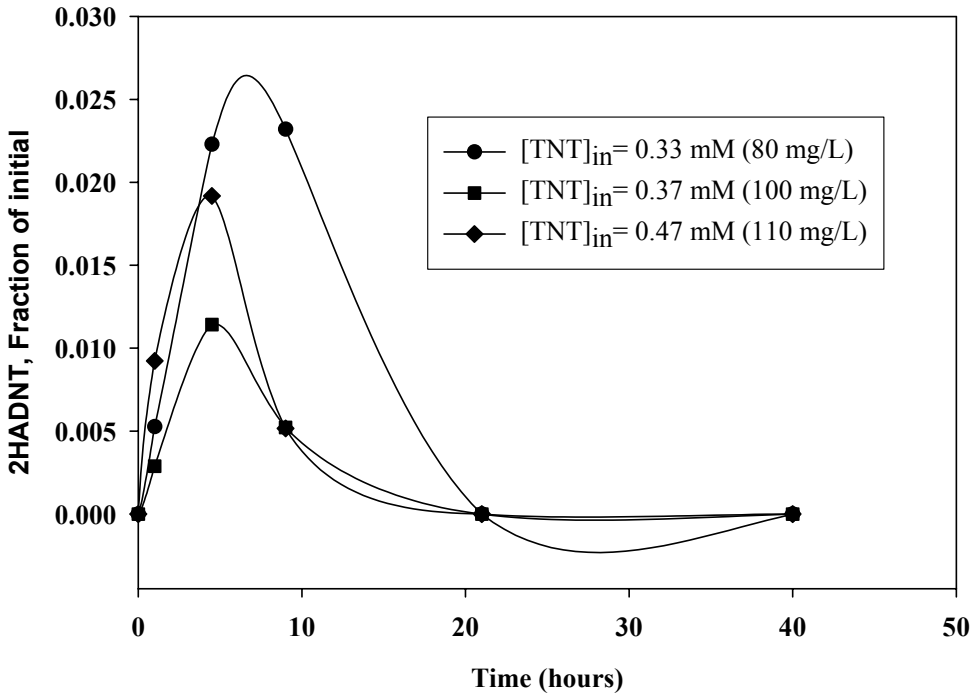


Figure 3.7: 2HADNT Profile in *C. roseus*: Transient extracellular concentrations of 2-hydroxylamine in axenic hairy roots of *C. roseus* in the high TNT concentration feeding experiment. This was the first instance of hydroxylamines being determined in an axenic phytoremediation study. In addition, previous studies with these hairy roots did not reveal the presence of hydroxylamines, because of their low levels and stability. High levels of initial TNT were used in the studies presented here, which revealed the presence of hydroxylamines. 2HADNT was formed at low levels, but disappeared rapidly within 20 hours. The highest levels of 2HADNT were seen in the lowest TNT-amended system, and vice-versa; this is due to the lower levels of TNT transformation in the highest TNT-amended system.

Table 3.1: 4HADNT Levels in *C. roseus*: Extracellular 4HADNT levels (in mg/L) in axenic *C. roseus roots* in the high-TNT concentration feeding experiment. Very low levels of the hydroxylamine were observed for up to 4.5 hours after TNT addition; 4HADNT disappeared below detection limit (1 mg/L, 3μM) rapidly from the system.

$[TNT]_{initial}$, mM	$[4HADNT]_{max}$, μM	Time since TNT addition, Hours
0.33	3.6	1
0.37	7.85	4.5

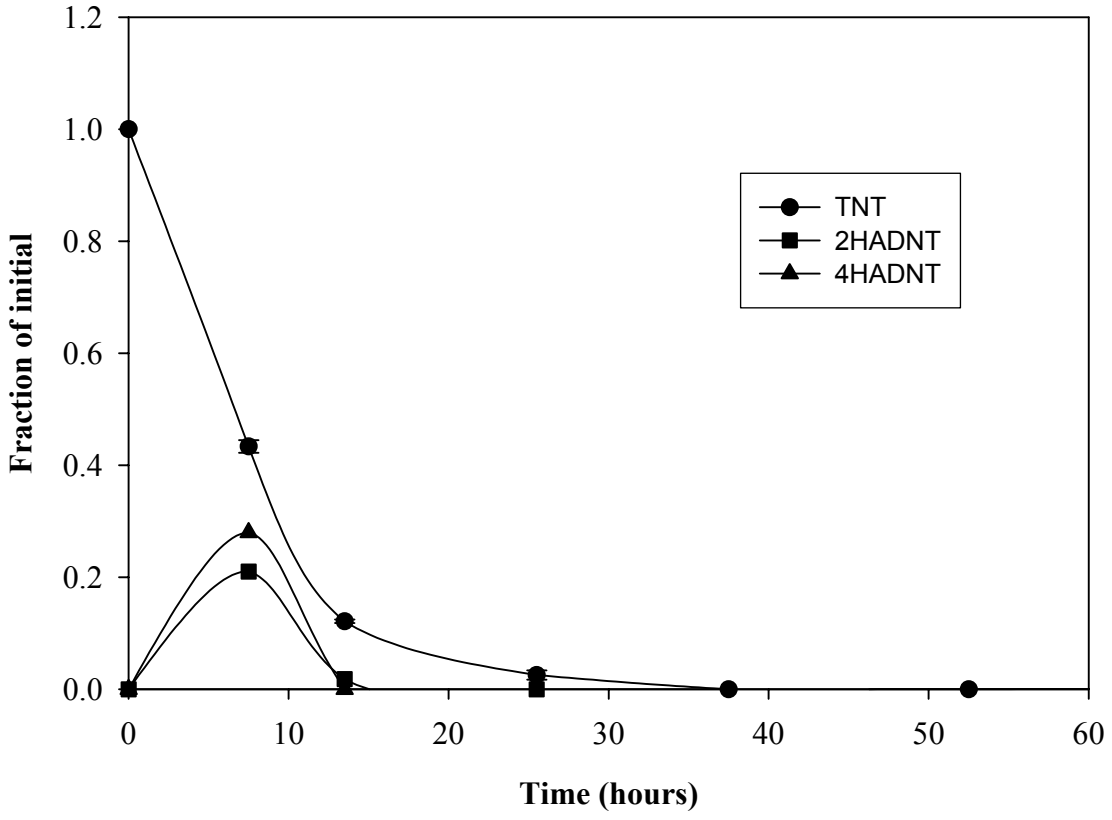


Figure 3.8: Transient Levels of Metabolite in Arabidopsis: Extracellular levels of TNT, 2HADNT and 4HADNT in Arabidopsis seedlings fed with 100 mg/L (0.44 mM) of TNT. The formation of hydroxylamines coincided with the removal of TNT, and they were present until 15 hours from TNT addition. The TNT concentrations are averages and standard deviations of triplicates, while the hydroxylamine concentration represents one seedling only. All three systems were analyzed for hydroxylamine concentrations, but due to their instability they degraded during the residence time. Only the first run samples showed hydroxylamines, and those are shown in the above figure. A rapid rate of both formation and degradation of hydroxylamines are seen. Apart from the *C. roseus* hairy roots, this is the only other axenic plant system where hydroxylamines have been observed.

stability in aqueous conditions, with over 80% degradation within a few hours. It should be noted that hydroxylamines present in an in-vivo system are faced with further reducing and oxidizing conditions that were not mimicked in our stability experiments. These additional driving forces will further reduce the stability of hydroxylamines, and cause their increased turnover. A combination of these factors could be responsible for the absence of hydroxylamines from previous axenic TNT phytotransformation studies

As mentioned earlier, two previous studies utilizing the non-axenic aquatic plants *Myriophyllum aquaticum* and *Myriophyllum spicaticum* have shown the formation of hydroxylamines during TNT transformation (Pavlostathis *et al.*, 1998; Wang *et al.*, 2003). Many bacterial systems, mainly anaerobic, have shown the formation of hydroxylamines (Esteve-Nunez *et al.*, 2001); it is possible that bacteria, existing in a symbiotic relationship with the plant, were responsible for the presence of hydroxylamines in these aforementioned studies. In studies presented here, two axenic plant systems, *C. roseus* hairy roots and Arabidopsis seedlings, did produce hydroxylamines. This proves the direct role of native plant enzymes in the formation of hydroxylamines. Both 2HADNT and 4HADNT were observed in both *C. roseus* and Arabidopsis, although their presence in both systems was short lived. Hydroxylamines, to the best of our detection scheme, appear in the first few hours after TNT addition and disappear within 12-18 hours. Hence, a narrow window exists for the identification of these metabolites. The significance of the hydroxylamine detection can be gauged from the numerous reactions this metabolite can undergo. Hydroxylamines can be biotically further reduced to monoamines (Corbett and Corbett, 1995), abiotically polymerized to azoxy compounds (Wang *et al.*, 2003) and also follow oxidative schemes (Bhadra, Spanggord *et al.*, 1999).

Given the variety of reactions hydroxylamines can undergo, they are central variables in the TNT transformation pathway and can potentially manipulate the direction of TNT phytotransformation. Hydroxylamines have shown to be more toxic and mutagenic than all other TNT metabolites in various studies (Tan *et al.*, 1992; Honeycutt *et al.*, 1996; Padda *et al.*, 2000; Tadros *et al.*, 2000), and hence their formation is of interest in TNT phytoremediation. Minimized residence time of hydroxylamines appears to be conducive to

better plant health, while their persistence in the system can cause plant health deterioration. This has been shown through various metabolite feeding studies, as discussed in Chapter 6.

3.5: In conclusion

Hydroxylamines have been detected as the primary metabolites during TNT transformation by plants as evidenced by their presence in two axenic, hydroponic systems. Their low stability in aqueous media and high reactivity causes a high turnover rate and hence they have not been detected in most previously published phytotransformation studies, and have never been identified in an axenic phytotransformation study. In their role as primary metabolites, hydroxylamines possess the ability to branch the TNT transformation pathway in many different directions, which makes them of special interest. In addition, their high toxicity and mutagenicity makes them of special interest from an implementation point of view. The next chapter delves into the specific role of hydroxylamines in the TNT pathway.

Chapter 4: Role of Hydroxylamines in the Phytotransformation of TNT

4.1: Objectives

While the previous chapter established the presence of hydroxylamines during the transformation of TNT by axenic plant systems, this chapter attempts to delineate their specific role in TNT transformation. This small chapter extends upon the results of the previous chapter. Previous feeding studies with TNT and ADNTs had indicated that hydroxylamines probably play a role in the formation of conjugates and oxidative metabolites (Bhadra, Spanggord *et al.*, 1999). In work presented here, confirmation for the role of hydroxylamines in the formation of conjugates was accomplished by feeding various TNT transformation metabolites to *C. roseus* roots or Arabidopsis seedlings, and analyzing the extracellular and intracellular metabolites. These results, coupled with previous inferences drawn from literature were combined to advance the understanding of the TNT transformation pathway.

4.2: Materials and Methods

4.2.1: Chemicals

Liquid HPLC standards of TNT, 2ADNT, 4ADNT, 2HADNT, 4HADNT and 4,4'-azoxy were purchased from AccuStandards (New Haven, CT). The 2HADNT, 4HADNT, 2ADNT and 4ADNT standards were also used in the low concentration metabolite feeding studies too, since the solid form of these compounds were not always available. All solvents, including 2-propanol, methanol, ethanol, ethyl ether and acetonitrile were purchased from Fisher Scientific.

4.2.2: Plants

Two different plant species were used in the studies described here. The first, hairy roots of *Catharanthus roseus*, which were grown as described in Hughes *et al*. (Hughes *et al.*, 1997), propagate solely as roots. *C. roseus* roots were grown in 50 ml of half strength

Gamborg B5 media in 250 ml flasks, and shaken at 100 rpm in the dark at 26 °C. Seven day old, early exponential phase roots were used in the TNT and metabolite feeding experiments.

The other plant system tested, *Arabidopsis thaliana*, was grown axenically from seeds. 50 seeds of the plant were surface sterilized with 20% bleach for 15 minutes and subsequently rinsed three times with sterile water. They were next transferred to 50 ml of Arabidopsis growth media (4.2 mg/L of MS media salts, 20 g Sucrose, 1 ml Gamborg B-5 vitamins, MES buffer, Potassium phosphate in 1 L of nanopurified water at a pH of 5.7) in a 250 ml Erlenmeyer flask and shaken at 100 rpm under light at 25 °C. Seven day old, early exponential phase seedlings were used in the TNT and metabolite feeding experiments. These seedlings consisted of both roots and leaves.

4.2.3: Analytical Methods

Reverse-phase HPLC was used for the separation and identification of metabolites from the phytotransformation studies. A Waters system with a 717 autosampler equipped with a PDA detector was used for these studies. Two types of columns were used, a NovaPak C8 and a NovaPak C18 column. An isocratic mobile phase of 82% water and 18% 2-propanol were used with the C8 column to isolate and identify TNT, 2ADNT, 4ADNT and the previously identified conjugates of TNT-1, TNT-2, 2A-1 and 4A-1 (the formation of these conjugates have been described in Chapter 2, section 2.4.3) (Bhadra, Wayment *et al.*, 1999). For the C18 column, conditions as described in Wang 2003, with a gradient mobile phase of 60% water and 40% acetonitrile ramped to 40% water and 60% acetonitrile, at a flow rate of 0.6 ml, was used. From minute 0 to 5, 75:25 of water: acetonitrile was used, from minute 5 to 12, 70:30 of water: acetonitrile was used, from minute 12 to 20, 40:60 of water: acetonitrile was used, and from minute 20 to 25, 75:25 of water: acetonitrile was used. This column was used to identify the metabolites 2HADNT, 4HADNT, 2,2'-azoxy and 4,4'-azoxy. Identification and quantification was done based on retention time and spectral matches of samples to standards.

4.2.4: Extraction of Intracellular Metabolites

At the completion of the experiment, at 50 hours, an analysis of products within the tissue was performed through an intracellular extraction procedure. This required the sacrifice of the seedlings and extraction of metabolites through a procedure of freeze-drying and sonication. Biomass, frozen at -20 °C, was freeze dried for 48 to 72 hours. The dried, friable biomass was then ground in a pestle and mortar in the presence of liquid nitrogen. The powdered biomass was sonicated in 15 to 20 ml of methanol twice, at 15 °C, for 36 to 48 hours to extract the metabolites. The methanol was centrifuged, decanted, evaporated down to 5 ml, and subsequently analyzed via HPLC.

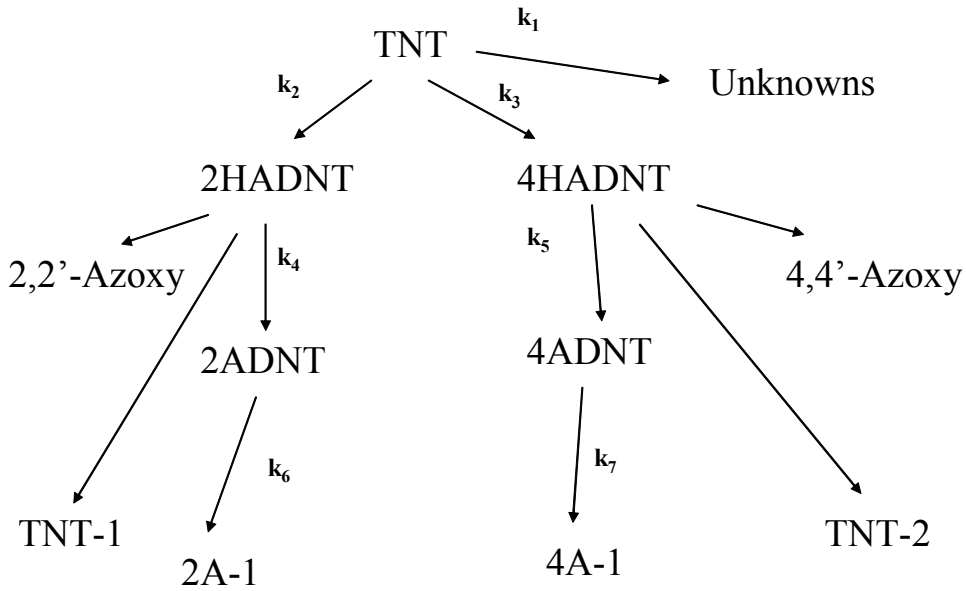
4.2.5: Metabolite Feeding Studies

2HADNT, 4HADNT, 2ADNT and 4ADNT were added to seven day old *C. roseus* roots at a concentration of 5 mg/L and the media analyzed for metabolite formation. In Arabidopsis, 2HADNT, 4HADNT, 2ADNT and 4ADNT were added to different seven day old axenic Arabidopsis seedlings at a starting concentration of 5 mg/L. 25 of Arabidopsis seeds per flask were used in these studies, and a volume of 25 ml of media was used per flask. All experiments were performed with duplicate samples.

4.2.6: Kinetics of the Pathway

First order kinetic rate of transformation for various metabolites in the pathway were calculated to provide relative rates between branches of the pathway. While the first order expression implicitly assumes a constant biomass, since early exponential phase Arabidopsis seedlings were used this assumption does not hold true. However, since the rate of biomass increase is the same for all metabolites, this approximate first order model provides relative rates between various transformations occurring in the pathway. The rate constant thus derived is referred to as Pseudo-first order rate constant. Figure 4.1 shows a schematic of the transformation pathway, and the various kinetic rate constants involved.

k_{TNT} was calculated directly from TNT transformation at an initial concentration of 60 mg/L (data from Chapter 6, section 6.2.6.2). From information on the formation of unknowns (described in Chapter 6, section 6.2.6.1), it was found that 33% of the initial TNT



Pseudo-1st Order Rate Constant	(hr^{-1})
k_1	0.04
k_2	0.03
k_3	0.04
k_4	0.41
k_5	1.97
k_6	0.05
k_7	0.16

Figure 4.1: Kinetics of TNT Transformation: Schematic of the TNT transformation pathway showing the various kinetic rate constants and their values in Arabidopsis.

was converted to unknowns. From 2HADNT and 4HADNT formation amounts, it was found that 22% of initial TNT went towards the formation of 2HADNT and 28% to 4HADNT (described in Chapter 6, section 6.2.6.2). Hence, k_{TNT} was split between these three metabolites to give k_1, k_2 and k_3. The degradation rates of 4HADNT and 2HADNT from the same experiment gave the value of k_4 and k_5, respectively. The transformation of 2ADNT and 4ADNT in the monoamine feeding studies (described in this chapter, section 4.2.5) gave the values of k_6 and k_7, respectively. Exponential fits on the data gave the value of the pseudo-first order rate constant.

The equations for the pseudo-first order metabolite kinetics were derived in equations (1) through (4), where

M is the metabolite of interest;

t represents the time since the tissue was amended with TNT or the metabolite.

The kinetic expression is assumed to first-order with respect to the concentration of the metabolite [M] and first-order with respect to plant biomass [P], as shown in figure (1), where k is the intrinsic rate constant.

$$d[M]/dt = k\times[P] \times[M] \qquad (1)$$

Equation (1) can be simplified to a pseudo-first order rate expression by assuming [P] to be constant, and hence a pseudo-first order rate constant $k_1= k\times[P]$.

Therefore, (1) is rewritten as:

$$d[M]/dt = k_1\times[M] \qquad (2)$$

Integrating both sides, with limits 0 to t, and $[M]_{in}$ to [M], where $[M]_{in}$ is the initial concentration of the metabolite M gives equation (3).

$$\int (d\,[M]/\,[M]) = \int k_1\times dt \qquad (3)$$

Upon integration, $\ln ([M]/[M]_{in}) = k_1\times t$, or

$$[M]= [M]_{in} \exp (k_1\times t) \qquad (4)$$

Hence an exponential fit of [M] versus t should provide the value of k_1 as the slope.

This calculation was performed for all the measured metabolites and TNT.

4.3: Results

When *C. roseus* roots were fed 2ADNT, they produced only the conjugate 2A-1 in the extracellular fraction; when they were fed with 2HADNT, they formed the conjugates 2A-1 and TNT-1, both in the extracellular fraction. Since 2HADNT is upstream of 2ADNT in the TNT transformation pathway, it follows that a portion of 2HADNT is being reduced to 2ADNT that is subsequently being conjugated to 2A-1; simultaneously a parallel branch of 2HADNT is being directly conjugated to form TNT-1. The flow of logic is summarized in Table 4.1 and explained schematically in Figure 4.2.

When 4HADNT was fed to the *C. roseus* roots no apparent metabolites in either the extracellular or the intracellular-extractable fraction were observed. 4ADNT fed roots showed the formation of the conjugate 4A-1 in the extracellular fraction. Hence, no conclusive evidence on 4HADNT conjugation was seen in *C. roseus*. A starting concentration of 5 mg/L HADNT was used in all the HADNT feeding experiments and the sensitivity of the HPLC in measuring metabolites and conjugates is at 1 mg/L. Therefore, the concentration of the metabolites and the conjugates were close to the detection limits of the instrument at all times. Therefore, the non-observance of 4-substituted metabolites and conjugates in the 4HADNT-fed roots could be a result of insufficient concentrations. All extracellular metabolites were detected in concentrations of 1 to 3 mg/L. No metabolites were observed in the intracellular-extractable fraction in any of the above experiments. Since the roots or seedlings were sacrificed at 50 hours it is expected that all metabolites and conjugates would have reached their final intracellular-bound form by this stage and hence be immeasurable. No azoxies were observed in these studies either, probably due to their low concentration levels.

When Arabidopsis seedlings were fed with 4ADNT they produced the conjugate 4A-1 extracellularly; when they were fed with 4HADNT they produced both the conjugates TNT-2 and 4A-1, both in the extracellular fraction. Similar to the logic pursued in *C. roseus* for 2ADNT and 2HADNT feeding studies, these results imply that 4HADNT is being directly conjugated to form TNT-2. The 4HADNT-fed seedlings showed the initial formation of TNT-2 and 4ADNT, in the media, within 2 hours of hydroxylamine addition. As 4ADNT disappeared from the media, the other conjugate 4A-1 began to accumulate in the media.

Table 4.1: Feeding Studies: Summary of feeding studies performed in Arabidopsis and *C. roseus*. Only experiments that produced useful finding have been tabulated. All the metabolites reported in this table were observed in the extracellular fraction. All experiments were performed in duplicate.

Expt.#	Plant Species	Compound Fed	Metabolites Observed, Extracellular
1	*C. roseus*	TNT	2HADNT, 2ADNT, 2A-1, TNT-1
2	*C. roseus*	2HADNT	2ADNT, 2A-1, TNT-1
3	*C. roseus*	2ADNT	2A-1
4	Arabidopsis	TNT	4HADNT, 4ADNT, 4A-1, TNT-2
5	Arabidopsis	4HADNT	4ADNT, 4A-1, TNT-2
6	Arabidopsis	4ADNT	4A-1

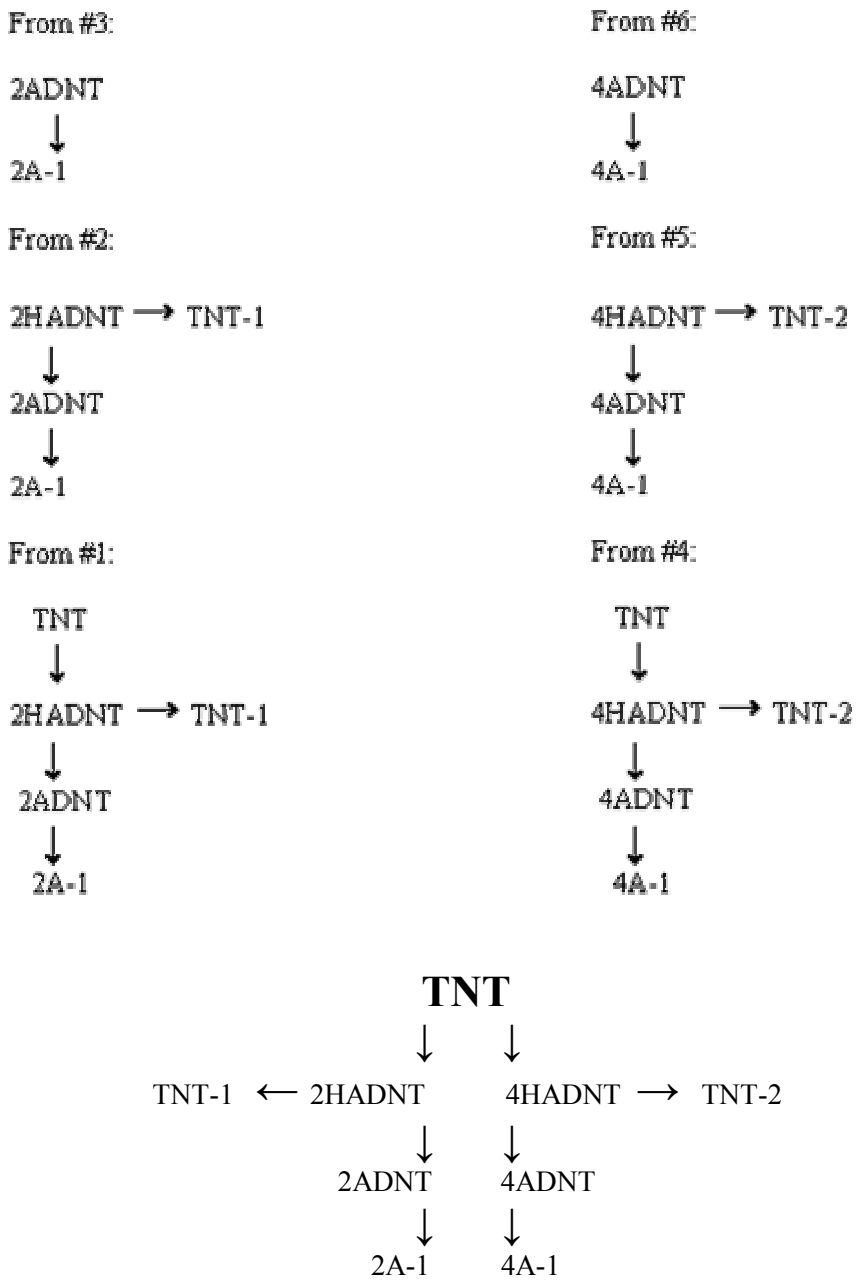


Figure 4.2: Logical Pathway Flow: Flow of logic showing the metabolite formation in the experiments listed in Table 4.1. These figures show how the various feeding studies taken in total prove the conjugative capacity of hydroxylamines.

This was in conformity to previous speculation that 4A-1 is formed by the conjugation of 4ADNT (Bhadra, Spanggord *et al.*, 1999). No metabolites or conjugates were observed in the intracellular-extractable fraction, probably due because they were bound to the biomass.

When 2HADNT was fed to the seedlings no extracellular or intracellular metabolites were observed in either of the duplicates. When 2ADNT was fed to the seedlings 2A-1 was measured in both the intracellular and extracellular fraction. Since no metabolites were detected in the 2HADNT-fed seedlings the conjugation of 2HADNT of was not proven in Arabidopsis seedlings. As with the case of *C. roseus*, low concentrations and the 1 mg/L detection limit of the HPLC may have been responsible for the non-observance of metabolites in the 2HADNT system. No azoxies were measured during these studies as well. All the extracellular concentrations were in the range of 1 to 3 mg/L. All experiments have been summarized in Table 4.1 and the flow of logic explained schematically in Figure 4.2. Based on previously published feeding studies (Bhadra, Spanggord *et al.*, 1999; Bhadra, Wayment *et al.*, 1999) we postulated that hydroxylamines can be directly conjugated (Subramanian and Shanks, 2003). However, thus far, no confirmatory studies had been performed for the same. The hydroxylamine feeding studies performed here provide a confirmation that hydroxylamines can indeed be directly conjugated without a further reduction of their hydroxylamine moiety.

4.4: Discussion

With the identification of hydroxylamines as the primary metabolites in TNT transformation, determining their role in pathway direction and kinetics became of interest. Previous studies had indicated that while oxidative products are seen during TNT transformation by plants, they were not formed when only monoamines were added. In addition, not all conjugates were seen when only the monoamines were added to the roots. It was suggested from these studies performed on *C. roseus* that hydroxylamines can directly be conjugated (Bhadra, Spanggord *et al.*, 1999; Bhadra, Wayment *et al.*, 1999). In those studies, TNT added to *C. roseus* roots showed the formation of the conjugates TNT-1, TNT-2, 2A-1 and 4A-1; however when 2ADNT was added to the roots, only 2A-1 was formed, and when 4ADNT was added to the roots, only 4A-1 was observed. Hence, it was speculated

that a metabolite upstream of the ADNTs was responsible for the formation of TNT-1 and TNT-2. In feeding studies presented here, ADNT-fed roots showed the formation of 2A-1 and 4A-1, while HADNT-fed roots showed the formation of all four conjugates. This confirmed that hydroxylamines are those upstream metabolites capable of direct conjugation. It has been shown that hydroxylamines can abiotically combine to form azoxies (Wang *et al.*, 2003); it has also been speculated that hydroxylamines are responsible for the formation of oxidative metabolites (Bhadra, Spanggord *et al.*, 1999), although this has not be corroborated. Given this variety of reactions hydroxylamines can undergo, they are central variables in the TNT transformation pathway and can be used for potential opportunities for the metabolic pathway manipulation of TNT transformation.

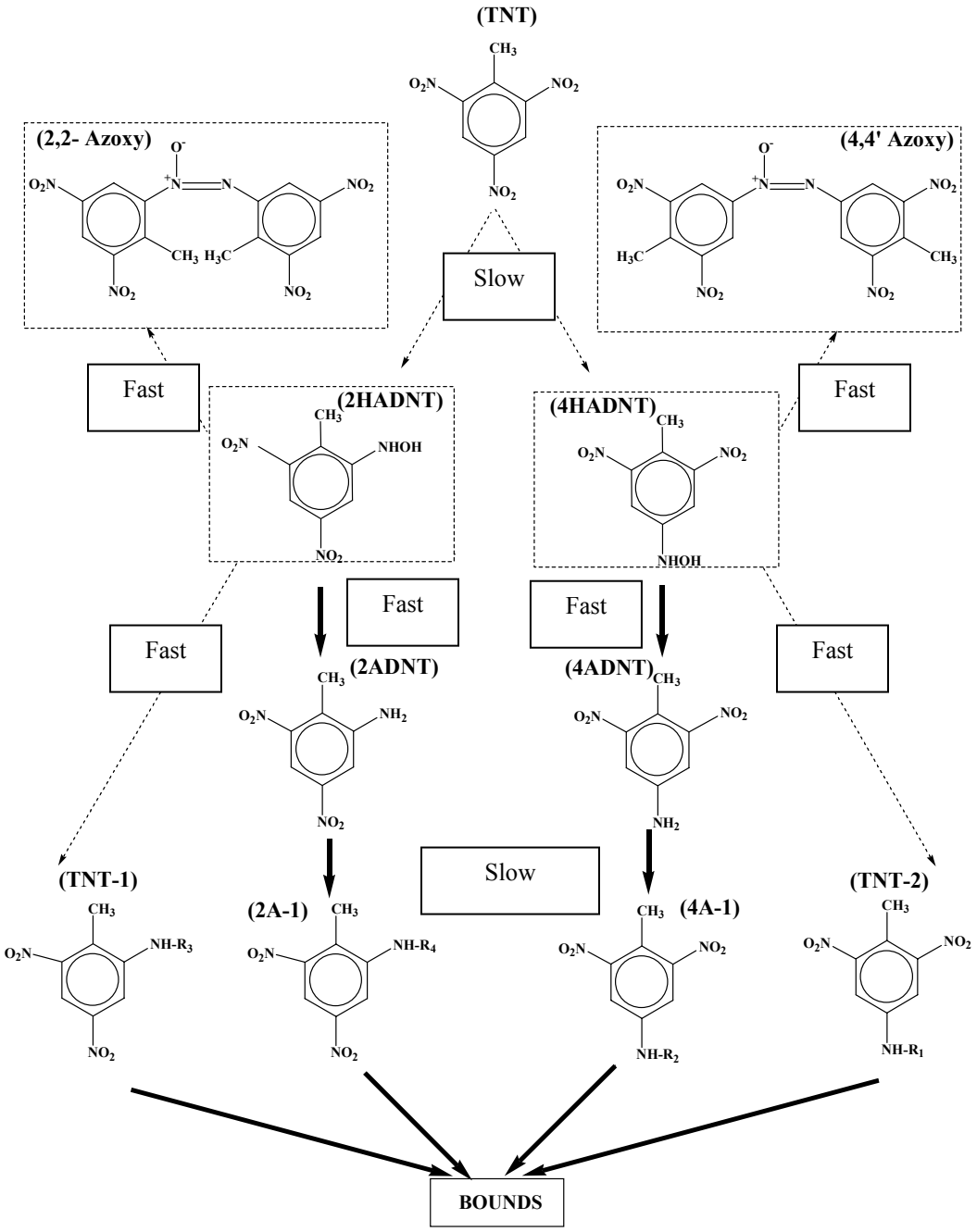
There appear to be, qualitatively and from a calculation of rates, two rate-limiting steps in the TNT transformation pathway- (1) TNT to hydroxylamines and (2) monoamines to conjugates. The data for the kinetic rates calculations in Arabidopsis was obtained from the TNT amendment experiment (Chapter 6, section 6.2.6.2) and the metabolite feeding experiments (this chapter, section 4.2.5). The concentration profiles for the TNT and the metabolites are given in Chapter 6 (sections 6.3.2 and 6.3.3). This section only presents a kinetic analysis of this data in Arabidopsis, with special relevance to hydroxylamines. When TNT is partially reduced, the product hydroxylamines are rapidly turned over. The pseudo first order rate constant for TNT reduction to 2HADNT, k_2, is 0.03 (hr^{-1}), and the rate of TNT reduction to 4HADNT, k_3, is 0.04 (hr^{-1}). In contrast, the rate of 2HADNT removal, k_4, is 0.41 (hr^{-1}), while the rate of 4HADNT removal, k_5, is 1.97 (hr^{-1}). Hence, the rate of 4HADNT removal is 53 times its formation, while the rate of 2HADNT removal is 14 times its formation from TNT. This indicates that the reduction of TNT to hydroxylamines is rate limiting in the initial part of the pathway. Hence, genes over-expressing enzymes that reduce TNT to hydroxylamines are potentially the most promising in terms of speeding up TNT removal. Monoamines are formed from the rapid reduction of hydroxylamines and are subsequently conjugated. The rate of HADNT removal can be used as a rough estimate for ADNT formation, and these rates were calculated to be 0.41 (hr^{-1}) (k_4) and 1.97 (hr^{-1}) (k_5) for 2ADNT and 4ADNT, respectively. In contrast, the rate of 2ADNT removal, k_6, was calculated as 0.05 (hr^{-1}), and 4ADNT removal, k_7, was found to be 0.16 (hr^{-1}). Hence the rate

of 2ADNT formation was 8 times its removal, while the number for 4ADNT is 12.7. These numbers, and the fact that the hydroxylamines disappear from the system within 20 hours, while the ADNTs persist for the length of the experiment (up to 120 hours), implies that ADNT turnover is rate-limiting in this section of the pathway. It has also been shown that hydroxylamines can be directly conjugated. Hence, knowledge of the genes and enzymes involved in hydroxylamine and amine conjugation can lead to the development of transgenics wherein the hydroxylamines are extensively conjugated, thereby avoiding the intermediary rate limiting amine step. The conjugates are transported into the cell and bound to the plant biomass. Wang (2003) also mention the possibility of directing the TNT flux from hydroxylamines toward azoxies and away from the monoamines. Since azoxies, like hydroxylamines, are rapidly turned over, there exists potential for overall faster TNT to bounds pathway. In essence, making conditions unfavorable for HADNT to ADNT reduction should channel the flux in directions away from the ADNTs. Sulfates and nitrates are two ubiquitous electron acceptors that can compete for the electrons required for the reduction of HADNT. Studies on the effect of nitrate concentration on RDX biotransformation show that high levels of nitrate (500 mg dm^{-3}) can significantly slow down the rate RDX removal (by 98%), because of the competition for electrons (Wani and Davis, 2003). Sulfates were also shown to have a delaying effect on RDX biotransformation (Wani and Davis, 2003). These seem to suggest that utilizing these electron competitors may impact the rate or nature of TNT phytotransformation. The RDX studies were performed with microbial slurries, hence impacts on rates may have been substantial; plants have more elaborate and compartmentalized metabolic systems, hence the effect of nitrates and sulfates on TNT transformation rates may marginal. Knowledge of the regular substrates of the enzymes catalyzing HADNT to ADNT reaction could also prove useful- these substrates could be added in conjunction with TNT to compete for the electrons, and thereby obstruct the HADNT to ADNT reaction. If nitrates, sulfates or substrates can be added to a plant system to modify the rate or pathway of TNT transformation, improvements in its phytotransformation can be obtained without any genetic manipulations. The hydroxylamines will, in this scenario, be compelled to go along the azoxy and conjugative branches of the

pathway, and hence the rate limiting step of monoamine conjugation will be avoided. This will lead to a faster formation of the final products- intracellular bounds.

Figure 4.3 delineates the TNT transformation pathway in axenic plant systems, as understood currently. Hydroxylamines play a key role in deciding the final fate and direction of the pathway. There exist significant portions of the TNT pathway that have not been identified, and it is probable that hydroxylamines are involved in the formation of those metabolites as well. To engineer phytoremediation systems with low final toxicities and high kinetic rates may entail metabolic manipulation; hydroxylamines may play an important role in these metabolic engineering aspects, hence their understanding in the TNT transformation pathway is important.

Figure 4.3: Updated TNT Transformation Pathway: TNT transformation pathway in plants, as currently understood, based on TNT and metabolite feeding experiments in *Arabidopsis* seedlings and *C. roseus* hairy roots. The dotted arrows represent the new steps delineated in the TNT transformation pathway by experiments performed in this chapter. These steps display the primary role of the hydroxylamines in the pathway, since they can be reduced, oxidized or conjugated into different metabolites. The hydroxylamines are hence branch points in the TNT transformation pathway and can potentially be used in genetic manipulation of the pathway. Moving the pathway along the newly elucidated branch of hydroxylamines to conjugates, for instance, can reduce the time required for complete immobilization of the foreign compound in the form of bounds. The slow and fast steps are illustrated in the pathway.


(TNT)
(2,2- Azoxy)
(4,4' Azoxy)
Slow
Fast
(2HADNT)
(4HADNT)
Fast
Fast
Fast
Fast
(2ADNT)
(4ADNT)
Slow
(TNT-1)
(2A-1)
(4A-1)
(TNT-2)
BOUNDS

Chapter 5: Identification of Metabolic Pathway Alterations in Transgenic Tobacco Transforming TNT

A condensed version of this chapter has been prepared for publication in the journal Plant Physiology in collaboration with Dr. Hannink (Cambridge, UK) and Dr. Bruce (York, UK).

5.1: Introduction

While most plants have some capacity to remediate soil and water of explosives, using genetically modified plants may improve the capacity and efficiency of pollutant removal. Limitations such as the slow speed of remediation and the toxicity of the pollutant to the plant can be overcome by using genetically modified plants over-expressing single or multiple enzymes. A liberal dose of caution is also warranted however, since the consequences of introducing genetically modified species in the environment is unknown. Many researched bacterial and fungal enzymes are shown to possess a faster rate of pollutant removal than plants; however bacteria and fungi do not produce sufficient biomass (unlike plants) for remediation of large land or water sites and hence their application can be an aesthetic and engineering challenge. Incorporating the advantages of the bacterial or fungal enzymes in a plant system by inserting multiple copies of those genes into plants will help in the development of new and efficient transgenics. This will help hasten the slow rate of remediation and hence reduce exposure times to the toxic pollutants which will reduce the toxic effects experienced by the plants. As mentioned in section 2.10 of Chapter 2, genetic information on explosive metabolism is scarce, although there is a growing body of information on the types of enzymes involved in the remediation processes. In addition, rapid strides have been made in plant molecular biology. Exploiting both these advances may help generate specific transgenic plants that show enhanced abilities to detoxify explosive wastes. Insertion of genes encoding for reductases and oxidases into plants is a current topic of intense interest, since these transgenic plants can detoxify explosive tracts much faster than wild-type plants, as shown in a few instances (French *et al.*, 1999; Doty *et al.*, 2000; Hannink *et al.*, 2001). This approach of engineering plants could pave the way in making phyto-remediation a sustainable and acceptable means of pollution abatement.

This chapter describes studies with tobacco transgenic plants, obtained kindly from Prof. Neil Bruce (University of York, York, UK) and Dr. Nerissa Hannink, (Cambridge University, Cambridge, UK). TNT transformation experiments were performed on two different transgenics: an NR3-2 and a PETN line; in addition a wild-type control was also tested. The NR3-2 line contained a modified nitroreductase from *Enterobacter cloacae* strain 96-3 (ATCC43560) (Bryant *et al.*, 1991), while the PETN transgenic line had a PETN reductase inserted from the same bacteria (French *et al.*, 1999). In order to understand the effect of the inserted genes on phytoremediation of TNT, with specific regard to the transformation pathway and hydroxylamine production, numerous transformation studies of the transgenics were performed. Nitroreductases in microorganisms can have one of many effects- they could cause the reduction of the nitro group to the hydroxylamine group ($NO_2 \rightarrow NHOH$), or they could lead to a complete reduction to the amino group ($NO_2 \rightarrow NHOH \rightarrow NH_2$). More details about the various roles of nitroreductases on explosive reduction are given in Section 2.8 of Chapter 2. In order to determine the specific effect of the inserted nitroreductases, attention was focused on the production of hydroxylamines and monoamines. Both low and high concentrations of TNT were used to examine the rate of hydroxylamine production in either situation.

As has been previously described (Section 2.4 of Chapter 2, Chapter 3, Chapter 4), plants follow the 'Green Liver Model' of transforming TNT, by first reducing them to hydroxylamines, and subsequently completely reducing them to monoamines. In addition, the hydroxylamines and the monoamines can conjugate by the addition of plant biomolecules to their functional moieties to aid in the detoxification of these metabolites. As has been explained in the previous chapters, four TNT-conjugates were observed in previous TNT phytotransformation studies- two with UV spectra similar to 2-ADNT and another two with spectra similar to 4ADNT. The first two were designated TNT-1 and 2A-1 that were spectroscopically similar to 2ADNT. Two other metabolites were designated TNT-2 and 4A-1 were spectroscopically similar to 4ADNT. Chemical or enzymatic hydrolysis was found to return the conjugate to the ADNTs (Bhadra *et al.*, 1999). In the transgenic tobacco seedlings, since the inserted nitroreductases could transform the nitro group of the TNT to either the hydroxylamine or monoamine moiety, measuring the concentration of hydroxylamine was

important. Since hydroxylamines are highly unstable in aqueous conditions, they tend to degrade during their HPLC separation. Hence, to obtain an accurate quantification of these metabolites, a stabilization derivatization procedure was adapted and optimized for our conditions. The derivatization procedure was used in the detection and quantification of hydroxylamines during the TNT transformation experiments.

5.1.1: Derivatization of Hydroxylamines as Detection Mechanism

Conventional HPLC detection of hydroxylamines is usually insufficient since these compounds tend to degrade in the mobile phase. Hence, low concentrations of hydroxylamines are never measured in phytoremediation studies. In addition, the concentrations of hydroxylamines measured using reverse-phased HPLC may be subject to significant error. To overcome some of these limitations, a derivatization procedure obtained from literature was used to stabilize the hydroxylamino group (Hughes *et al.*, 1998; Wang and Hughes, 1998). However, prior to the application of this procedure, optimization of the derivatization scheme was necessary to enable its implementation in a phytoremediation system with low hydroxylamine concentrations.

The derivatization of hydroxylamines consists of adding acetic anhydride to the sample, under highly acidic conditions. This leads to the formation of an acetoxyacetamido moiety, which is more stable than its parent hydroxylamine (Figure 5.1). Two carbonyl groups (CH_3CO) from acetic anhydride attack the hydroxylamine group (NHOH) in a nucleophilic substitution, with the release of acetate. Sodium acetate is added to end the reaction and stabilize the product by increasing the pH and thereby preventing hydrolysis of the acetoxyacetamido group. The acetoxyacetamido substituted group can subsequently be detected via conventional reverse-phase HPLC. In order to successfully utilize the derivatization procedure for our system, it was also necessary to optimize the process with respect to the various reactants added. The materials and methods section describes in greater detail the optimization procedure used to determine ideal quantities of acetic anhydride and sodium acetate.

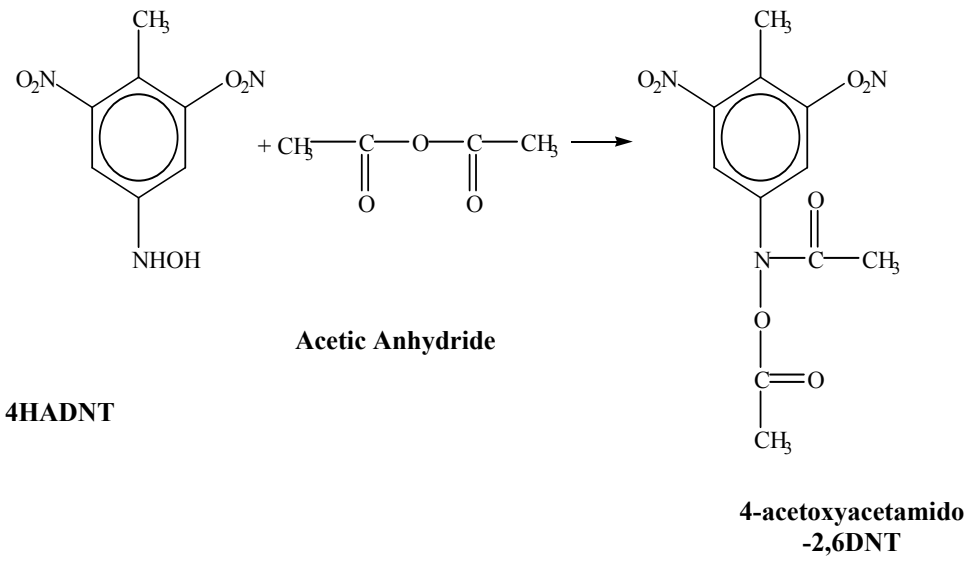


Figure 5.1: Derivatization of 4HADNT: Derivatization of 4-hydroxylamine-2,4DNT using acetic anhydride and sodium acetate, at pH 1.

5.2: Materials and Methods

5.2.1: Plant Material

17-day-old tobacco cultures were used for all the experiments. They were cultured using sterile techniques. For each flask, 50 seeds were counted and placed in a sterile eppendorf. 1 ml of a sterilizing solution, consisting of sterile water with 10% bleach and 0.1% SDS, was added to the seeds. The seeds were incubated with the sterilizing solution for half-an-hour, after which they were triple rinsed with sterile water and then transferred to the flask. Each 250 ml flask contained 100 ml of Murashige and Skoog media (4.4g of the media, 15 g of sucrose, pH to 5.6) and all flasks were grown at 25°C under fluorescent light, while being shaken at 100 rpm. 13 flasks of the NR 3-2 line, 11 flasks of the PETN line, and 13 flasks of wild-type control were used for the experiments.

5.2.2: Chemicals

Solid TNT (minimum 35% water), 2ADNT and 4ADNT were obtained from ChemService, while all the liquid standards (TNT, 2ADNT, 4ADNT, 2HADNT, 4HADNT) were obtained from Accustandards. A mixture of HADNTs was also synthesized in the lab using the methods described in Beelen *et al.* (Beelen and Burris, 1995). Acetic anhydride, sodium acetate, zinc powder, ammonium chloride and methanol were obtained from Fisher Chemicals. All solvents used were degassed using helium prior to use, to reduce the air/oxygen content in them. Previously isolated and purified samples of conjugates were used for conjugate identification (Bhadra *et al.*, 1999).

5.2.3: Analytical Methods

A Waters HPLC, equipped with an autosampler and a PDA detector was used to identify all compounds. Reverse phased HPLC with a Nova-Pak C8 column, with a mobile phase of 82:18, water: 2-propanol was used. Metabolite identification was done via a UV-vis spectral match. The HPLC was automated using Empower, a Waters software; EPA method 8330 was used for all separation and analysis. Dr. Chaunyue Wang, from Dr. Joe Hughes lab at Rice University, kindly provided the spectra for 4-acetoxyacetamido-2,6-dinitrotoluene, the product from 4HADNT derivatization. Figure 5.2 shows the spectra of 4-acetoxyacetamido-2,6-dinitrotoluene and 4HADNT and 4ADNT.

5.2.4: Preparation of Hydroxylamines

A mix of 2 and 4HADNT was prepared in the lab by chemical reduction of TNT in the presence of zinc and ammonium chloride (Beelen and Burris, 1995) under anaerobic conditions. A 25 ml flask was evacuated using a vacuum pump, and then filled with Nitrogen gas. 10 ml of 5 mM TNT in ethanol was added to the flask, followed by 2.3 g on Zn powder and 1.7 g ammonium chloride. The flask was again purged with nitrogen gas, to create an inert atmosphere, and then stirred for 36 hours at room temperature. The resultant product was filtered and a sample analyzed via the HPLC, and the remainder stored at –20°C.

5.2.5: Derivatization of Hydroxylamines

The procedure described has been adapted from (Hughes *et al.*, 1998), but been modified for different conditions. The modified-derivatization procedure begins with acidification of the sample containing the hydroxylamine to a pH < 1. Methanol was added to aqueous samples of lab synthesized HADNTs to further increase the stability of the hydroxylamines and followed by a drop-wise addition of acetic anhydride. The entire mixture was chilled in an ice-bath, and the products are allowed to equilibrate for 5 to 10 minutes, following which sodium acetate was added to quench the reaction.

5.2.6: Optimization of Derivatization

Since the derivatization procedure has many solvents and reactants that need to be used, an optimization procedure was used to determine their optimal amounts. Optimization experiments were carried out with respect to acetic anhydride and sodium acetate with lab synthesized HADNT. Five amounts of acetic anhydride- 0.25 ml, 0.5 ml, 1 ml, 2 ml and 3 ml were added to 0.05 ml HADNTs, 0.2 ml methanol, 0.2 ml water and 0.04 ml HCl (11N). The reaction was allowed to progress on ice for 10 minutes, after which 0.5 ml sodium acetate (25 g/L) was added. Optimization was performed with respect to sodium acetate too, five amounts of the same- 0.25 ml, 0.5 ml, 1 ml, 2 ml and 3 ml were added to 0.05 ml HADNTs, 0.2 ml methanol, 0.2 ml water, 0.04 ml HCl (11N) and 1 ml acetic anhydride after completion of the reaction.

5.2.7: TNT Transformation Experiments

15 flasks of the NR 3-2 line, the PETN line and the wild-type were cultured from sterile seeds. After 17 days, 10 flasks of the PETN transgenics and 13 flasks of the wild-type and NR 3-2 transgenics were used for transformation studies, as the discarded flasks either were contaminated or had insufficient biomass for the experiments. Three concentrations and a control were used in the PETN transgenics, while four concentrations and a TNT-free control were used in the NR3-2 transgenics and the wild-type. Samples were taken every few hours for metabolite analysis by HPLC. Samples were also simultaneously taken for derivatization for hydroxylamine analysis. Sterility was maintained through out the

experiment. Biomass free controls were also included to monitor TNT degradation due to photocatalytic effects. Biomass measurements of the mutants and wild-type were done to compare phytotoxicity of TNT across transgenic lines. Some flasks were sacrificed at 10 hours, freeze-dried, extracted and analyzed for intracellular-extractable metabolite levels. The experiment was stopped by freeze drying all the samples, 120 hours after initial exposure to TNT, and intracellular metabolites were analyzed. An extraction procedure, which consisted of freeze-drying the biomass, followed by sonication of the biomass in 20 ml methanol was followed. The sonication was done for 12 hours, and repeated three times with the same biomass. The supernatants from all the sonications, for each biomass sample, were collected and evaporated down to 5 ml. A sample of this was run through the HPLC for analysis of intracellular-extractable metabolites. Dry and wet weight measurements of the biomass were made at every stage.

5.2.8: Derivatization of Samples

In order to detect the presence of hydroxylamine in the system, a 0.5 ml sample was derivatized at each time step, for all the flasks. Both intracellular-extractable and extracellular samples were derivatized. The procedure consisted of mixing 0.5 ml of the sample with 0.5 ml of methanol (pH=1) and 1 ml acetic anhydride. This reaction mixture was kept in ice for 10 minutes, following which 0.5 ml of 25 g/L sodium acetate was added to each sample to quench the reaction, and stabilize the products. The derivatized product was run through the HPLC for immediate analysis. The amounts of acetic anhydride and sodium acetate used in the derivatization procedure were obtained from the optimization protocols. A calibration curve for the derivatization procedure was also developed. Four concentrations of 4HADNT from 1 mg/L through 20 mg/L were used in the calibration. Various amounts of pure 4HADNT was added to methanol (total volume of hydroxylamine plus methanol was 1 ml), 1 ml acetic anhydride and 0.5 ml sodium acetate. These samples were then analyzed for concentrations via the HPLC.

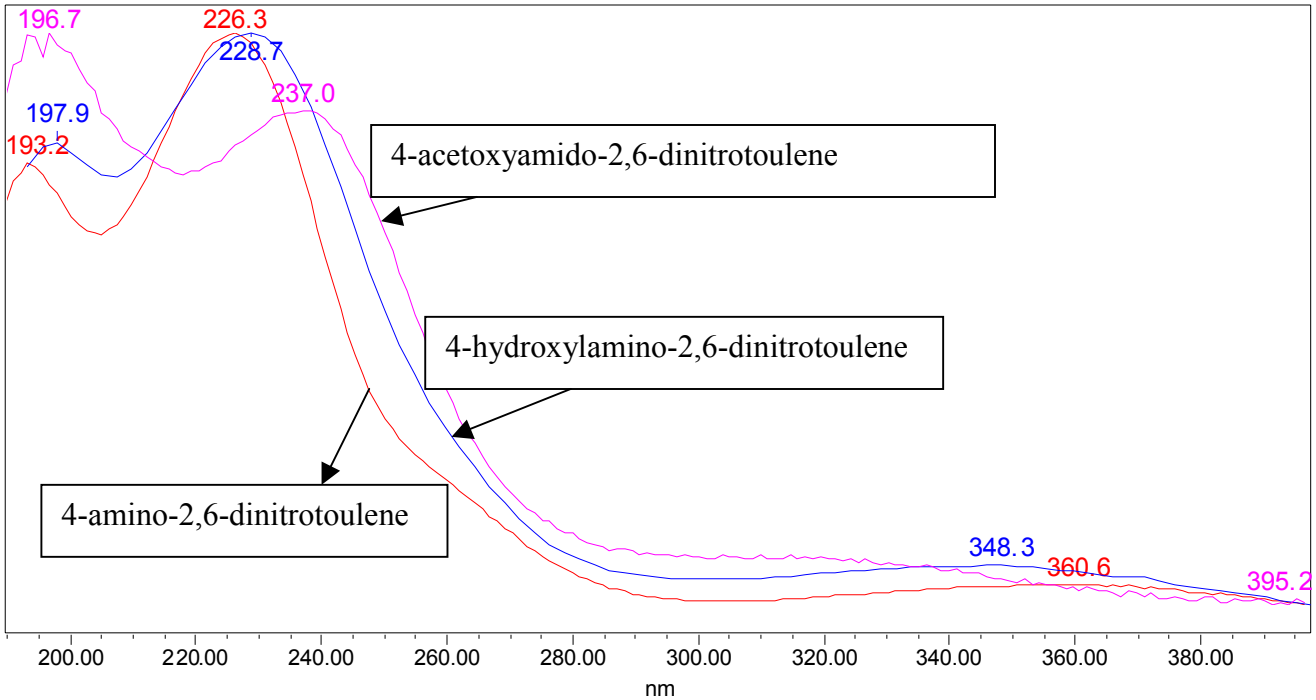


Figure 5.2: Derivatization Spectra: UV-vis spectra of 4-acetoxyamido-2,6-dinitrotoluene, 4-hydroxylamine-2,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene as determined by PDA detection. 4-acetoxyamido-2,6-dinitrotoluene, the derivatization product of 4-hydroxylamine-2,6-dinitrotoluene, has a much higher stability than its parent compound and hence is used in the detection and quantification of 4HADNT.

5.3: Results

5.3.1: Optimization of Derivatization

Using the spectra provided by Dr. Chaunyue Wang (Rice University) the derivatized 4-acetoxyacetamido-2,6DNT was identified at an elution time of 17 minutes when a mobile phase of 82:18, water:2-propanol was used (Figure 5.2). An unidentified byproduct was also formed, which eluted at 30 minutes for the same mobile phase conditions. Sodium acetate was found to be a very important reagent, since it appeared to stabilize the products. In the absence of sodium acetate, the derivatized product quickly degraded. The derivatization of 2HADNT also produced a compound that is speculated to be the derivatized product, but since a spectrum is unavailable a definite confirmation could not be made.

The first optimization runs were designed to identify amounts of acetic anhydride to be added for complete reaction. Since, the derivatization needs to proceed to completion to fully quantify the hydroxylamines, an excess of acetic anhydride and sodium acetate had to be added. However, large amounts of excess can dilute the derivatized product and lowers the concentration below detection levels; hence, an optimization was required for the amounts. In addition, methanol was added to all the reaction mixtures to stabilize the hydroxylamines since they show a higher stability in methanol than water (Section 3.3.1 of Chapter 3). Figures 5.3A and B show the optimization results for acetic anhydride and sodium acetate. As seen in the graph, 1 ml acetic anhydride and 0.5 ml sodium acetate appear to give the highest amounts of 4-acetoxyacetamido-2,6-DNT. Hence, these values were used in the experiments to quantify hydroxylamines in phytoremediation systems.

5.3.2: Application of Derivatization

The optimized derivatization procedure was applied to measure hydroxylamine concentrations during the TNT transformation experiments, and the results obtained were compared to hydroxylamine levels obtained from running samples directly through the HPLC. Samples from the TNT transformation by tobacco were run through the HPLC to analyze for metabolite concentrations. The samples were stored at -80°C, and then run

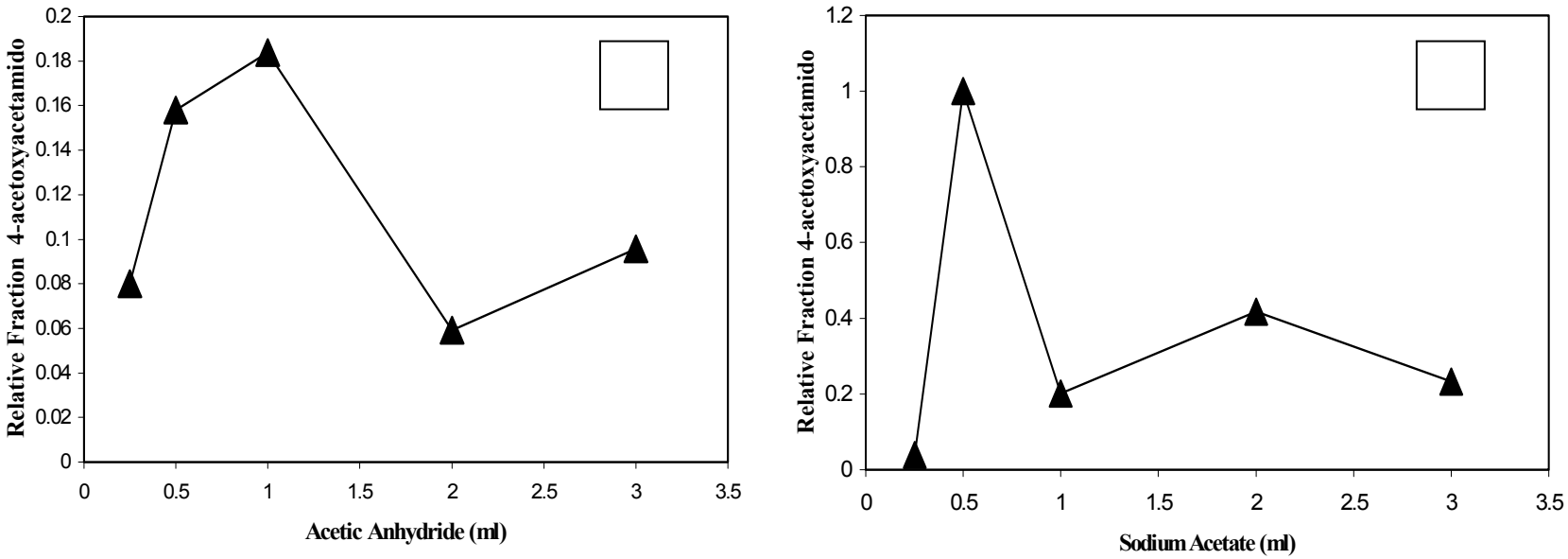


Figure 5.3: Optimization of Derivatization: The derivatization procedure was optimized with respect to acetic anhydride (A) and sodium acetate (B). Different amounts of each compound were added to 4HADNT (separately) and the resultant 4-acetoxyacetamido measured to determine the amount of reactant that resulted in the maximum production of the acetoxyacetamido. 1ml of acetic anhydride and 0.5 ml of sodium acetate were the optimum amounts required for the derivatization of 4HADNT.

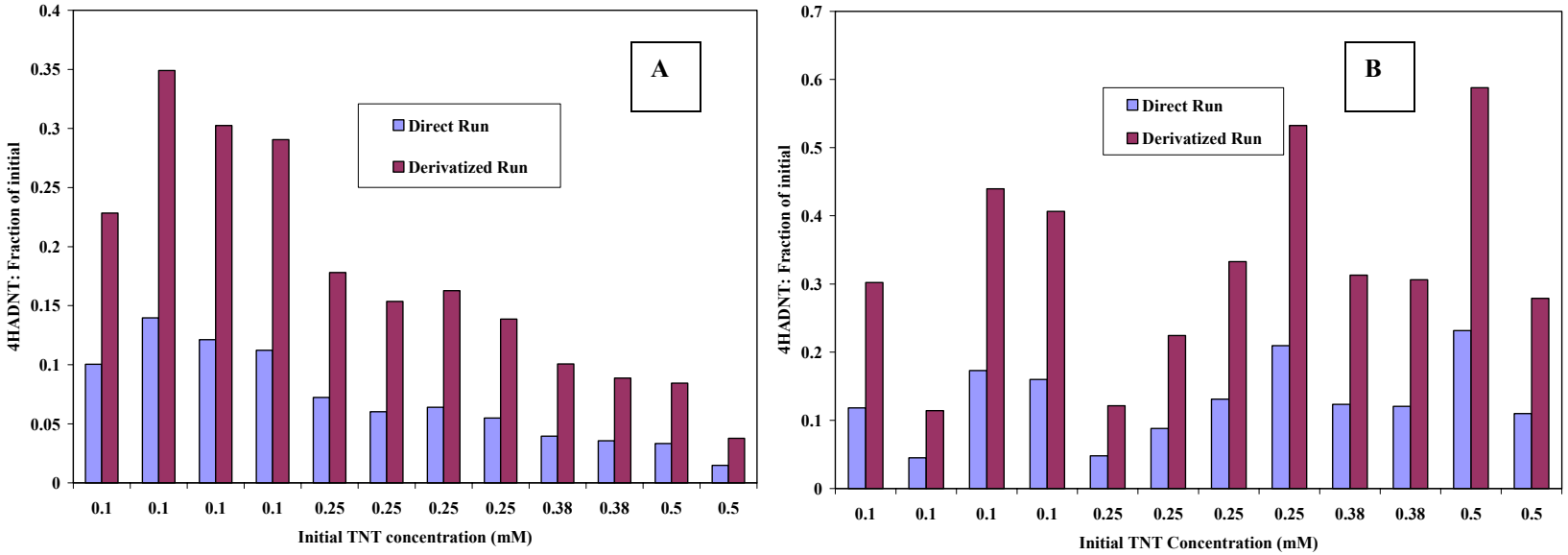


Figure 5.4: Role of Derivatization: Comparison of 4HADNT concentrations between direct run and derivatized run samples at 2 hours (A) and 6 hours (B) for NR 3-2 transgenic seedlings. The direct run samples represent those concentrations determined from directly running the media samples through a C8 column on an HPLC, while the derivatized runs represent samples that were first derivatized, and subsequently analyzed. Direct and derivatized runs were performed for all samples, and it is seen from the figure that direct running of samples results in considerable degradation of the hydroxylamine. Hence, derivatization of samples helps is more accurate quantification of HADNT levels.

immediately through the HPLC, with no lag time. This was done to minimize the degradation of hydroxylamines in the system. In addition, a sample of 0.5 ml was taken out at each time step and derivatized and then run through the HPLC. Using the developed calibration curve for derivatization, the hydroxylamines were quantified. The comparisons between the direct-run and derivatized-run samples are shown in Figures 5.4A and 5.4B. Derivatization of the sample stabilizes the hydroxylamines and shows higher concentrations and appears to work successfully for this system.

5.3.3: Phytotoxic Effects of TNT during Vegetative Growth

Simultaneous with the TNT transformation experiment, biomass weights of the various transgenic lines and wild-types were monitored to measure phytotoxic effects experienced. After 18 days of growth from seeds, initial weights were measured, TNT added and final weights measured 5 days hence. Table 5.1 summarizes all the weights measured, and calculates a growth ratio- measuring final weight over initial weight; a growth ratio over 1 implies positive growth, a below 1 growth ratio implies loss of biomass. It was observed that at 0.1 mM TNT, the wild-type seedlings had a growth ratio of 1.2, the NR 3-2 seedlings 1.8 and the PETN seedling 2.0; hence, at this concentration of TNT, the NR 3-2 and PETN seedlings appeared to be more resistant than the wild-type seedlings. A single factor ANOVA analysis gave a null factor probability of P= 0.12 between NR 3-2 and wild-types. This implies that there is a 0.12 probability of the difference in growth ratios between the two systems being due to random variations. All P values have been summarized in Table 5.1. At a concentration of 0.25 mM wild type plants lost weight (Growth ratio of 0.9) and were seen to exhibit severe chlorosis and were necrotic at the end of the five-day study. In contrast, the transgenic line, NR 3-2 had gained weight (Growth ratio of 1.7) and did not appear chlorotic after five days. PETN seedlings did slightly better than wild-type seedlings with a growth ratio of 1.1. Wild type plants lost considerable weight (Growth ratio of 0.5) at 0.38 and at 0.5mM TNT and were again necrotic after five days. In contrast, the transgenic line, NR 3-2 gained weight at both 0.38mM and 0.5mM TNT concentrations (Growth ratio of 1.4 and 1.5, respectively). PETN seedlings were not tested at these concentrations. Control plants grown without TNT in the medium exhibited no visible signs of toxicity such as chlorosis, an

Table 5.1: Toxic Effects of TNT: Toxic effects of TNT on the seedlings were quantified by measuring their biomass before (initial weights) and five days (final weights) after TNT amendment. Results are the mean and standard error of the mean from duplicated results except 0.38 and 0.5mM which are individual results. A growth ratio that calculates the relative change in biomass has also been included. A growth ratio above 1 implies positive growth, while a growth ratio below 1 implies loss of biomass. The P values represent the probability that the difference in the means between the transgenic and wild-type were due to random variations (Single Factor ANOVA). Hence, a low value of P (P close to zero), implies the difference in means were due to an actual difference in characteristics. A P close to 1 implies the difference in means was due to random variations.

Initial TNT [mM]	**Wild Type Plants**			**NR 3-2 Plants**			**PETN Plants**			**Null Value Hypothesis**	
	Initial Weight (g)	**Final Weight (g)**	**Growth ratio**	**Initial Weight (g)**	**Final Weight (g)**	**Growth ratio**	**Initial Weight (g)**	**Final Weight (g)**	**Growth ratio**	P_{NR-WT}	$P_{PETN-WT}$
0	2.3 ± NA	4.3 ± NA	1.8 ± NA	3.3 ± NA	6.0 ± NA	1.8	2.8 ± NA	2.7 ± NA	0.9 ± NA	-	-
0.1	3.7 ± 0	4.6 ± 2.26	1.2 ± 0.5	3.9 ± 0.28	6.8 ± 1.2	1.8 ± 0.27	2.0 ± NA	4.0 ± NA	2.0 ± NA	0.12	-
0.25	3.4 ± 0.14	3.35 ± 0.49	0.9 ± 0.1	3.75 ± 0.07	6.5 ± 0.7	1.7 ± 0.16	5.3 ± 1.0	6.0 ± 5.9	1.1 ± 0.9	0.00	0.62
0.38	3.3 ±NA	1.6 ± NA	0.5 ± NA	4.1 ±NA	5.7 ±NA	1.4	4.1 ± NA	9.4 ± NA	2.3 ± NA	-	-
0.5	3.2 ±NA	1.5 ±NA	0.5 ± NA	8.9 ±NA	13.6 ±NA	1.5				-	-

indication that the toxic effects were due to the presence of TNT and not submersion in the growth medium. Growth ratios for the control plants are also given in Table 5.1.

5.3.4: TNT Transformation characteristics

The objectives of the experiments were to verify the capacity of the tobacco transgenics to take up and transform TNT, understand the phenotypical differences between the transgenics and wild-type seedlings, and use the derivatization procedure to quantify hydroxylamines. The TNT concentration measurements proved that both the NR3-2 and PETN transgenics are capable of absorbing and transforming TNT, as seen in Figures 5.5 and 5.6. Figure 5.7 shows the performance of wild-type seedlings in TNT transformation. While the NR transgenics remove all TNT, even at the highest concentration, from the extracellular medium within 40 hours, the PETN and wild-type seedlings appear incapable of transforming concentrations of TNT above 0.38 mM (86 mg/L). Figures 5.8 and 5.9 compare the TNT removal efficiency by all the three systems. At 0.1 mM (23 mg/L), wild-type seedlings took 120 hours to remove all the TNT, while the PETN lines required 72 hours. In contrast, the NR 3-2 line removed all the TNT from the medium within 25 hours (Figure 5.8A). At 0.25 mM, it took the wild-type seedlings 120 hours (end of experiment) to remove 48% of the TNT, by which time these seedlings were necrotic and stunted. For PETN and NR 3-2 seedlings the time to remove 50% of the TNT was 6 hours and 10 hours respectively, while the time required to remove TNT completely from the medium was 48 hours and 60 hours respectively (Figure 5.8B). Since the initial weight of the PETN seedlings were 5.3 ± 1.0g, while the initial weights of the NR 3-2 seedlings were 3.75 ± 0.07g the intrinsic rate of TNT removal was higher in NR 3-2 than PETN seedlings. Wild-type and PETN seedlings exposed to 0.38 mM (86 mg/L) of TNT showed signs of phytotoxicity with severe signs of phytotoxicity being seen in the wild-types and PETN lines. At this concentration, wild-type seedlings removed 31% of TNT from the medium in 120 hours, while the PETN lines removed 40% of the TNT (Figure 5.9A). The PETN cultures turned brownish red as the experiment proceeded, which is probably due to the formation of hydride-meisenheimer complexes (Nerissa Hannink, personal communication). The NR 3-2 seedlings performed the best, removing all the TNT within 50 hours of addition. At the highest TNT concentration

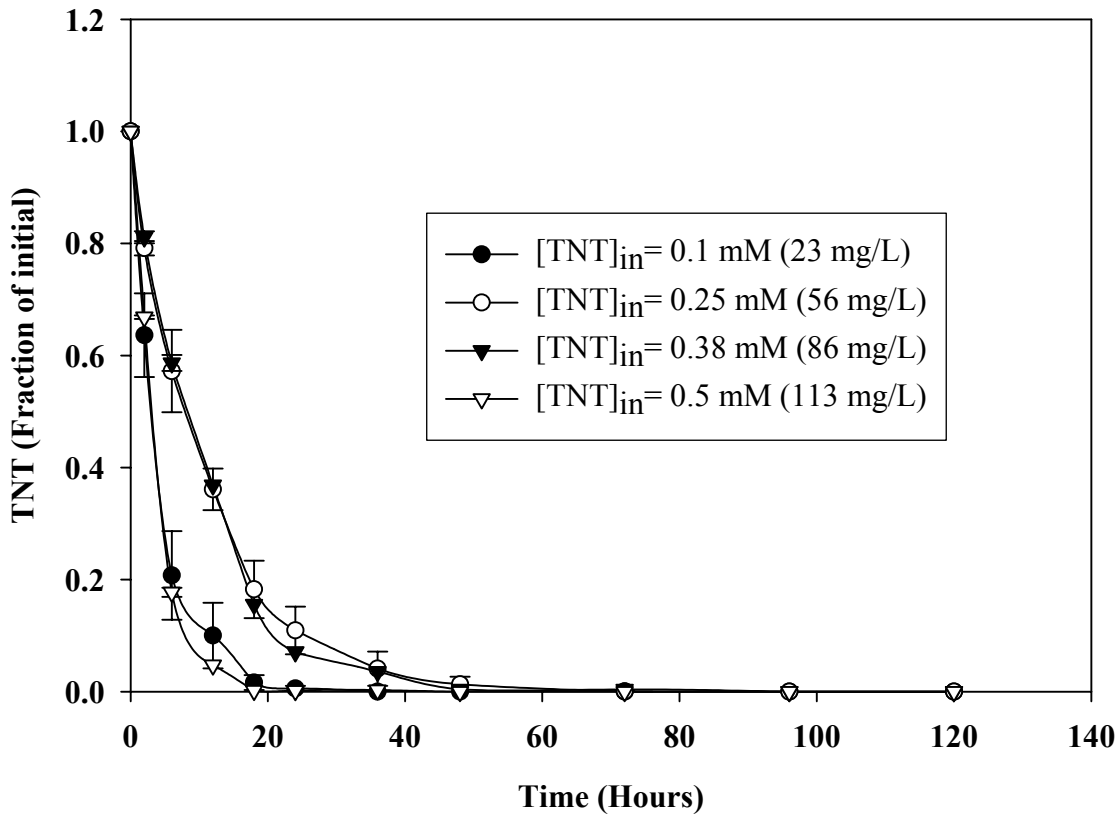


Figure 5.5: TNT Removal by NR3-2: 2-week old axenic NR 3-2 transgenic seedlings transformed TNT efficiently, with all of the TNT being removed within 50 hours, at the highest concentration. The results are averages and standard deviations of duplicates for the 0.1 mM and 0.25 mM TNT amended systems. For the 0.38 mM and 0.5 mM systems, the results are from duplicates until 12 hours, after which they represent single flasks.

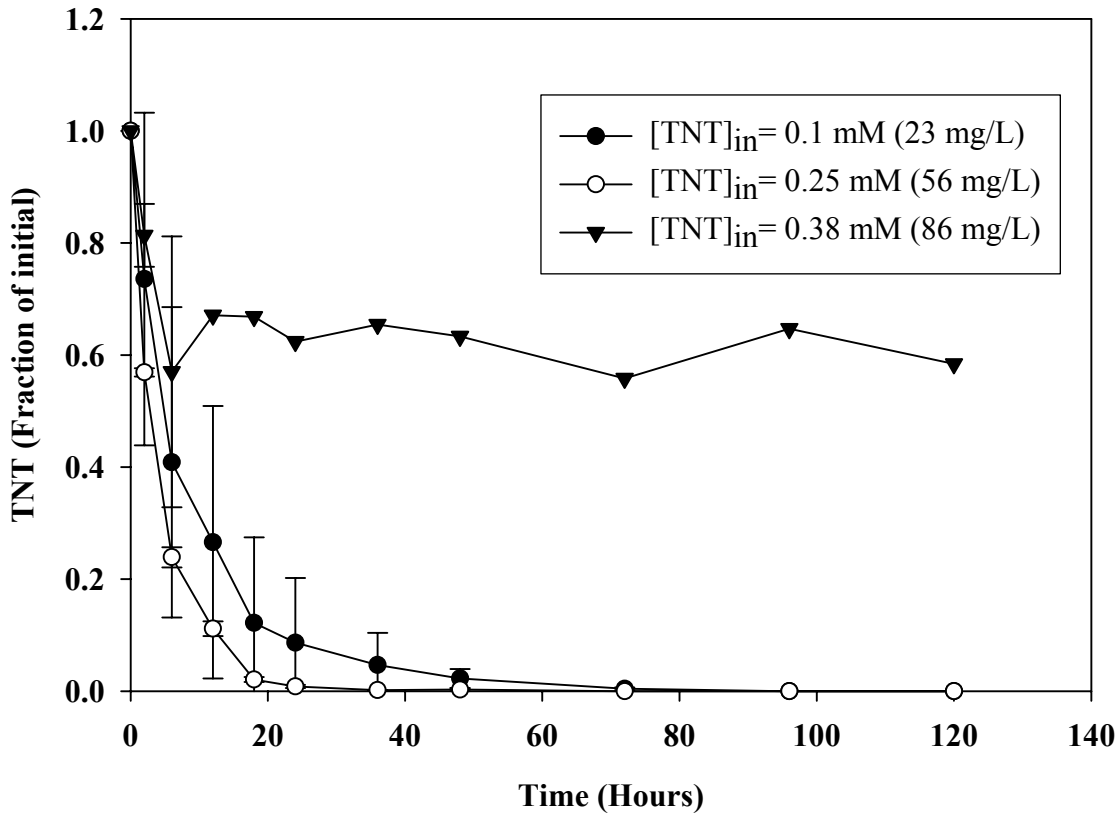


Figure 5.6: TNT Removal by PETN: 2-week old axenic PETN transgenic seedlings transformed TNT efficiently at the low concentrations of 0.1 and 0.25 mM. At the highest concentration of tested (0.38 mM), these seedlings were unable to transform most of the TNT, with over 60% remaining after 120 hours. The results are averages and standard deviations of duplicates for concentrations of 0.1 mM and 0.25 mM TNT amended systems. For the 0.38 mM, the results are from duplicates until 12 hours, after which they represent single flasks.

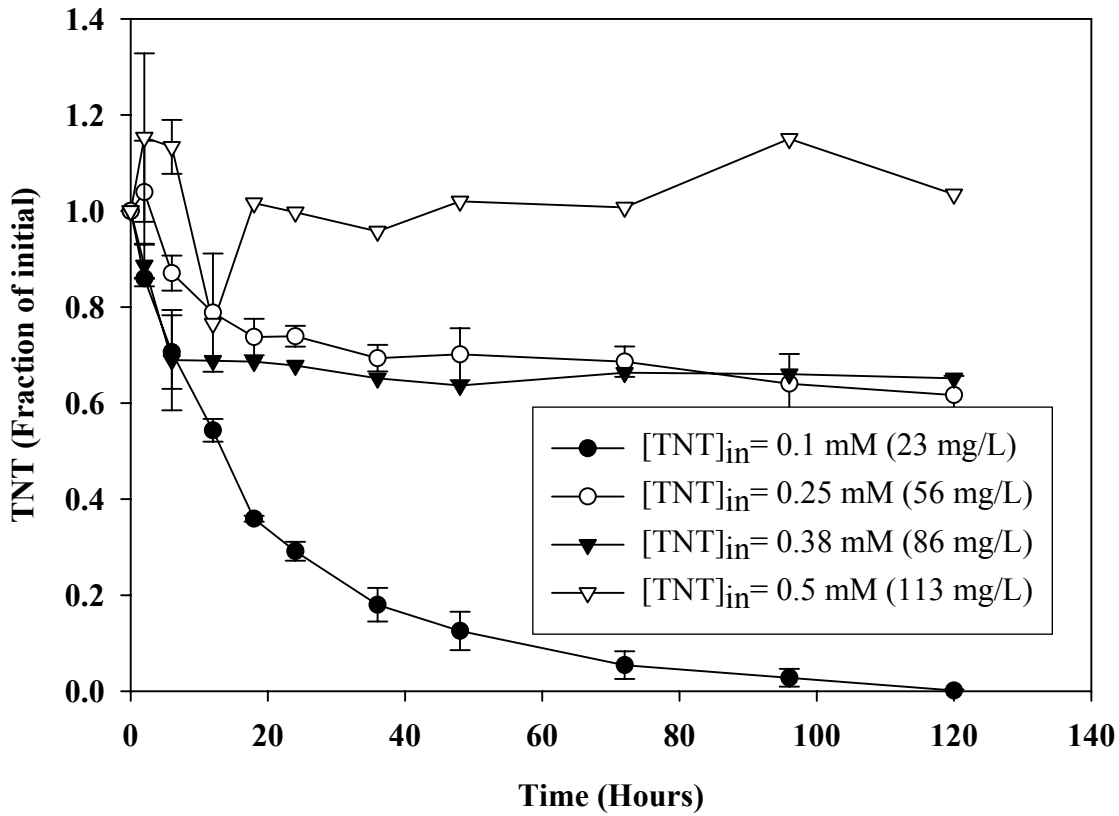


Figure 5.7: TNT Removal by Wild-Type Seedlings: 2-week old axenic wild-type seedlings transformed TNT only at the lowest concentration of 0.1 mM. At all higher concentrations, these seedlings were stunted by TNT and could not remove it from the system in significant amounts. In the 0.5 mM flask, TNT levels were increasing, which was probably due to the heterogeneous absorption of water and an apparent increase in TNT levels. The results are averages and standard deviations of duplicates for initial concentrations of 0.1 mM and 0.25 mM. For the 0.38 mM and 0.5 mM systems, the results are from duplicates until 12 hours, after which they represent single flasks.

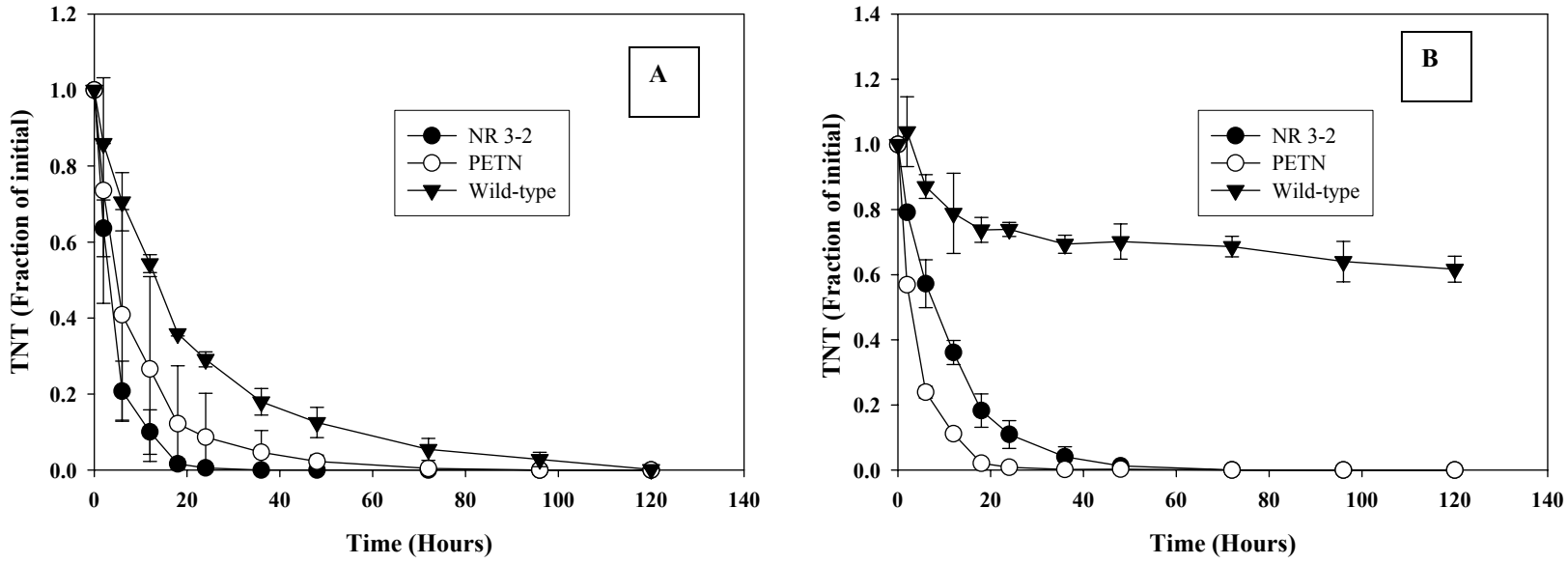


Figure 5.8: Relative TNT Removal at 0.1 and 0.25 mM: Comparative extracellular TNT removals by both the transgenics and wild-type seedlings are shown for the 0.1 mM (A) and 0.25 mM (B) initial TNT level. Values shown are averages and standard deviations of duplicates. The initial biomass was 3.7 ± 0g for the wild-type seedlings, 3.9± 0.28g for the NR 3-2 seedlings and 3.6 ± 2.3g for the PETN seedlings in the 0.1 mM system. In the 0.25 mM system the biomass for wild-type, NR 3-2 and PETN seedlings were 3.4 ± 0.14g, 3.75 ± 0.07g, 5.3 ± 1.0g, respectively. In the 0.1 mM system, NR 3-2 removes the TNT fastest from the system, while the PETN seedlings also appear to rapidly remove the TNT. In the 0.25 mM system, PETN seedlings remove the TNT fastest followed by the NR 3-2 seedlings, although the rate needs to be corrected to account for PETN seedlings higher initial biomass.

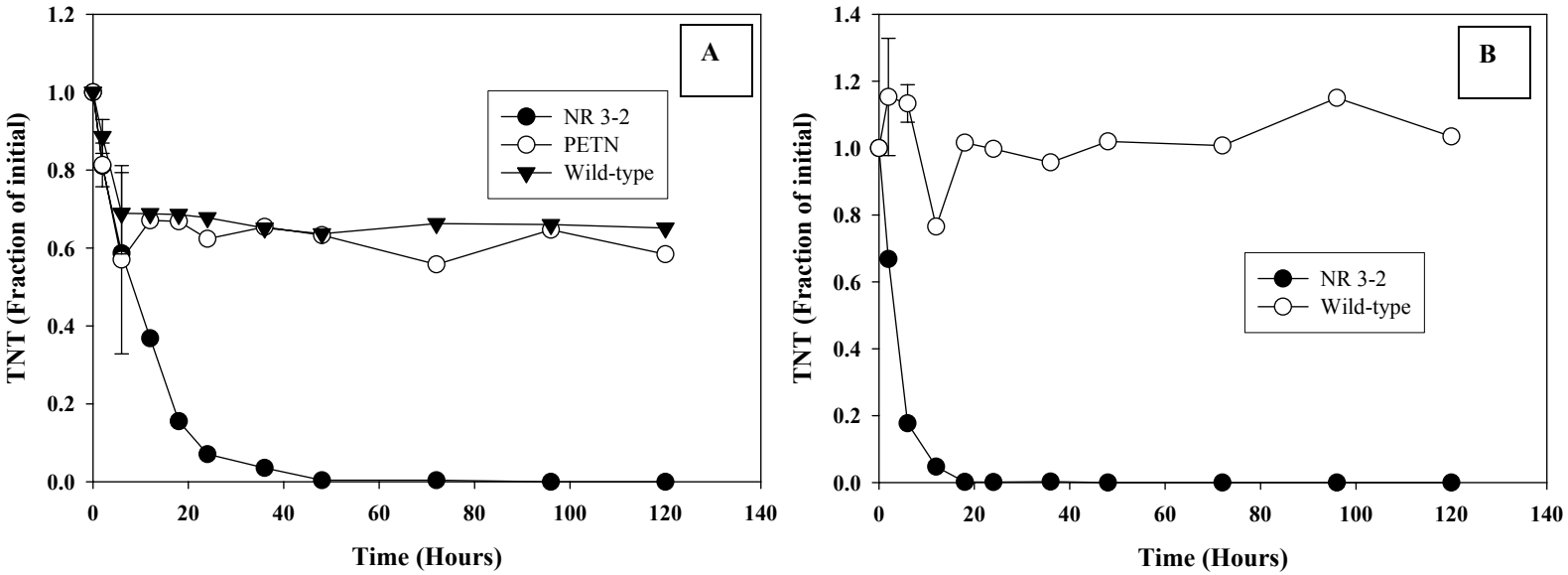


Figure 5.9: Relative TNT Removal at 0.38 mM and 0.5 mM: Comparative extracellular TNT removals by the transgenics and wild-type seedlings are shown for the 0.38 mM (A) and 0.5 mM (B) initial TNT level. Values shown are averages and standard deviations of duplicates up to 12 hours, following which they are for single flasks. The initial biomass was 3.3g for the wild-type seedlings, 4.1g for the NR 3-2 seedlings and 4.1g for the PETN seedlings in the 0.38 mM system. In the 0.5 mM system the biomass for wild-type and NR 3-2 seedlings were 3.2 and 8.9, respectively. In the 0.38 mM system, the clear superiority in NR 3-2 seedling in TNT removal is visible, since it is the only system capable of removing all of the TNT from the system. The PETN transgenic and wild-type seedlings appear incapable of removing significant amounts of TNT. In the 0.5 mM system, the NR 3-2 seedlings remove all the TNT from the system within 20 hours, while the wild-type seedlings cannot remove any of the TNT even after 120 hours.

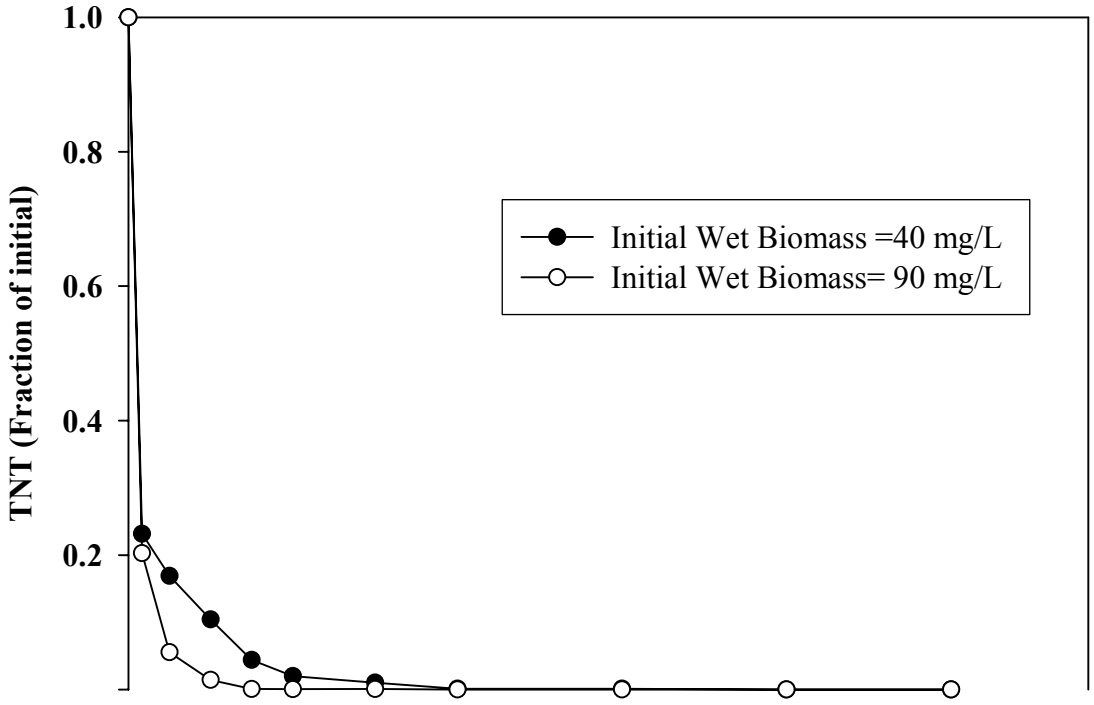


Figure 5.10: Biomass Effect on TNT Removal: The effect of biomass on the efficiency of TNT transformation at an initial concentration of 0.38 mM is seen; a higher biomass results in a faster removal of TNT. Hence, for comparison of any TNT removal characteristics, biomasses have to be equalized prior to the start of the experiment.

tested of 0.5 mM (113 mg/L), wild-type seedlings removed 25% of TNT from the medium in 120 hours, while NR 3-2 seedlings with the highest biomass needed 50 hours to remove all the TNT from the media (Figure 5.9B). Control flasks of TNT without plant biomass showed no significant loss of TNT (data not shown). The flask containing 0.5mM (113mg/L) TNT might have solubility issues as the aqueous solubility limit of TNT is 132mg/L in water at 25 °C (Lynch *et al.*, 2001)and TNT crystals were visible in the flask for the wild-type. Figure 5.10 gives the effect of biomass on the rate of TNT transformation: as expected higher biomass facilitated faster TNT removal.

5.3.5: Extracellular Metabolite Production: Hydroxylamines and Monoamines

Media samples were periodically analyzed under the HPLC for TNT and metabolite concentrations to determine trends and kinetics of transformation. These samples are referred to as extracellular measurements.

The NR 3-2 lines produced high concentrations of extracellular 4HADNT and much lower concentrations of 2HADNT. Concentrations as high as 0.2 mM (43 mg/L) of 4HADNT were seen in NR 3-2 at the 0.5 mM TNT system, which is the first time that such elevated concentrations of any hydroxylamine have been observed in a plant system. The NR 3-2 line transformed a maximum of 28, 17, 32 and 40% of the initial TNT to 4HADNT at 0.1, 0.25, 0.38 and 0.5mM TNT, respectively (Figures 5.11 and 5.12). The 4HADNT concentrations rapidly declined in the system and were followed by an increase in the 4ADNT levels. 4HADNT had completely disappeared from the transgenic system within 12 hours, while 4ADNT was present until the end of the experiment at less than 5% of the initial TNT added. Very low levels of 2HADNT were observed (less than 1% of the initial TNT added) at the 0.38 mM and 0.5 mM addition levels.

In contrast to the results observed in the NR 3-2 seedlings, the PETN transgenic plants were found to produce low levels of 2HADNT and no 4HADNT; 2HADNT was found in concentrations less than 0.02 mM (5 mg/L). The maximum amount of TNT transformed to 2HADNT was 7% at the 0.38 mM TNT concentration. Both 2ADNT and 4ADNT were produced in PETN seedlings, at low levels, below 5% of the initial TNT added.

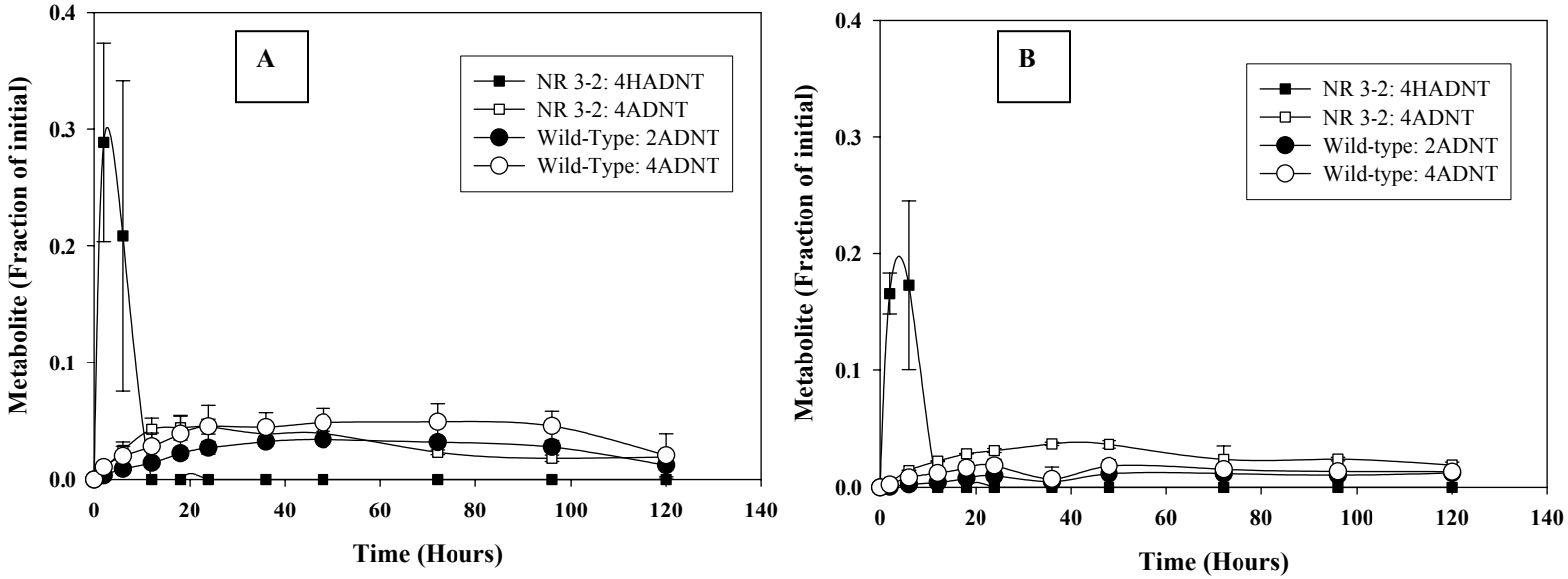


Figure 5.11: Mono and Hydroxylamines at 0.1 mM and 0.25 mM: Extracellular levels of HADNTs and ADNTs formed during TNT transformation by NR 3-2 and wild-type seedlings exposed to 0.1 mM (A) and 0.25 mM (B) of TNT are shown. The values shown are averages and standard deviation of duplicates. The HADNT levels shown were determined by derivatization. High levels of 4HADNT and an absence of 2-substituted metabolites are the characteristic of NR 3-2, while the wild-type shows low levels of both ADNTs and an absence of HADNTs. In the 0.1 mM system, around 30% of the initial TNT appears to be transformed to 4HADNT in NR 3-2 seedlings, due to the cloned bacterial enzyme, while the number is close to 20% in the 0.25 mM system.

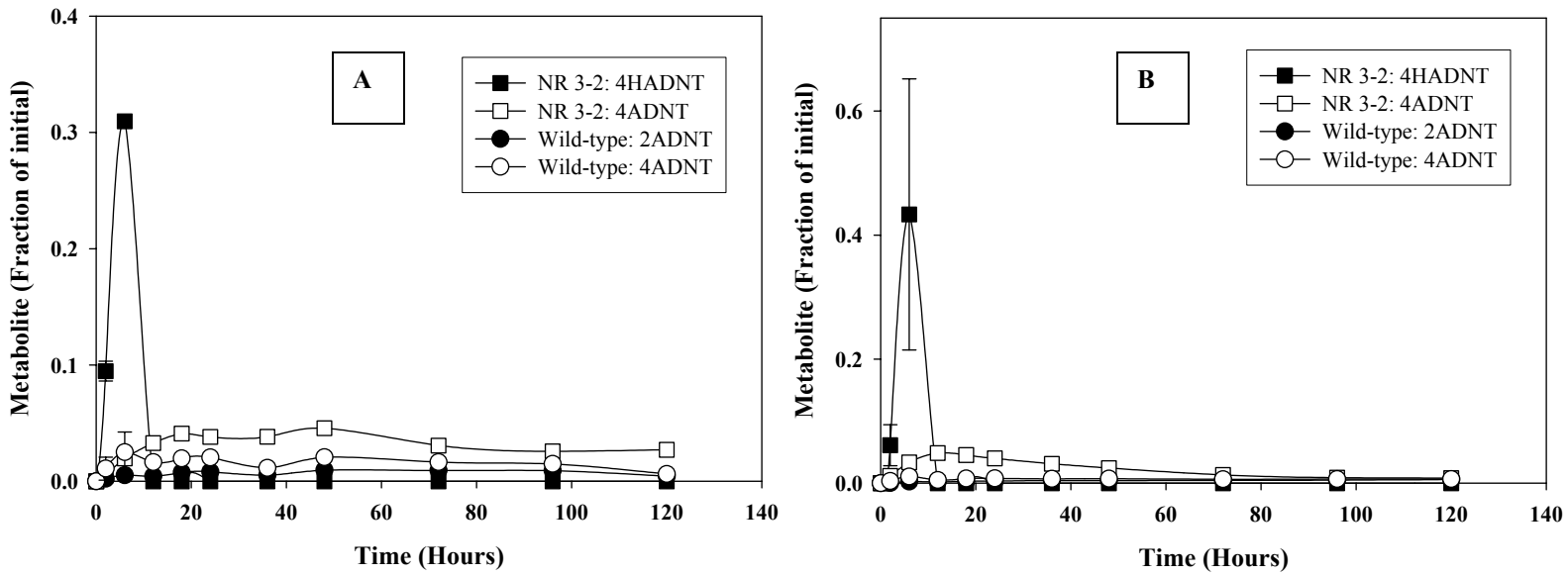


Figure 5.12: Mono and Hydroxylamines at 0.38 mM and 0.5 mM: Extracellular levels of HADNTs and ADNTs formed during TNT transformation by NR 3-2 and wild-type seedlings exposed to 0.38 mM (A) and 0.5 mM (B) of TNT are shown. Data are averages and standard deviation of duplicates until 10 hours, following which they are from single flasks. The HADNT levels shown were determined by derivatization. High levels of 4HADNT and an absence of 2-substituted metabolites are the characteristic of NR 3-2, while the wild-type shows low levels of both ADNTs and an absence of HADNTs. About 30% and 40% of the initial TNT is converted to 4HADNT in the NR 3-2 seedlings, in the 0.38mM and 0.5 mM systems respectively.

The wild-type seedlings produced no hydroxylamines, which is consistent with the low rate of TNT transformation in those seedlings. Very low levels of monoamines, below 5% of initial TNT added were observed at all concentrations. Overall, the greatest percentages of metabolites were observed in NR 3-2, followed by PETN and the lowest amount was seen in the wild-type seedlings. This is in direct correlation to the rate of TNT removal and final amount of residual TNT in the media, the NR 3-2 being the fastest, while the wild-types being the slowest. Figures 5.11 and 5.12 compare the relative levels of the hydroxylamines and monoamines in NR 3-2 and wild-type seedling for all the initial TNT concentrations.

5.3.6: Intracellular-Extractable Metabolite Production

At 10 hours and 120 hours (end of experiment), plant seedlings were sacrificed and extracted with methanol to analyze the presence of metabolites within the tissue (termed intracellular-extractables). The NR 3-2 seedlings showed very low levels of 4HADNT and 4ADNT production in the tissue- all below 1% of initial TNT added; no 2ADNT was observed. A high residence time sonication is involved in the extraction of metabolites from the plant tissue and perhaps significant 4HADNT degradation occurred during its extraction. High levels of conjugates were also observed in the intracellular-extractable phase. No TNT was observed in the tissue at 120 hours, thereby indicating its complete transformation.

The PETN tissue samples showed no hydroxylamine production, but less than 1% of 2ADNT and 4ADNT production. In wild-type seedlings, 2ADNT and 4ADNT were the only products observed in the plant tissue extracts, with the 4ADNT at slightly higher concentrations. Interestingly, concentrations of ADNTs were greater at 120 hours (compared to 10 hours) at 0.1mM TNT. In the tissue of wild-type seedlings, TNT was found to the compound of greatest abundance at 0.25, 0.38 and 0.5mM TNT with only trace amounts of the transformation products.

5.3.7: Conjugate Production

Previous TNT transformation studies have revealed the formation of conjugates by the addition of plant macromolecules to the metabolites. The production of conjugates was

investigated in this study too, with specific emphasis on determining any influence of the bacterial enzyme on conjugation. Both extracellular and intracellular-extractable fractions of the seedlings were analyzed for conjugate production. Previously isolated conjugates were used here to identify compounds of TNT transformation from tobacco (Bhadra *et al.*, 1999). The concentrations of the conjugates obtained should be regarded as approximations to the actual values, since degradation of the standards may have occurred.

NR 3-2 transgenics showed a preference to form TNT-2, a previously identified conjugate (Bhadra *et al.*, 1999), that had a plant molecule substituted in the 4-position on the benzene ring; previous studies (Chapter 3 and Chapter 4) have shown that this conjugate is formed by the direct conjugation of 4HADNT. Extracellular concentrations of TNT-2 were less than 2 mg/L, while 4A-1 (another 4-substituted conjugate) was detected, extracellularly, at certain time-points at low levels. A complete absence of any 2-substituted conjugates (TNT-1 and 2A-1) was a distinguishing feature of the NR 3-2 transgenic line. TNT-2 was the only conjugate detected in the intracellular-extractable phase and high concentrations of this conjugate were seen in the samples sacrificed at 10 and 120 hours. Figures 5.13, 5.14 and 5.15 compare the transient levels of all metabolite, intracellular and extracellular in NR 3-2 and wild-type seedling at the levels of 0.1, 0.25 and 0.38 mM TNT amendment.

The PETN transgenic line showed a more balanced production split with all four conjugates being formed in low concentrations, in the extracellular phase. In the intracellular phase, TNT-1 and TNT-2 were formed at high concentrations (Figure 5.16). Wild-type seedlings showed behavior similar to the PETN transgenic line as it produced all four conjugates in the extracellular phase, and TNT-1 and TNT-2 in the intracellular phase. In addition to the previously identified metabolites, new 4-substituted and 2-substituted conjugates were formed in all three systems. Although these metabolites were not isolated and conclusively identified by MS and NMR as conjugates, evidence supports the possibility that they are conjugates. They share spectra that are similar to TNT-1 (and 2A-1) and TNT-2 (and 4A-1). In addition, their low (4-7 minutes) retention times are similar to that of the previously identified conjugates, and hence these metabolites are more polar than TNT. Tables 5.2 lists the conjugates seen in the extracellular fraction while Table 5.3 shows the

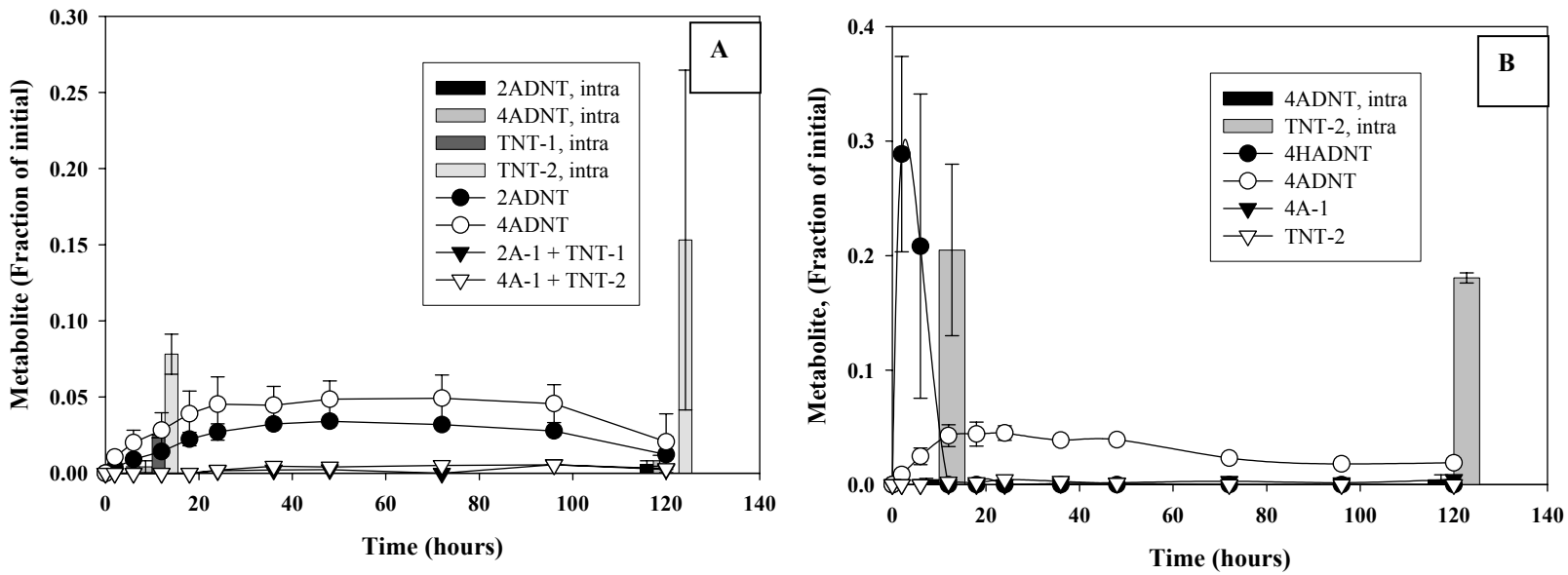


Figure 5.13: Transient Metabolite Profile at 0.1 mM: Profiles of both intracellular-extractable and extracellular metabolites for wild-type seedlings (A) and NR 3-2 seedlings (B) exposed to 0.1 mM of TNT are shown. The intracellular levels are shown for 10 hours and 120 hours, the two time steps when sacrifices were made. The HADNT levels shown were determined by derivatization. All concentrations are averages and standard deviations of duplicates. In the wild-type seedlings, low levels of ADNTs are the most common metabolites in both the media and the tissue. In the NR 3-2 system, high levels of 4HADNT and a high level of the conjugate TNT-2 at 12 hours are observed. TNT-2 is formed from the direct conjugation of 4HADNT, hence the steep drop in 4HADNT level is followed for a peak for the intracellular level of TNT-2. This conjugate is also seen, intracellularly, at 120 hours, the end of the experiment.

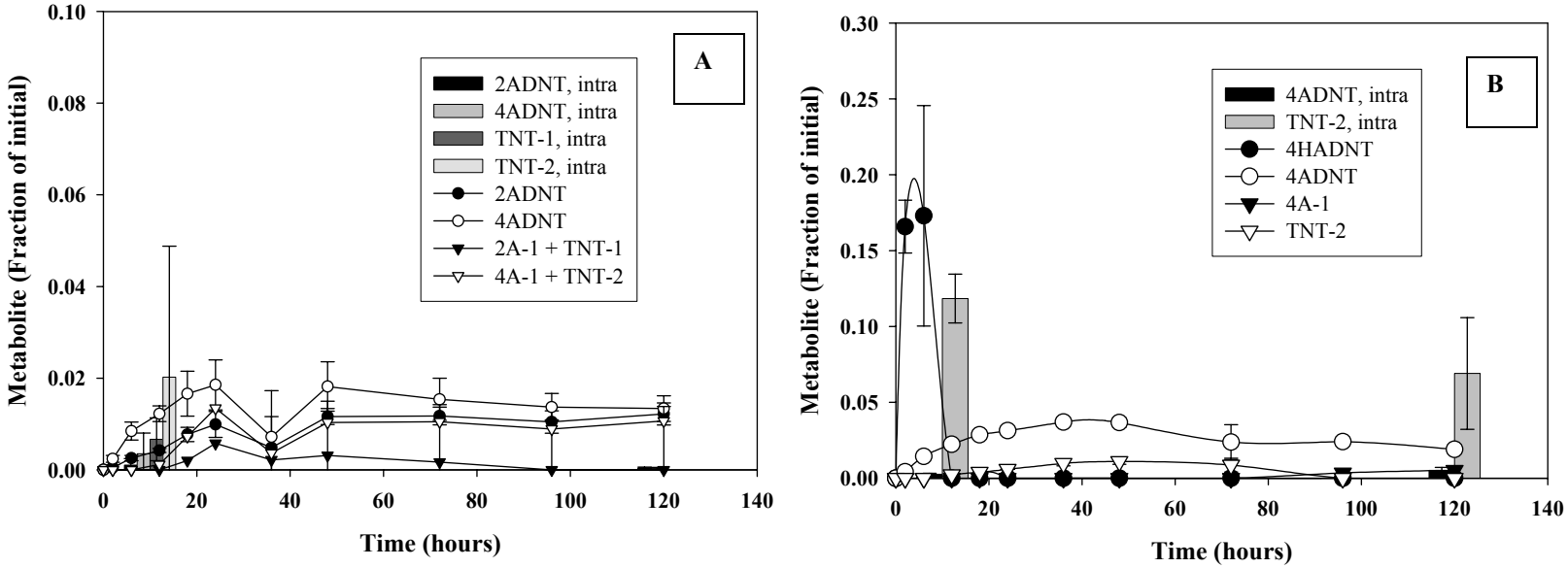


Figure 5.14: Transient Metabolite Profile at 0.25 mM: Profiles of both intracellular-extractable and extracellular metabolites for wild-type seedlings (A) and NR 3-2 seedlings (B) exposed to 0.25 mM of TNT are shown. The intracellular levels are shown for 10 hours and 120 hours, the two time steps when seedlings were sacrificed. The HADNT levels shown were determined by derivatization. All concentrations are averages and standard deviations of duplicates. In the wild-type seedlings, low levels of ADNTs are the most common metabolites in both the media and the tissue. The conjugates TNT-1 and TNT-2 are also seen, although their precursor metabolites- the hydroxylamines were not detected, presumably due to their low concentration. In the NR3-2 system, high levels of 4HADNT and a high level of the conjugate TNT-2 at 12 hours are observed. TNT-2 is formed from the direct conjugation of 4HADNT, hence the steep drop in 4HADNT level is followed by a peak in the intracellular level of TNT-2. This conjugate is also seen intracellularly at 120 hours, the end of the experiment.

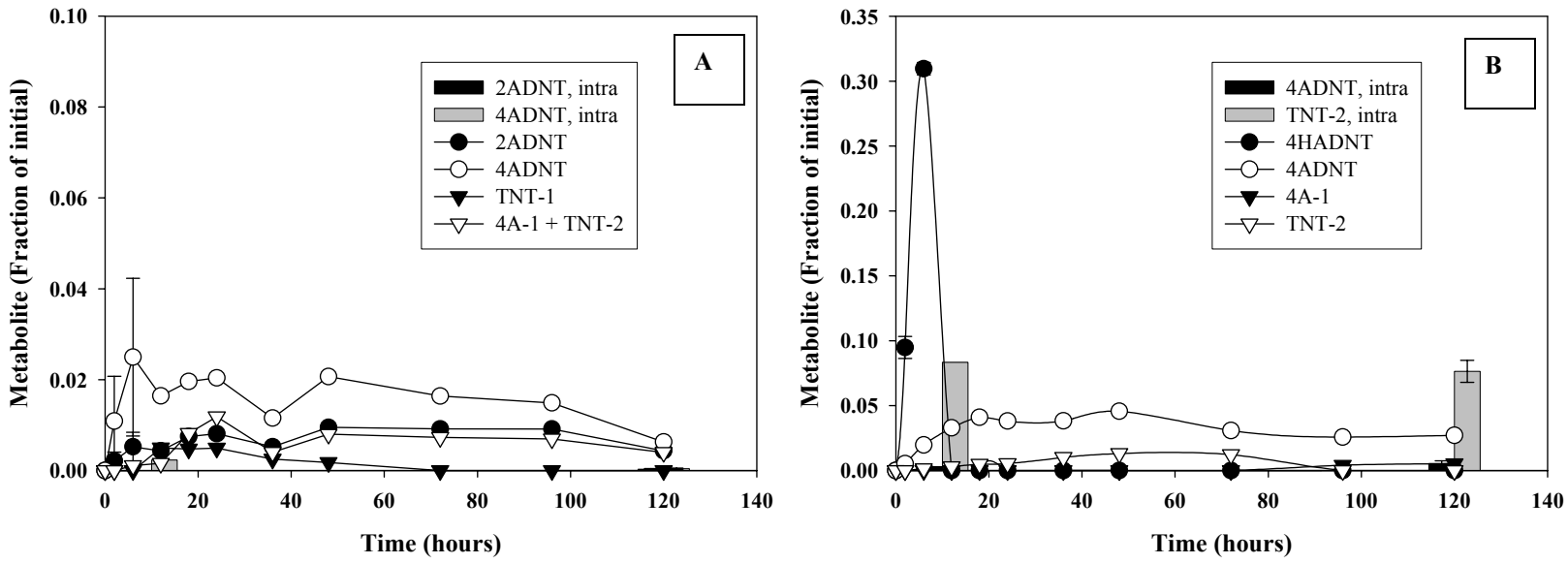


Figure 5.15: Transient Metabolite Profile at 0.38 mM: Profiles of both intracellular-extractable and extracellular metabolites for wild-type seedlings (A) and NR 3-2 seedlings (B) exposed to 0.38 mM of TNT are shown. The intracellular levels are shown for 10 hours and 120 hours, the two time steps when sacrifices were made. All intracellular concentrations and the extracellular concentrations up to 10 hour are averages and standard deviations of duplicates. The HADNT levels shown were determined by derivatization. In the wild-type seedlings, very low levels of ADNTs were the most commonly observed metabolites in both the media and the tissue. Less than 2% of the initial TNT was converted to these metabolites, due to the really low levels of TNT transformation by these seedlings. In the NR3-2 system, high levels of 4HADNT and a high level of the conjugate TNT-2 at 12 hours are observed.

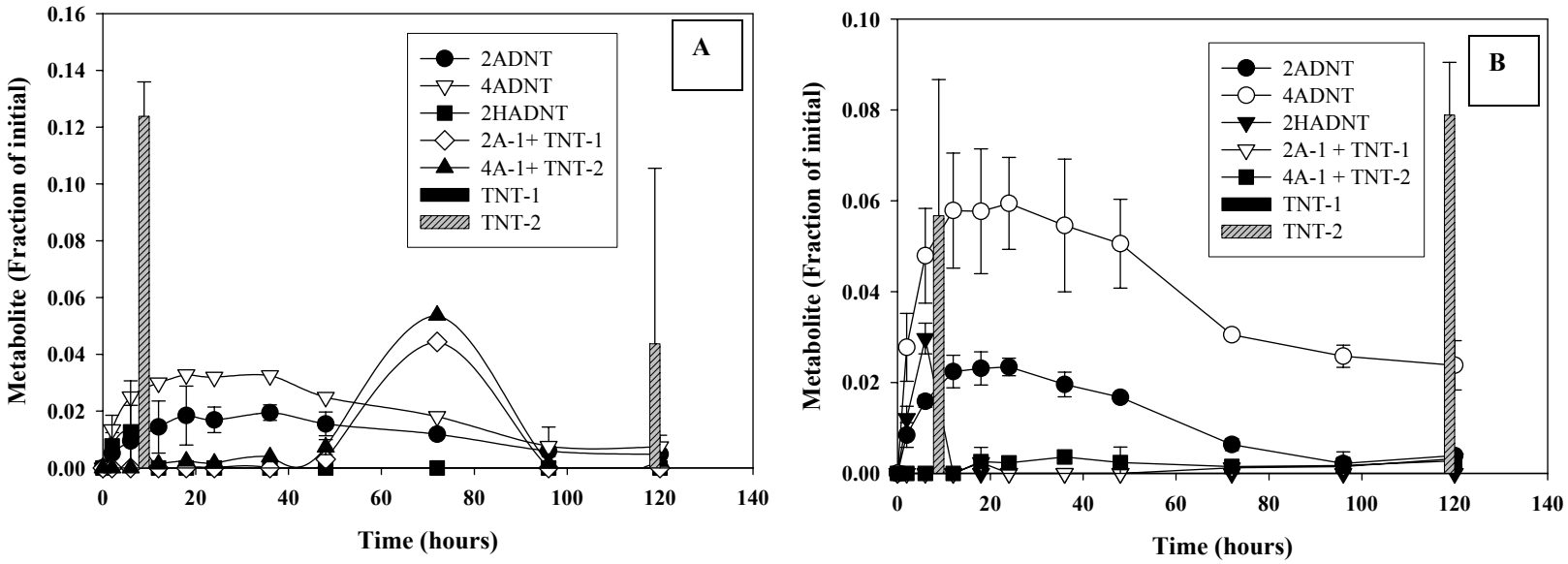


Figure 5.16: Transient Metabolite Profile in PETN: Transient extracellular and intracellular-extractable concentrations of metabolites in PETN seedlings exposed to 0.1 mM (A) and 0.25 mM (B) of TNT show are shown. The intracellular levels are shown for 10 hours and 120 hours, the two time steps when sacrifices were made. Low levels of 2HADNT, determined by direct HPLC runs, and low levels of monoamines are observed. All concentrations are averages and standard deviations of duplicates.

Table 5.2: Extracellular Conjugation Products: Eighteen day-old seedlings were incubated with TNT for five days at which time a sample of growth medium was taken and analysed for products of TNT conjugation by HPLC. Results are taken from two independent samples (results recorded where both samples contained the conjugate) for the 0.1 and 0.25 mM TNT amended seedlings and individual results from 0.38 and 0.5mM TNT amendment to media.

Initial TNT Concentration	Wild-Type Seedlings	NR 3-2 Seedlings	PETN Seedlings
0.1 mM 23 mg/L	TNT-1 TNT-2 2A-1 4A-1	TNT-2 4A-1	TNT-1 TNT-2 2A-1 4A-1
0.25 mM 56 mg/L	TNT-1 TNT-2 2A-1 4A-1	TNT-2 4A-1	TNT-1 TNT-2 2A-1 4A-1
0.38 mM 86 mg/L	TNT-1 TNT-2 4A-1	TNT-2 4A-1	TNT-1 TNT-2 4A-1
0.5 mM 113 mg/L	TNT-1 TNT-2 4A-1	TNT-2 4A-1	-

Table 5.3: Intracellular-extractable Conjugation Products: Eighteen day-old seedlings were incubated with TNT for five days at which time products of TNT conjugation were extracted from plant tissue and analysed by HPLC. Results are taken from two independent samples (results recorded where both samples contained the conjugate) for the 0.1 and 0.25 mM TNT amended seedlings and individual results from 0.38 and 0.5mM TNT amendment to media.

Initial TNT Concentration	Wild-Type Seedlings	NR 3-2 Seedlings	PETN Seedlings	TNT-2 mass ratio (NR3-2/WT)
0.1 mM 23 mg/L	TNT-1 TNT-2	TNT-2	TNT-1 TNT-2	1.6 ± 0.95
0.25 mM 56 mg/L	TNT-1 TNT-2	TNT-2	TNT-1 TNT-2	115 ± 51.4
0.38 mM 86 mg/L	TNT-1 TNT-2	TNT-2	TNT-1 TNT-2	144
0.5 mM 113 mg/L	None observed	TNT-2		>180*

* TNT-2 for WT, 0.5 mM, taken as 1 ppm in order to calculate ratio.

relative levels of conjugates formed in the intracellular-extractable fractions of all the three systems.

5.3.8: Transformation of TNT by *Enterobacter cloacae* Nitroreductase:

Note: This particular experiment was performed in University of Cambridge, UK, by our collaborators. A brief mention of the results is presented below, since they are relevant to the experiments and the ensuing discussion.

Enterobacter cloacae nitroreductase, the enzyme encoded by the gene inserted in the NR 3-2 transgenic line, was obtained by expressing this enzyme in *E. coli* and purifying the resultant broth. This enzyme was incubated with TNT along with NADH and a cofactor recycler in phosphate buffer at 25^0C, and the metabolites analyzed using a HPLC-time course assay. It was found that 0.04 mM of TNT was transformed to 4HADNT over 100 minutes, which proves that the nitroreductase enzyme in NR 3-2 forms a HADNT end-product (Figure 5.17).

5.4: Discussion

5.4.1: Modification of TNT Transformation and Phytotoxic Effects

The main objective of the TNT transformation studies on the transgenic seedlings and the wild-type plants was to determine the influence of the inserted bacterial gene on the performance of the seedlings in transforming TNT, and the resultant impact on the metabolic pathway. This was accomplished with the low and high-level TNT feeding experiments, and correlating those results with TNT transformation by the NR 3-2 native enzyme. Many instructive results were obtained regarding the efficiency of TNT transformation by the seedlings, the nature of the TNT transformation pathway, the toxic effects exerted by TNT on the seedlings, and the nature of bacterial nitroreductase.

The NR 3-2 transgenic was found to remove TNT much more efficiently than the PETN seedlings and the wild-type seedlings. They removed TNT completely within 20 and 50 hours for 0.1 mM and 0.25 mM initial TNT added, respectively. The PETN seedlings took 72 and 48 hours for the same concentrations, while the wild-type seedlings took 120 hours to

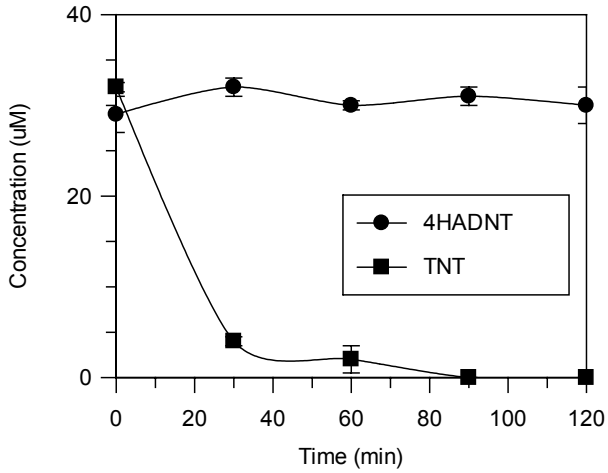


Figure 5.17: Native *E. cloacae* NR 3-2 Function: TNT was incubated with *E. cloacae* NR 3-2, NADH and cofactor recycler in phosphate buffer at 25^0C. Samples were taken every 30 min and analyzed by HPLC. Results are the averages and standard deviations of duplicates. It is seen that the native enzyme forms 4HADNT from TNT, which is consistent with the results obtained for the NR 3-2 seedling metabolite studies. No 2HADNT was detected. Figure adapted from Hannink (2003).

remove the TNT from 0.1 mM system and could not transform a significant amount of TNT in the 0.25, 0.38 or 0.5 mM systems (Figures 5.8 and 5.9). The higher TNT removal rates in the NR 3-2 seedlings appeared to correlate directly with better health of the seedlings. TNT phytotoxicity was measured according to the gain or loss of weight by the plant lines in the presence of a range of TNT concentrations (Table 5.1). Growth suppression and chlorosis have been observed as symptoms of TNT phytotoxicity in Eurasian watermilfoil (*Myriophyllum spicatum*) between concentrations of 5.9 μM (1.3mg/L) and 23 μM (5.22 mg/L) and 0.1 mM (25 mg/L) (Pavlostathis *et al*., 1998). In our experiments, a faster TNT removal rate appeared to reduce the phytotoxic effects of TNT towards the seedlings by reducing the time of exposure. Since the metabolites formed upon TNT transformation are less toxic than TNT itself, the transformation of TNT results in reduced toxicity. The monoamines and conjugates have significantly lower toxicities than TNT, and the NR 3-2 seedlings showed the formation of 4ADNT within 2 hours of TNT addition and the conjugate TNT-2 was formed just 6 hours after TNT addition (Figure 5.11 and 5.12). This progression along the pathway (Green Liver model) helped minimize the phytotoxic effects on TNT on the seedlings. In contrast to the NR 3-2 seedlings, the wild-type seedlings were exposed to TNT for much longer time periods, and in the case of 0.25, 0.38 and 0.5 mM systems, TNT was present for the length of the experiment (120 hours). The longer exposure time appeared to manifest itself in terms of enhanced chlorosis and stunting of growth. PETN seedlings showed more toxic effects than NR 3-2 and less toxic effects than the wild-type seedlings, which is consistent with its mid-level TNT removal rate. At the high initial TNT concentrations of 0.25, 0.38 and 0.5 mM levels, residual TNT was found in the plant tissues of the wild-type seedlings at 120 hours. This appeared to indicate that while these seedlings were experiencing severe phytotoxic effects, they were capable of taking up the TNT and transforming small amounts of it. This has previously been observed in other studies (Pavlostathis *et al*., 1998), and suggests that TNT uptake is a physical and a biological process. When the health of the seedling is good, uptake is superior, but when biological uptake is stunted due to toxicity, a residual physical uptake remains.

5.4.2: Pathway Characteristics

In addition to monitoring the TNT concentrations in all the three systems, the presence and concentration of other metabolites were monitored too. This was to help identify precise portions of the pathway affected by the enzyme insertion, and determine the positive or negative role these pathway modifications would have on the entire process of phytotransformation. The PETN and wild-type seedlings showed typical behavior, similar to characteristics observed during TNT transformation by other plant systems. Monoamines were formed and were subsequently conjugated, and the conjugates disappeared over time (Figure 5.16). The PETN seedlings showed the formation of hydroxylamines as the primary metabolites, while the wild-type seedlings failed to reveal their presence. Low levels of monoamines, below 5% of the initial TNT added, were the main feature of the transformation pathway. These trends were also reflected in results of the plant tissue extractions. PETN and wild-type seedlings again produced both 2ADNT and 4ADNT transformation products of TNT and as in the growth medium samples, but no HADNT products were observed. The ADNT transformation products were found in plant tissue at higher amounts in 120-hour samples (compared to the 10-hour samples) only at 0.1mM (23mg/L). This may be due to the observation that at higher concentrations, wild type plantlets were necrotic by 120 hours and presumably unable to transform TNT to any degree.

In sharp contrast to PETN and wild-type seedlings, the NR 3-2 seedlings showed two salient features- high levels of 4HADNT and an almost complete absence of 2-substituted metabolites and conjugates (2HADNT, 2ADNT, 2A-1 and TNT-1). The high levels of 4HADNT have not been observed in any other study, and appear to be characteristic of NR 3-2 (Figures 5.13, 5.14 and 5.15). The derivatization procedure used in the studies helped demonstrate the much higher 4HADNT concentrations, than would have been obtained from running the samples directly through the HPLC. Figures 5.4A and B show the differences in amounts observed when the samples were first derivatized and run, as opposed to directly analyzed via the HPLC. This is the first application of the derivatization procedure in a TNT-phytotransformation study, which can be used in any plant system since it is species independent.

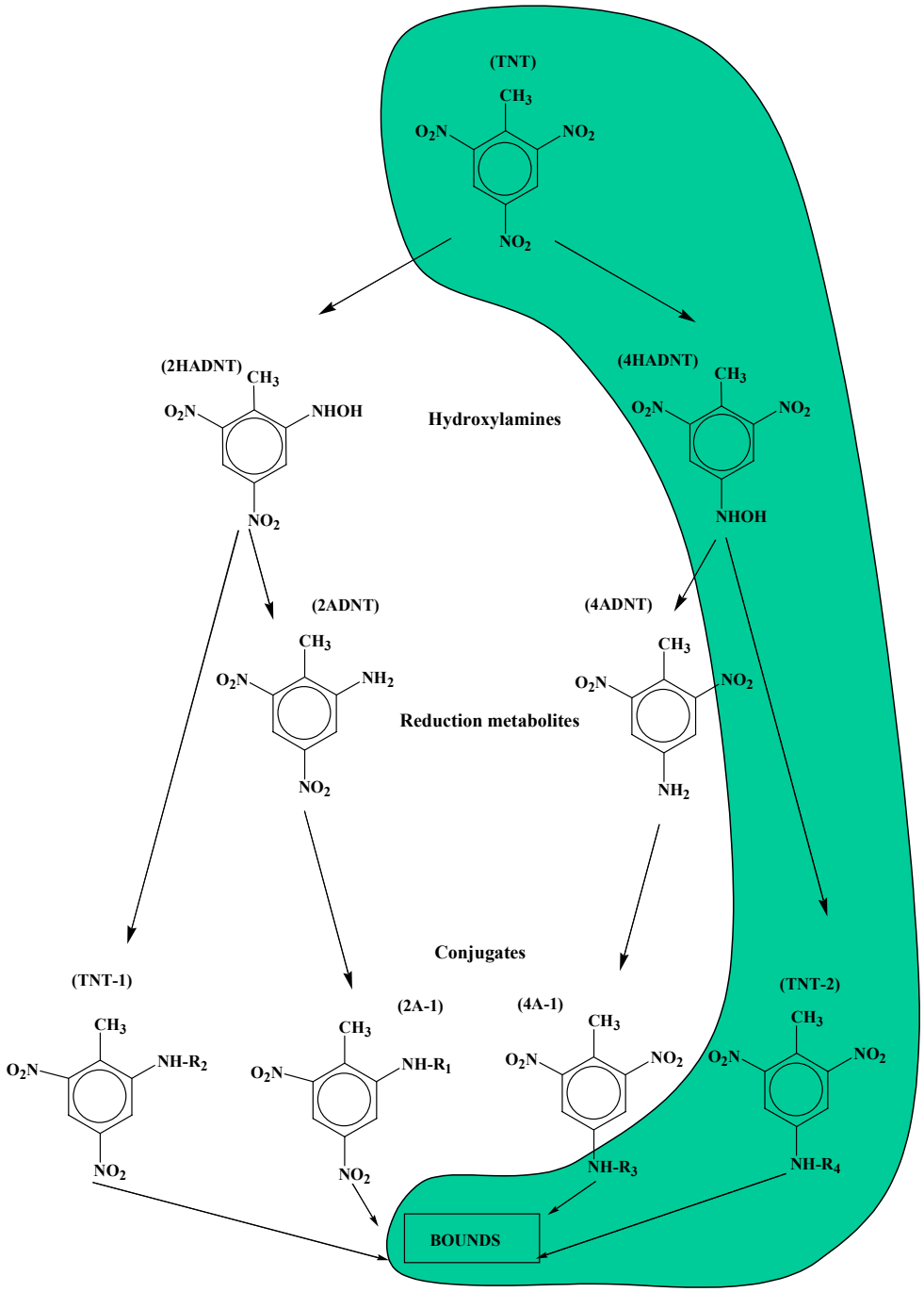
Hydroxylamines have rarely been detected in TNT transformation studies, and have not been seen in non-aquatic plant species (Pavlostathis *et al.*, 1998; Wang *et al.*, 2003). The recent study by Wang (2003), details the production of hydroxylamines in an aquatic species, *Myriophyllum aquaticum*, in a TNT transformation study. In previous work with *Myriophyllum spicatum* (Eurasian water milfoil) 4HADNT was observed at low concentrations when 50 μM of TNT was used (Pavlostathis *et al.*, 1998). However, in spite of the very rare detection of hydroxylamines it has been speculated that they are formed during TNT transformation even in terrestrial species (Burken *et al.*, 2000).

Monoamines have been seen in almost all TNT phytotransformation studies to date and are presumably formed from the reduction of hydroxylamines. In addition, the conjugates TNT-1 and TNT-2 are formed by direct conjugation of hydroxylamines to a six-carbon plant macromolecule (Chapter 4). The non-observance of hydroxylamines is probably because of their unstable nature in aquatic, aerobic media, and their high turnover rate (Section 3.3.1). As the analytical methods used here did detect production of 4HADNT in the transgenic seedlings, it would be expected that HADNTs would also be detectable in wild type plants if produced at a high enough level.

The 4HADNT produced here by NR 3-2 had a very short life (high turnover rate) and was quickly transformed to 4ADNT and TNT-2. 4HADNT is presumably either acted upon by different plant nitroreductases, or in some instances conjugated or oxidized or polymerized. These competing reactions and the natural instability of the hydroxylamine lead to its quick turnover. Hence, not only does the transgenic plant line remove TNT faster from the growth medium, the 4HADNT produced is also turned over very rapidly. This result is manifested in the rapid formation of the conjugate, TNT-2, which is formed from the direct conjugation of 4HADNT. The initial reduction of almost all of the TNT to 4HADNT therefore biases the pathway and leads it along the 4-substituted metabolite branch (4HADNT, 4ADNT, TNT-2), with very low levels of 2HADNT, 2ADNT and 2A-1 being observed (Figure 5.18). These 2-substituted metabolites were observed due to the endogenous plant nitroreductases that transformed TNT to 2ADNT.

Confirmation of the specific role of the bacterial nitroreductase was provided by incubating TNT with the native NR 3-2 enzyme, along with NADH and a cofactor recycler in

Figure 5.18: Tobacco-TNT transformation pathway: TNT transformation pathway as understood in tobacco seedlings, from our experimental results. While the PETN and wild-type seedlings used the complete pathway to metabolize TNT to irreversibly polymerized bounds, the NR 3-2 seedlings used mainly the shaded portion of the pathway, going from TNT to 4HADNT to TNT-2 to bounds. This was due to the overexpression of the enzyme that catalyzed TNT to 4HADNT. NR 3-2 also showed the formation of 4ADNT, but that portion of the pathway was minor. PETN showed the formation of the hydroxylamines, whereas the wild-type seedlings did not, presumably due to their low levels and quick degradation.


(TNT)
CH3
O2N
NO2
NO2
(2HADNT)
CH3
O2N
NHOH
NO2
Hydroxylamines
(4HADNT)
CH3
O2N
NO2
NHOH
(2ADNT)
CH3
O2N
NH2
NO2
Reduction metabolites
(4ADNT)
CH3
O2N
NO2
NH2
Conjugates
(TNT-1)
CH3
O2N
NH-R2
NO2
(2A-1)
CH3
O2N
NH-R1
NO2
(4A-1)
CH3
O2N
NO2
NH-R3
(TNT-2)
CH3
O2N
NO2
NH-R4
BOUNDS

a phosphate buffer. Those results showed the formation of 4HADNT corresponding to a decline in TNT concentration (Figure 5.17). This confirmed that the nitroreductase from *Enterobacter cloacae* is a Type II nitroreductase (Bryant *et al.*, 1991) that only partially reduces the nitro group to a hydroxylamino group ($NO_2 \rightarrow NHOH$) (Figure 5.19); hence during the whole seedling studies, 4HADNT was the main metabolite observed in the NR 3-2 transgenic. Since 4HADNT led to the formation of 4ADNT and TNT-2, the preponderance of 4-substituted metabolites formed in the pathway was a direct result of the inserted bacterial gene.

High levels of the conjugate TNT-2 were observed in the NR 3-2 system, which is directly consistent with the flow of pathway flux along the 4-substituted metabolites. TNT-2 was formed within 6 hours at the 0.38 and 0.5 mM initial TNT and by 12 hours in the 0.1 and 0.25 mM initial TNT seedlings. This early formation of TNT-2, at significant levels, helps in a rapid reduction of toxicity, and hence the NR 3-2 seedlings always displayed better health than the PETN and wild-type seedlings (Figure 5.13, 5.14 and 5.15). The conjugates, in conformity with the "Green Liver model", appear to be accumulated intracellularly, and subsequently bound to the plant (Section 2.5 in Chapter 2).

5.5: In Conclusion

The experiments described in this chapter were designed to understand the effect of the genetic manipulation of tobacco on TNT transformation. This was accomplished by TNT transformation experiments, monitoring the metabolic pathway, monitoring the health of the seedlings, and finally determining the role of the native enzyme on TNT. In addition, a derivatization procedure was used to stabilize the hydroxylamines and provide for reliable quantification. The inserted nitroreductase enzyme was found to vastly enhance the efficiency of TNT transformation in NR 3-2, and have a marginal effect in PETN seedlings. In NR 3-2, the inserted nitroreductase caused the preferential reduction of TNT to 4HADNT, which was observed in high concentrations. This also led to the formation of mainly 4-substituted metabolites and conjugates, and to the accumulation of TNT-2 in the tissues. These studies have demonstrated the advantages to be gained from genetic engineering of plants for phytoremediation, and the importance of understanding the metabolic pathway

involved. The catalyzing of the TNT to HADNT step will probably have the greatest impact on the efficiency of TNT phytotransformation, since this is one of the rate limiting steps (Section 4.4 of Chapter 4). Hence, this is an ideal genetic manipulation from a practical perspective. Further research into other metabolic manipulations, such as the over-expression of genes involved in the polymerization of bounds, and the preferential channeling of the hydroxylamine flux towards conjugates and azoxies have the potential to generate tailored and efficient genetic phytoremediation systems.

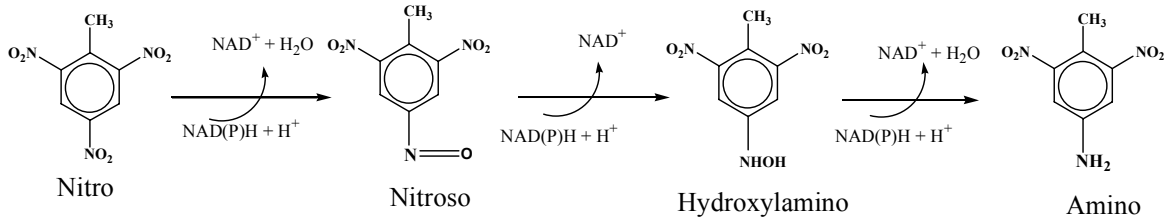


Figure 5.19: Nitroreductase Pathway: Reduction of the nitro group on the TNT to a nitroso, hydroxylamino and finally amino by nitroreductase with the supply of NAD(P)H. Figure adapted from Hannink (2003).

Chapter 6: TNT Transformation Characteristics in Arabidopsis

6.1: Introduction

While Chapters 3 and 4 elucidated the role of hydroxylamines in TNT transformation by Arabidopsis and *C. roseus*, this chapter will present a complete analysis of the TNT transformation pathway in Arabidopsis. Chapter 3 discussed the presence of hydroxylamines in the pathway, while Chapter 4 dealt with the role of these hydroxylamines, and the structure of the pathway. This chapter provides an analysis of the TNT transformation pathway, and determines its salient aspects in Arabidopsis. In addition, a complete mass balance on all branches of the pathway will be presented using [ring-U ^{14}C] TNT. Radiolabeled mass balances determine if any mineralization of the parent compound occurs and quantifies the final fate of the energetic compound. As has been discussed (Chapter 2, section 2.3; Chapter 3, section 3.1), Arabidopsis is an important system used in current research on phytoremediation of explosives, because of the powerful genetic tools available to isolate the role of specific genes. Mutants of Arabidopsis have also been generated by a variety of means and groups, and these may provide insights in the genetics of TNT and other explosive phytoremediation. However, prior to analysis of mutants for TNT remediation, it is important to understand the baseline of TNT transformation by wild-type Arabidopsis. This chapter attempts to provide a basic picture of TNT phytotransformation for comparison studies dealing with transformation characteristics of Arabidopsis mutants.

6.2: Materials and Methods

6.2.1: Chemicals

Solid TNT for feeding experiments was purchased from ChemServices (West Chester, PA), while liquid HPLC standards of TNT, 2ADNT, 4ADNT, 2HADNT, 4HADNT and 4,4'-Azoxy were purchased from AccuStandards (New Haven, CT). [ring-U ^{14}C]2,4,6-trinitrotoluene of 5 mCi activity in solid form was ordered from Perkin-Elmer Life Sciences (Boston, MA). The 2HADNT, 4HADNT, 2ADNT, 4ADNT and 4,4'-Azoxy standards were used in the low concentration metabolite feeding studies too, since their solid forms were not

available. All solvents, including 2-propanol, methanol, ethanol, ethyl ether and acetonitrile were purchased from Fisher Scientific. The scintillation cocktails Ultima Flo* M, Ultima Gold MV, Insta Fluor, PermaFluor and CarboSorb were purchased from Packard Instruments (Boston, MA).

6.2.2: Plants

Arabidopsis thaliana was grown axenically from seeds. 50 seeds of the plant were surface sterilized with 20% bleach for 15 minutes and subsequently rinsed three times with sterile water. They were next transferred to 50 ml of Arabidopsis growth media (4.2 mg/L of MS media salts, 20 g Sucrose, 1 ml Gamborg B-5 vitamins, MES buffer, Potassium phosphate in 1 L of nanopurified water at a pH of 5.7) in a 250 ml Erlenmeyer flask and shaken at 100 rpm under light at 25 ºC. Seedlings one week or 14 days old were used in the TNT and metabolite feeding experiments as mentioned during the experimental description. These seedlings were in the early (one-week) or mid (two weeks) exponential phase of growth.

6.2.3: Arabidopsis Growth Curve Generation

Determination of the growth characteristics of hydroponic Arabidopsis seedlings was accomplished by cultivating them from seeds as described in the previous section. Seedlings were grown at 25 ºC under light at 100 rpm for seven days, following which three samples were sacrificed for dry weight measurements. The dry weights were obtained by freeze drying the biomass for 48 hours. Triplicate samples were subsequently sacrificed at periodic time intervals to allow for the determination of dry weights at various time steps, until 35 days.

6.2.4: Analytical Methods

Reverse-phase HPLC was used for the separation and identification of metabolites from the phytotransformation studies. A Waters system with a 717 autosampler equipped with a PDA detector was used for these studies. Two types of columns were used, a NovaPak C8 and a NovaPak C18 column. A mobile phase of 82% water and 18% 2-propanol (volume

basis) were used with the C8 column to isolate and identify TNT, 2ADNT, 4ADNT and the previously identified conjugates of TNT-1, TNT-2, 2A-1 and 4A-1 (Bhadra, Wayment *et al.*, 1999). For the C18 column, conditions as described in Wang (2003) with a gradient mobile phase of 60% water and 40% acetonitrile ramped to 40% water and 60% acetonitrile was used. This column was used to identify the metabolites 2HADNT, 4HADNT, 2,2'-Azoxy (4,4',6,6'-tetranitro-2,2'-azoxytoluene) and 4,4'-Azoxy. Identification and quantification was done on the basis of retention time and spectral matches of samples to standards. Ion-suppressed HPLC separation with a mobile phase of 82% 50 mM H_3PO_4 to 18% propanol (volume basis) on a NovaPak C8 column was performed to detect any oxidative metabolites (Bhadra, Spanggord *et al.*, 1999).

In the radiolabeled TNT feed experiment, quantification of radioactivity under the HPLC peaks was accomplished by having a Packard 505 Flow Scintillation Counter in serial attachment to the PDA detector. A ratio of 1:1.5 of mobile phase to Ultima Flo* M scintillation cocktail was used. Figure 6.1 shows an overlay of the HPLC and FloScin traces; the hot (radioactive) peaks are seen on both traces, while the cold peaks are seen only on the HPLC trace. The hot peaks originate from the radiolabeled parent compound (^{14}C TNT in this case) while the cold peaks are probably plant-derived metabolites, which are not of interest. Hence, overlaying the two traces helps identify peaks of interest. Quantification of the radioactivity of extracellular, intracellular-extractable and intracellular- bounds fractions were done in a Packard 2900 TR Scintillation counter. A ratio of 1:5 of sample to Ultima Gold MV cocktail was used for aqueous samples, while a ratio of 1:5 of sample to Insta Fluor was used for organic samples. Residual radioactivity in the plant biomass was measured by combusting a portion of the biomass in an OX700 Harvey Biological Oxidizer. Oxygen was used to complete combustion, while Nitrogen was used to flush out residual radioactivity. 15ml CarboSorb was used to collect the radioactive CO_2 from the combustion chamber, which was combined with PermaFluor E^+ in a ratio of 1:5 and analyzed under the scintillation counter.

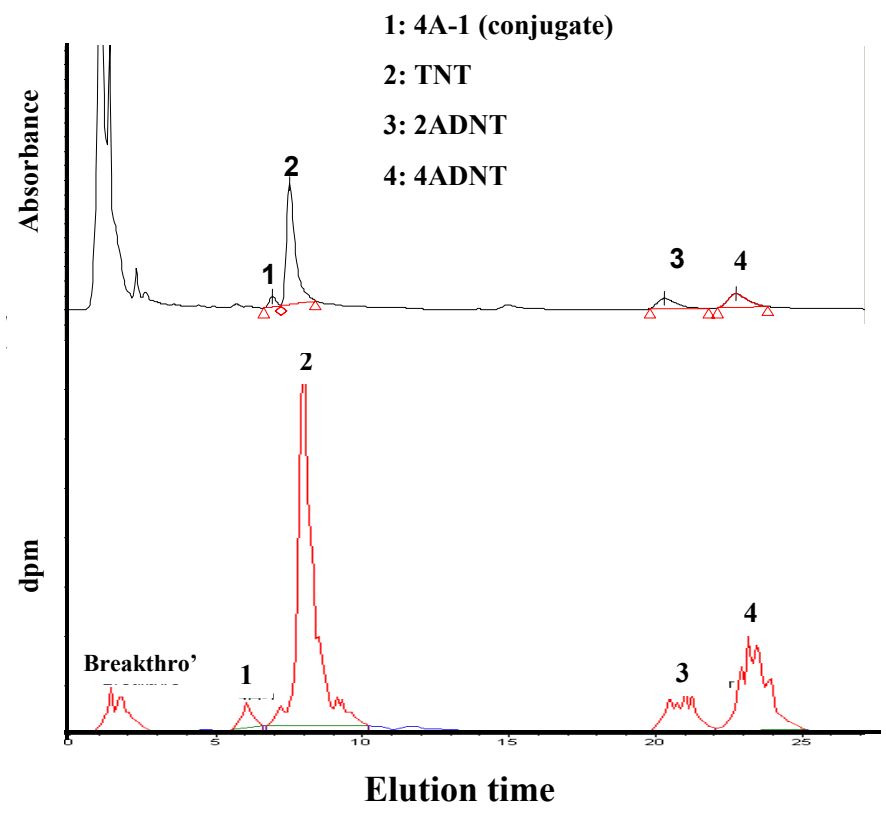


Figure 6.1: PDA and FloScin Comparison: An overlay between PDA (HPLC detector) and 505 (FloScintillation detector) detectors connected in serial. While the PDA detects any peaks separated by the column, the 505 identifies only radiolabeled peaks obtained from TNT transformation. Absorbance is proportional to concentration; dpm (disintegrations per minute) is the unit of radioactivity measurement in the FloScin.

6.2.5: Extraction of Intracellular Metabolites

When identification and quantification of the metabolites inside the plant biomass was necessary, an organic extractive procedure was used. This required the sacrifice of the seedlings and extraction of metabolites through a procedure of freeze-drying and sonication. Biomass, frozen at -20 ºC, was freeze dried for 48 to 72 hours. The dried, friable biomass was then ground in a pestle and mortar in the presence of liquid nitrogen. The powdered biomass was sonicated in 15 to 20 ml of methanol twice, at 15 degree °C, for 36 to 48 hours to extract the metabolites. The methanol was centrifuged, decanted, evaporated down to 5 ml, and subsequently analyzed via HPLC. In the radiolabeled TNT mass balance experiments, the residual radioactivity in the biomass was analyzed in a bio-oxidizer.

6.2.6: Transformation Experiments

6.2.6.1. TNT Mass Balance Study in Arabidopsis. Two-week old Arabidopsis seedlings were separately fed [ring-U ^{14}C] TNT at an initial concentration of 15mg/L (0.07 mM) and 50 mg/L (0.22 mM). The radioactivities were 0.06 μCi/ml and 0.2 μCi/ml for each system, respectively. Radiolabeled TNT was diluted with cold TNT to give the final concentration and activity. Complete mass balances were performed at every time step. Media samples were taken and volumes measured to calculate the extracellular radioactivity. Seedlings were sacrificed at every time step too; the sacrificed seedlings were subjected to the intracellular extraction process. The solvent thus obtained was used to quantify radioactivity and concentrations of the intracellular-extractable fractions. The remainder biomass was incinerated in a bio-oxidizer, as described, to determine the intracellular-bound fraction radioactivity. The experiment was continued until 168 hours with periodic removal of samples.

6.2.6.2. TNT Pathway Analysis Studies. In Arabidopsis seedlings, TNT was added to 14-day-old hydroponic seedling cultures and media samples taken periodically until 120 hours. Initial TNT concentrations ranged from 20 to 125 mg/L (0.09 to 0.55 mM) per flask. The flasks were constantly shaken at 100 rpm, and were placed under light. A control with heat-killed biomass and a biomass free control were also amended with similar TNT concentrations. The biomass-killed control was to isolate the effect of absorption of TNT by

the biomass, while the biomass-free control was used to identify the photodegradative and evaporative effects on TNT. A solely evaporative control was maintained by placing biomass-free TNT spiked media at 100 rpm in the dark. All controls had an initial TNT concentration of 50 mg/L (0.22 mM). Plants were sacrificed at 12 hours and at the end of the experiments (120 hours) and analyzed for their intracellular metabolites. In a separate experiment, three flasks of one-week old Arabidopsis seedlings were exposed to 105 mg/L of TNT, and a metabolite profile constructed out of the same.

6.2.6.3. Metabolite Feeding Studies. This section includes some experiments described previously in Chapter 4 (Section 4.2.5). These repeated experiments have been included to describe results not dealt with in Chapter 4. 2HADNT, 4HADNT, 2ADNT, 4ADNT and 4,4'-Azoxy were fed to one-week old axenic Arabidopsis seedlings, grown from 25 seeds in 25 ml of media at a concentration of 5 mg/L (0.02 mM). The uptake of the parent compound and formation of metabolites were measured for 55 hours.

6.2.6.4. Kinetic Analysis. First order kinetics was assumed with respect to TNT removal by the Arabidopsis seedlings. It was assumed that the biomass of Arabidopsis did not change significantly in the 20 hours it took to remove all the TNT from the system. Exponential fitting of the TNT removal plot verified this assumption. The first order rate constant was determined from the equation satisfying this curve. The mathematic treatment is similar to that used in section 4.2.6, wherein the first order kinetic equation was integrated with respect to time and concentrations.

From equation (3) of section 4.2.6,

$[M]= [M]_{in} \exp (k_1 \times t)$ can be further modified.

Taking the natural logarithm on both sides and using the notation M= TNT

$$\ln[TNT]= \ln[TNT]_{in} + k_1 \times t, \qquad (4)$$

Hence, a log plot of TNT versus time gives k_1 as the slope of the line.

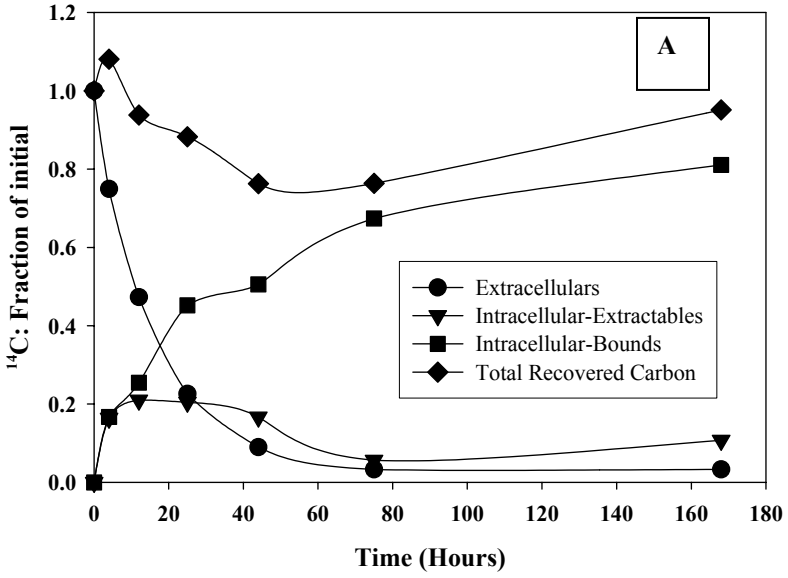
Both these methods were used to determine the values of k' for various initial TNT concentrations.

6.3: Results

6.3.1: TNT Mass Balance Studies

^{14}C radiolabeled TNT was taken up and transformed efficiently by the Arabidopsis seedlings. Initially, carbon from the TNT was observed completely in the extracellular portion, but is quickly taken up by the seedlings. In the low concentration system (15 mg/L , 0.07 mM of initial TNT), the extracellular radioactivity fell to less than 5% by the end of the experiment (168 hours), while the intracellular fraction peaked at around 20% within 20 hours of TNT addition, and then steadily declined to around 10% by 168 hours (Figure 6.2A). The amount of intracellular-bounds, the portion of the radiolabel that could not be extracted by sonication in methanol, rose steadily to more than 80% by 168 hours. A similar trend was observed in the high concentration system (50 mg/L, 0.22 mM of initial TNT), wherein the extracellular radioactivity (consisting predominantly of TNT) fell to less than 20% within 40 hours and was steady at less than 10% at 168 hours. The intracellular-extractable radioactivity rose sharply to 20% within 5 hours, and stayed at that level until 40 hours, from where it steadily declined to 10% within 168 hours (Figure 6.2B). The intracellular-bounds fraction was nearly 40% of the initial label in 40 hours, and increased to greater than 60% by 168 hours. The intracellular-bounds represent the final fate of the carbon from TNT, and its increasing number signifies complete TNT transformation. The complete mass balance for both the systems is also shown in Figures 6.2A and B. Complete recovery of the label is observed at the end point (168 hours) in both systems, which indicates that all the carbon from the TNT is shared between the extracellular, intracellular-extractable and intracellular-bound phases, and mineralization (conversion of TNT to CO_2) does not occur. In the 15 mg/L (0.07 mM) system, greater than 80% of the label is shown to be recovered at all times, while for the 50 mg/L (0.22 mM) system that number is 65%.

Figures 6.3A and B show the progression of the transformation pathway at 44 and 168 hours for the 50 mg/L (0.22 mM) TNT system. The proportion of intracellular-bounds is seen to increase from 35 to 69%, while the levels of all other fractions decreased correspondingly. The fraction termed “unknowns” represents the total quantified label minus TNT, ADNTs, conjugates and bounds; the amount of unknown label decreased from 35.9 to



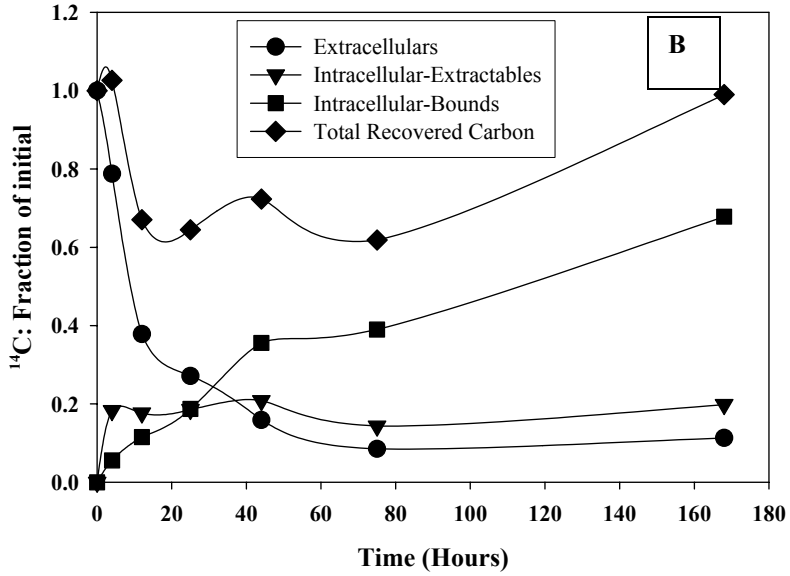


Figure 6.2: Radiolabeled TNT Mass Balances: Complete mass balance on 2-week-old axenic *Arabidopsis* seedlings exposed to [ring-U ^{14}C] TNT at an initial concentration of (1) 15 mg/L (0.07 mM) and initial radioactivity of 0.06 µCi/ml (Figure 6.2A) and (2) 50 mg/L (0.22 mM) and initial radioactivity of 0.2 µCi/ml (Figure 6.2B). Nearly 100% recovery of the C-14 label was seen by the end of the both experiments at 160 hours. Greater than 80% and 60% recovery of the label was observed in the 15 mg/L and 50 mg/L system at all times, respectively.

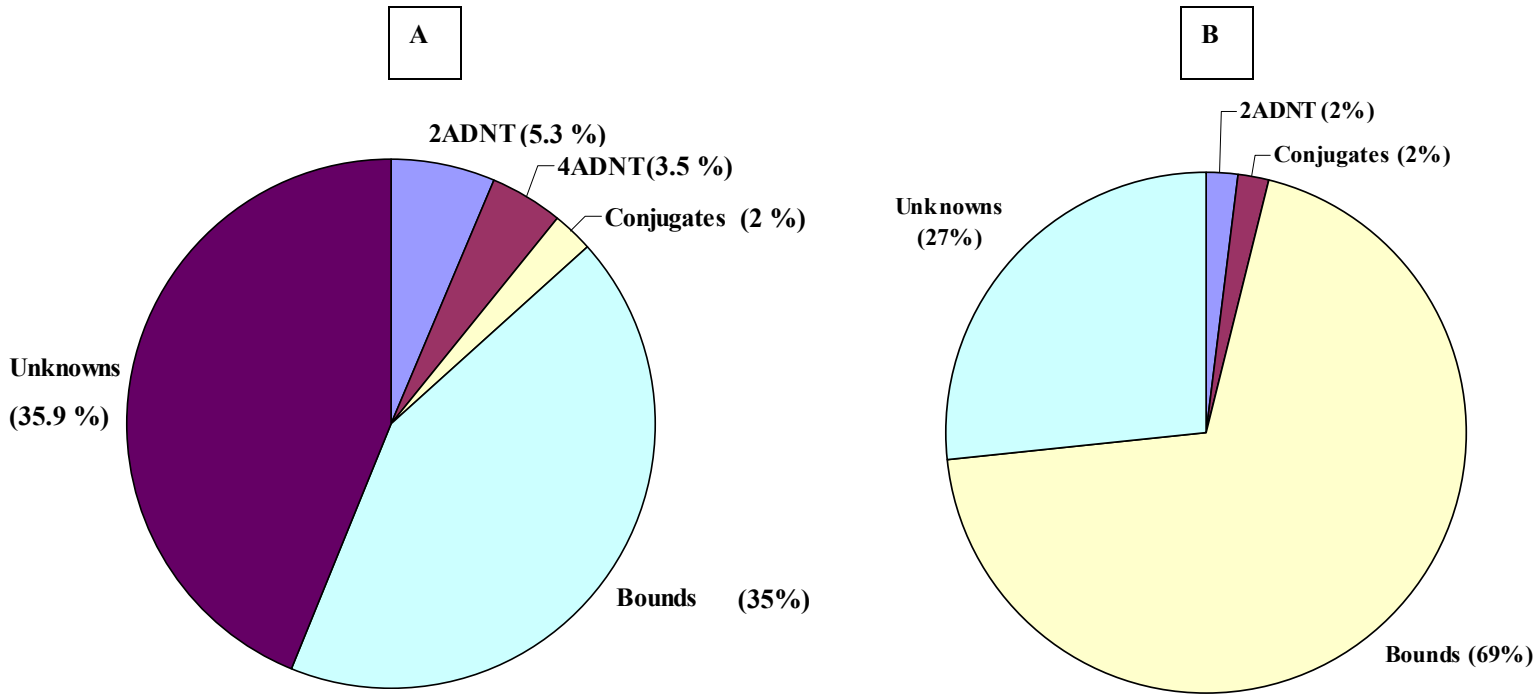


Figure 6.3: Snapshots of Metabolite Fractions: Metabolite fractions in Arabidopsis 44 hours (A) and 168 hours (B) after amendment with [ring-U ^{14}C] TNT at an initial concentration of 50 mg/L (0.22 mM) and initial radioactivity of 0.2 µCi/ml. The progression of the pathway is observed, as the proportion of bounds increases over time, with the amount of all other metabolites declining. The bounds represent a final form of the initial TNT.

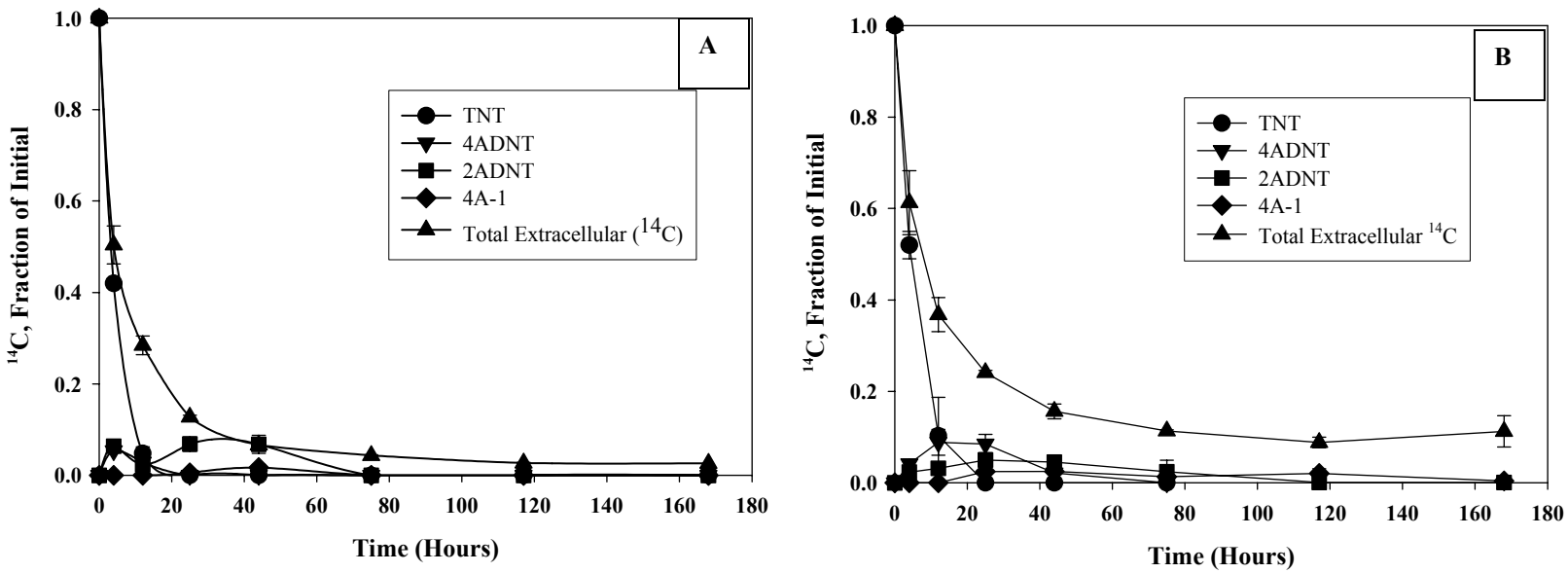


Figure 6.4: Extracellular Mass Balances: Extracellular metabolites formed when 2-week old axenic Arabidopsis seedlings are exposed to [ring-U ^{14}C] TNT at an initial concentration of (1) 15 mg/L (0.07 mM) and initial radioactivity of 0.06 µCi/ml (Figure 6.4A), and (2) 50 mg/L (0.22 mM) and initial radioactivity of 0.2 µCi/ml (Figure 6.4B). TNT is observed to be the biggest extracellular metabolite, with small amounts of ADNTs and the conjugate 4A-1. The fraction of extracellular metabolites falls to below 5% within 50 hours in the 15 mg/L system and falls below 15% within 75 hours in the 50 mg/L system.

27%. Figures 6.4A and B display the extracellular mass balances in both the systems. As seen in both these figures, TNT comprises the bulk of the extracellular label, and as TNT levels decline, the total extracellular ^{14}C count also falls. 2ADNT, 4ADNT and the conjugate 4A-1 are the other constituents of the extracellular fraction, and do not rise above 10% of the initial label. There are very low levels of unidentifieds in the extracellular fraction which seems to imply that most of the unknowns reside in the intracellular-extractable fraction.

6.3.2: TNT Transformation by Arabidopsis seedlings

Analysis of samples revealed very efficient uptake and transformation of TNT by Arabidopsis at low and medium levels of TNT addition. TNT was completely removed from the system within 20 hours for most of these concentrations. At much higher concentrations, from 110 to 125 mg/L (0.48 mM to 0.55 mM), TNT was not completely removed from the system (Figure 6.5). The heat killed controls showed 40% removal of TNT due to absorption, while the evaporative control showed an apparent increase in concentration, due to evaporation of the media. The control that combined evaporation and photodegradation showed a small decline of around 10% in TNT concentration. While evaporation of the media caused an increase in TNT concentration, phototransformation of TNT brought down the same. At the highest initial TNT concentrations (110 and 125 mg/L), the seedlings suffered severe TNT-induced phytotoxic effects. Stunted growth and browned leaves characterized these seedlings, while in extreme cases the plant completely died. The toxic effect of TNT did not manifest itself up to concentrations of around 60 mg/L. The first order kinetic rate constant ranged from 4.55×10^{-4} to 3.50×10^{-4} (L/g*hr) for initial TNT concentrations from 5 to 60 mg/L (0.02 mM to 0.26 mM). In contrast, seedlings exposed to higher levels of TNT, from 100 to 120 mg/L (0.44 mM to 0.52 mM), had kinetic rate constants in the range 4.34×10^{-5} to 2.34×10^{-5} (L/g*hr), an order of magnitude lower. Table 6.1 summarizes the TNT transformation kinetic rate constants.

In a separate experiment, wherein duplicates of 105 mg/L (0.46 mM) of TNT were added to one-week old axenic Arabidopsis seedlings, the metabolites 2HADNT, 4HADNT, 4,4'-Azoxy, 2ADNT and 4ADNT were detected. In addition, the previously identified conjugates 4A-1, TNT-1, TNT-2 and 2A-1 were also observed. Figure 6.6 shows the

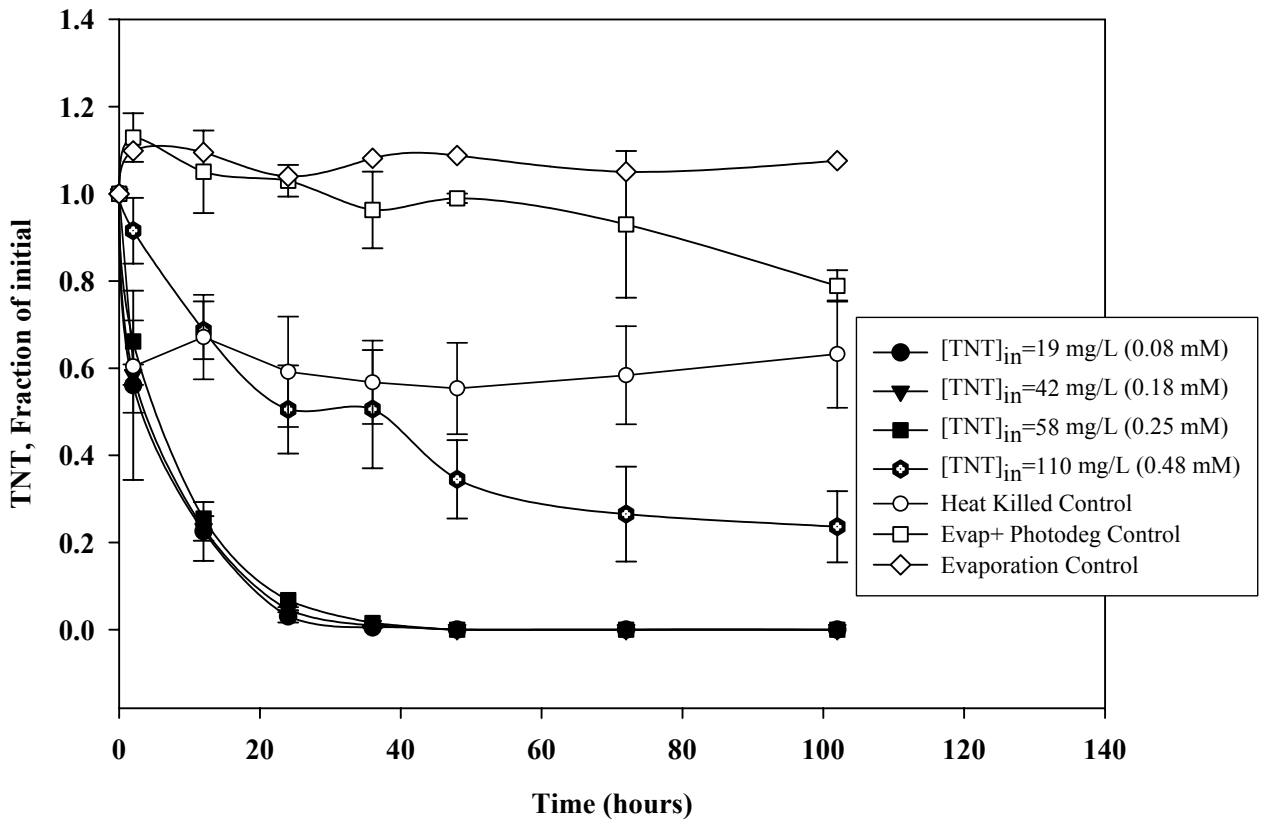


Figure 6.5: TNT Removal by Arabidopsis: Extracellular TNT levels in 14-day-old *Arabidopsis* seedlings, as determined by reverse-phased HPLC. Values for the 19, 42 and 58 mg/L are averages and standard deviations of duplicates, as are values for the heat killed and evaporation + photodegradation control. The toxic effect of TNT on the seedlings in seen to increase with increasing initial concentrations. Up to concentrations of 58 mg/L, there is no noticeable toxic effect; at concentrations of 110 mg/L, the rate of TNT removal is noticeably reduced due to the toxic effects of TNT and its metabolites.

Table 6.1: Rate Constants for TNT Transformation: First order kinetic rate constants for the uptake and transformation of TNT in 2-week old axenic *Arabidopsis* seedlings. Two schemes for determination of the rate constant were used, as described in section 6.2.6.4; the values obtained from both calculations are very close. A difference of one order of magnitude is observed for systems below 58 mg/L and those above 110 mg/L initial TNT.

Determined from direct exponential fits (equation 3):

Initial TNT Concentration		**First order rate constant, k (L/g*hr)**
mg/L	**mM**	
7.5	0.03	4.55×10^{-4}
19	0.08	4.21×10^{-4}
30	0.13	4.15×10^{-4}
42	0.18	3.92×10^{-4}
58	0.25	3.50×10^{-4}
110	0.48	4.34×10^{-5}
125	0.55	2.34×10^{-5}

Determined from plot of ln[TNT] vs. time (equation 4):

Initial TNT Concentration		**First order rate constant, k (L/g*hr)**
mg/L	**mM**	
7.5	0.03	4.55×10^{-4}
19	0.08	4.31×10^{-4}
30	0.13	4.18×10^{-4}
42	0.18	3.93×10^{-4}
58	0.25	3.55×10^{-4}
110	0.48	4.40×10^{-5}
125	0.55	2.40×10^{-5}

transient profile of TNT and all the metabolites formed, while Figure 6.7 shows just the metabolite formation. The hydroxylamines comprised greater than 20% of the initial TNT, while 4ADNT was formed between 10 to 15% (fraction of initial), while the 2ADNT was formed in amounts below 5% of the TNT added. 4HADNT was formed in higher levels than 2HADNT; 4ADNT was formed in levels higher than 2ADNT and the conjugate 4A-1 was the only conjugate observed in the system after 60 hours. A key difference between the response of Arabidopsis and *C. roseus* to TNT was the low levels of 2-substituted conjugates (2A-1 and TNT-1) and preponderance of 4-substituted conjugates (4A-1 and TNT-2) formed in Arabidopsis. The observation of the 4,4'-Azoxy metabolite was unique in an axenic TNT phytotransformation system. This metabolite, formed by the abiotic condensation of hydroxylamines (Wang *et al.*, 2003), was observed briefly at one time step at 4 hours and subsequently disappeared. No previous *C. roseus* studies have shown the formation of this metabolite.

6.3.3: TNT Pathway Analysis Studies

As has been described in Chapter 4 (Section 4.4), hydroxylamine and monoamine feeding studies in Arabidopsis seedlings have shown the direct conjugative capacities of the former. In addition, these studies revealed the preference Arabidopsis seedlings displayed for the 4-isomer over the 2-isomer. During the hydroxylamine feeding studies in Arabidopsis, it was observed that the rate of formation and removal of 2ADNT was much lower than that of 4ADNT (Figure 6.8B). The rate of 2ADNT formation was 0.41 (hr^{-1}), while the rate for 4ADNT formation was 1.97 (hr^{-1}). Similar observations were seen in the ADNT feeding experiments, wherein 2ADNT was removed at a slower rate compared to 4ADNT (Figure 6.8A). The rate of 2ADNT removal was found to be 0.05 (hr^{-1}), while 4ADNT removal rate was found to be 0.16 (hr^{-1}). These rate constants were calculated as described in section 4.2.6 in Chapter 4. In addition to these observations, the 2ADNT-fed seedlings did not show the formation of any extracellular conjugates, although the conjugate 2A-1 was detected in the intracellular-extractable component of the biomass. 4ADNT-fed seedlings showed the extracellular formation of 4A-1 (data not shown). From these observations, it

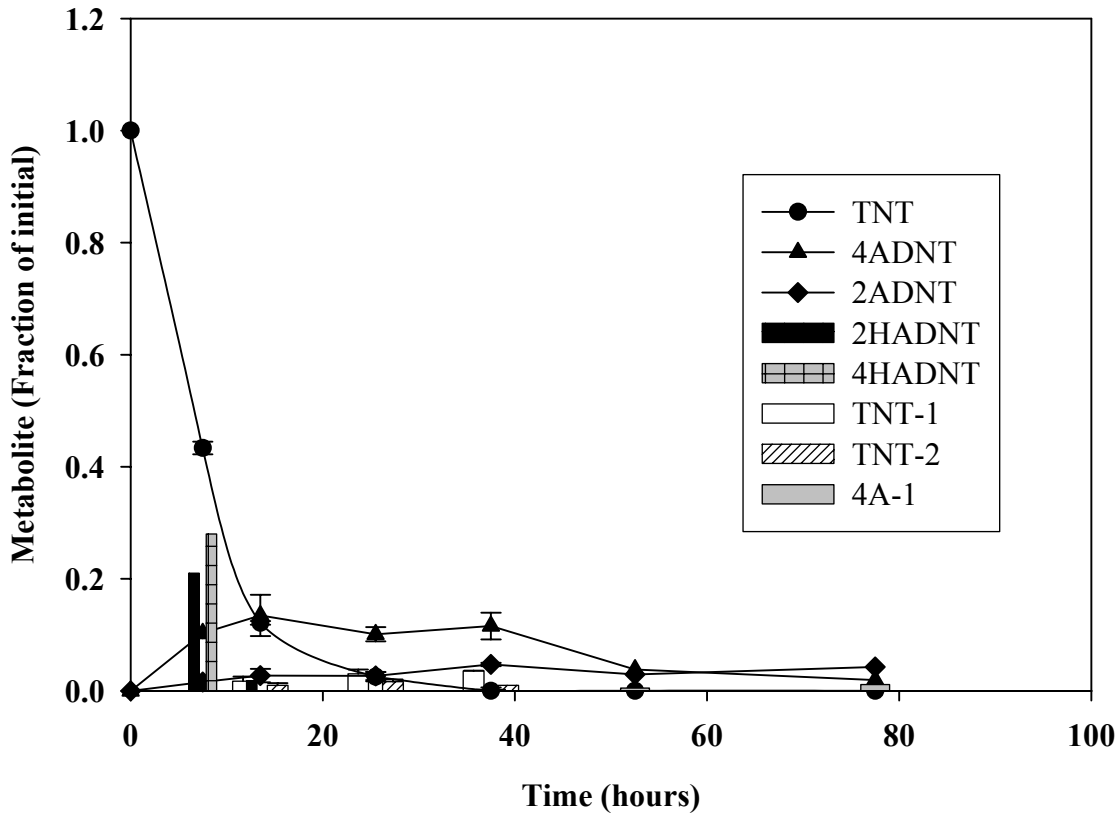


Figure 6.6: Transient Concentration of TNT and Metabolites: Extracellular, transient concentrations of metabolites and conjugates when 7-day-old *Arabidopsis* seedlings were amended with 105 mg/L (0.46 mM) of TNT. Values of 2ADNT, 4ADNT, TNT-1 and TNT-2 are averages and standard deviations of duplicates while the TNT values are averages and standard deviations of triplicates. The conversion of TNT to the various metabolites is seen to be below 25% for the hydroxylamines and below 20% for the monoamines.

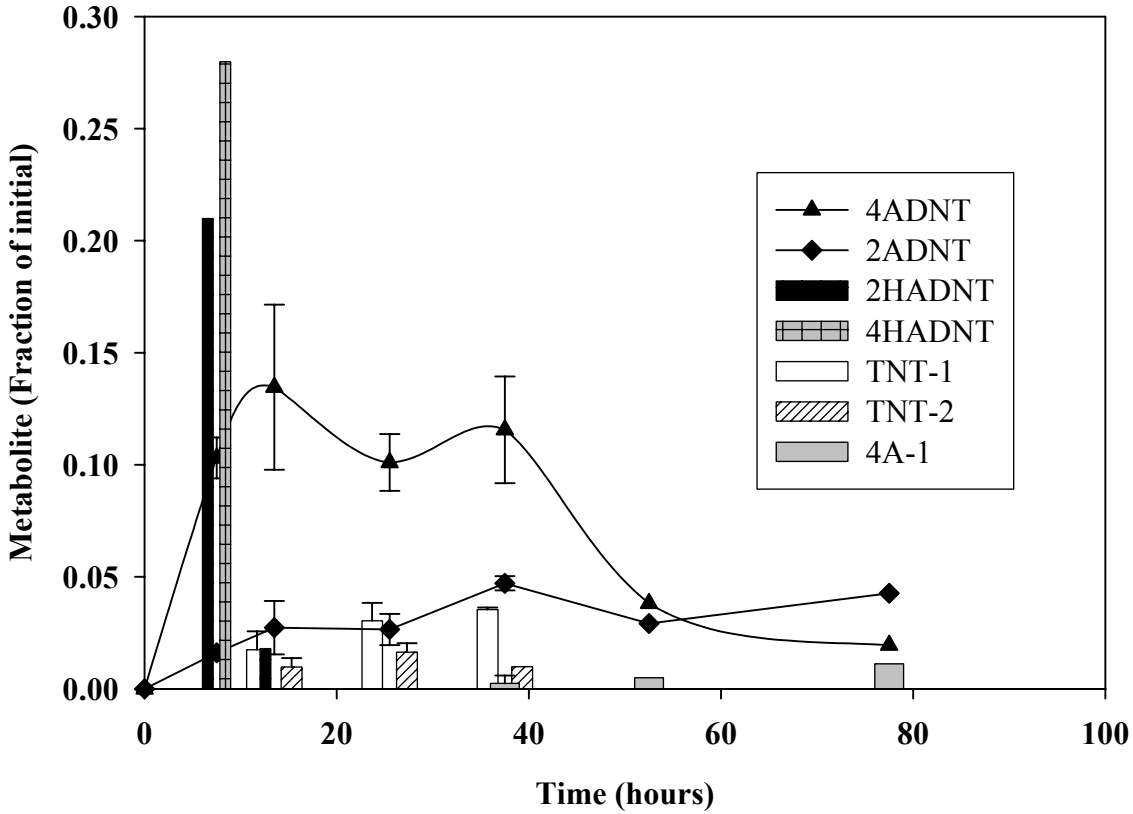


Figure 6.7: Transient Metabolite Concentrations: Extracellular, transient concentrations of metabolites and conjugates when 7-day-old axenic *Arabidopsis* seedlings were amended with 105 mg/L (0.46 mM) of TNT. Values of 2ADNT, 4ADNT, TNT-1 and TNT-2 are averages and standard deviations of duplicates, while the HADNT levels represent single measurements. An initial spike in HADNT levels is followed by the rapid decline; the ADNT concentrations follow a flatter profile, while three of the four previously identified conjugates are observed.

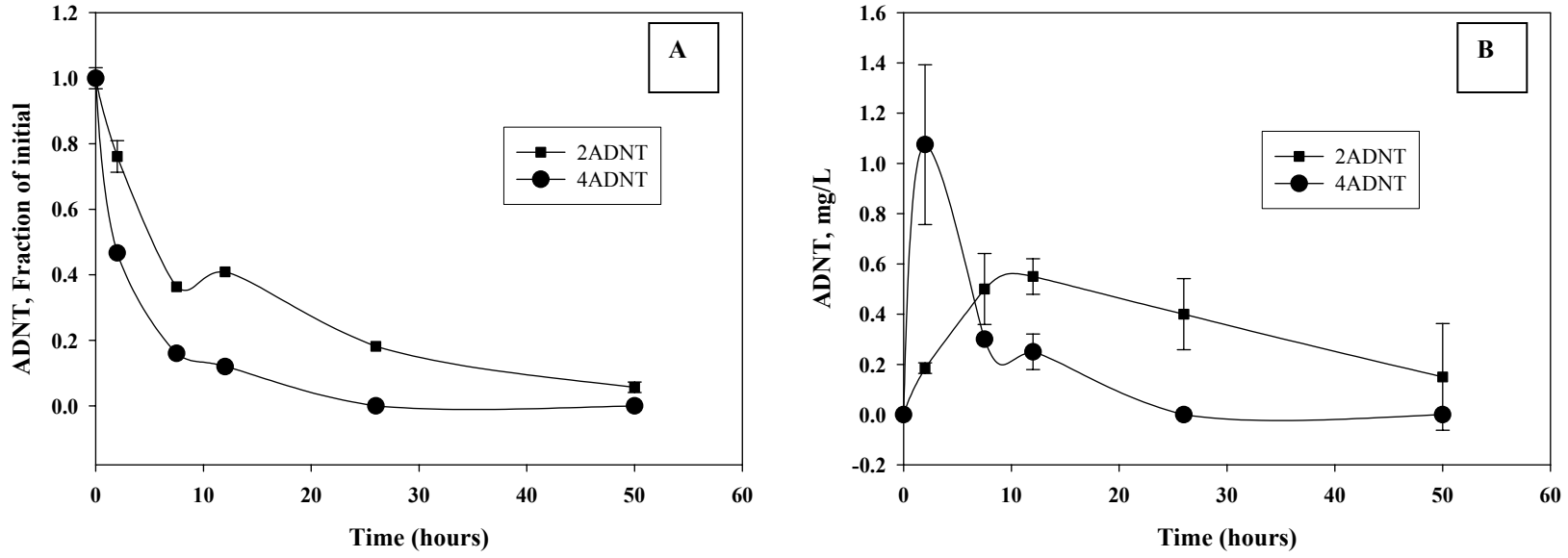


Figure 6.8: Monoamine Concentrations: Extracellular 2ADNT and 4ADNT concentrations in 14 day old *Arabidopsis* seedlings upon being fed 2ADNT and 4ADNT respectively (A) and extracellular 2ADNT and 4ADNT concentrations in 14 day old *Arabidopsis* seedlings upon being fed 2HADNT and 4HADNT respectively (B). The initial concentration of the ADNTs and HADNTs fed to the seedlings was 5 mg/L. Values shown are averages and standard deviations of duplicates. In the ADNT feeding studies, 4ADNT appears to be taken up more efficiently than 2ADNT. In the HADNT feeding studies, the rate of formation of 4ADNT is greater than that of formation of 2ADNT and the rate of 4ADNT removal is greater than that of 2ADNT removal.

appears that Arabidopsis seems to be better equipped to handle 4-substituted metabolites than their 2-substituted isomers.

4,4'-Azoxy was also added to one-week old seedlings, but was taken up completely by the seedlings within two hours. No additional metabolite formation was observed. Hence, while the azoxies were taken up efficiently by the seedlings, their fate remains unknown.

These feeding studies were also used to observe toxic effects exerted by the metabolites on the seedlings. Figure 6.9 shows the fresh (wet) weight of the seedlings 55 hours after being exposed to the metabolite. As seen in the figure, 2HADNT, 4HADNT and 4,4'-Azoxy exert a significantly higher toxic effect than TNT and the ADNTs. The weight of the seedlings exposed to these metabolites is around 60% of the weights of the control seedlings not exposed to any foreign compounds. An ANOVA analysis gave a null hypothesis probability, P= 0.002, which indicated that the variation in wet weights was due to the different toxicities exerted by the xenobiotics and not due to random variations.

6.3.4: Arabidopsis Growth Curve Studies

The effect of growth phase on TNT removal rate is demonstrated by Figure 6.10, wherein two identical systems exposed to the same TNT concentration demonstrated dramatically different characteristics. Both these systems had equal amounts of biomass at the start of the experiment. While one system removed all the TNT within 6 hours, the other system took 25 hours to remove all the TNT. Since the biomass of the both the systems were nearly identical, the only difference between the two systems was the variability due to growth phases. To minimize variability between samples, stationary phase Arabidopsis seedlings can be used for the transformation studies. To determine the time required to reach the stationary phase, growth curve studies were performed as described in section 6.2.3. Figure 6.11 shows the measured dry weights at various time steps, and it is observed that stationary phase was achieved 20 days after culturing. Exponential phase was observed to last from day 10 to day 20. In addition, variability between triplicates was high during the exponential phase, as seen in the standard deviations at day 15. However, by the 20th day after culturing of the seeds, variability between samples was less than 4% and biomass levels had reached a plateau, signifying the arrival of the stationary phase.

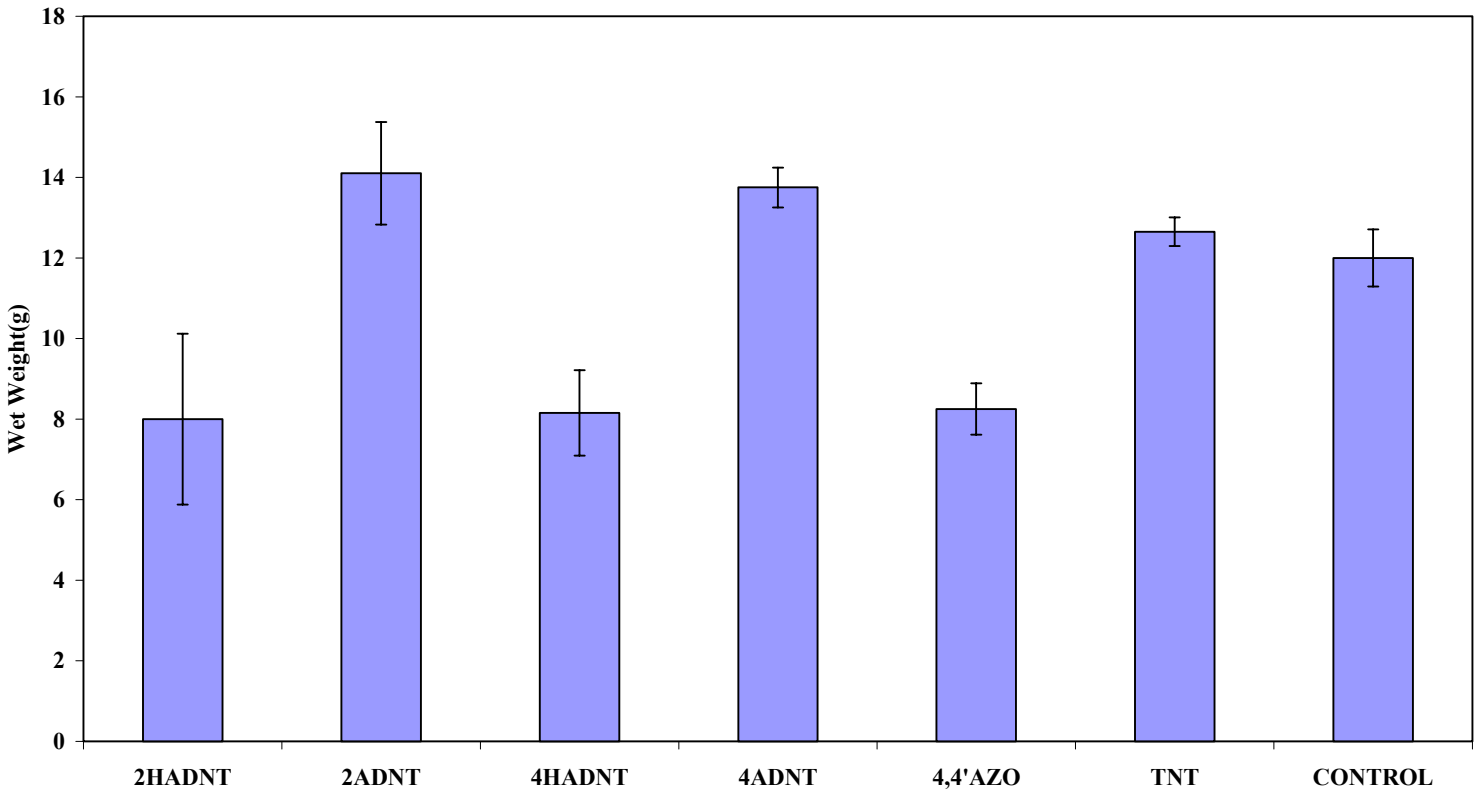


Figure 6.9: Comparison of Metabolite Toxicity: Wet weights of *Arabidopsis* seedlings exposed to various TNT transformation metabolites. The weights are reported 55 hours after exposure of the one-week old seedlings. The weights are averages and standard deviations of duplicates. As observed, 2HADNT, 4HADNT and 4,4'-Azo metabolites appear to detract TNT growth, while TNT, 2ADNT and 4ADNT do not have a noticeable toxic effect. The initial concentration of all the metabolites was 5 mg/L, except TNT which was fed at 75 mg/L, and the control which did not have any metabolites added to it. An ANOVA analysis of all the wet weight means gave P= 0.002, which indicates the difference in wet weights are due to toxic differences

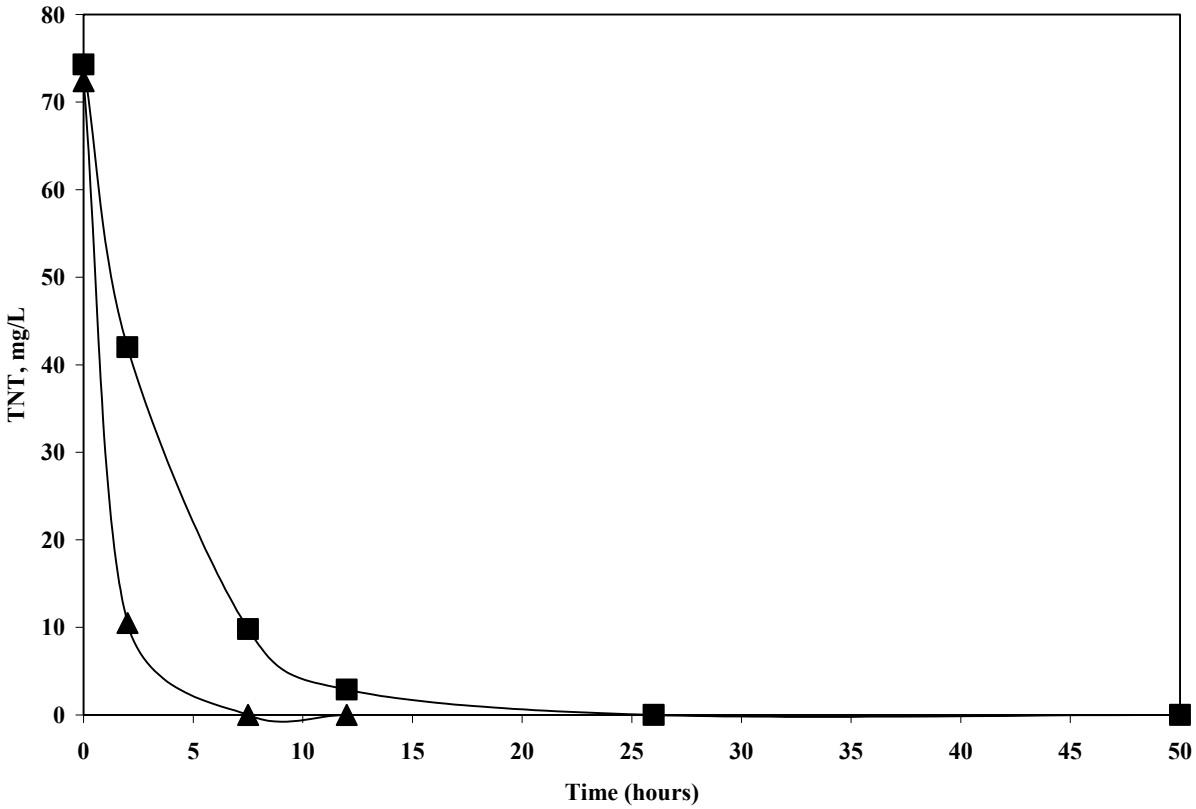


Figure 6.10: Effect of Growth Phase on TNT Removal: Both the systems shown above are one-week old cultures of axenic Arabidopsis seedlings grown from 25 seeds in a 125 ml flask with 50 ml of media, with the same fresh weight. There is, however, a dramatic difference in the TNT uptake capacity between the two systems; the first system removes 100 mg/L of TNT within 7 hours, while the other system requires 25 hours for the same. This is probably due to the difference in exponential phase behavior and seed germination between the two systems.

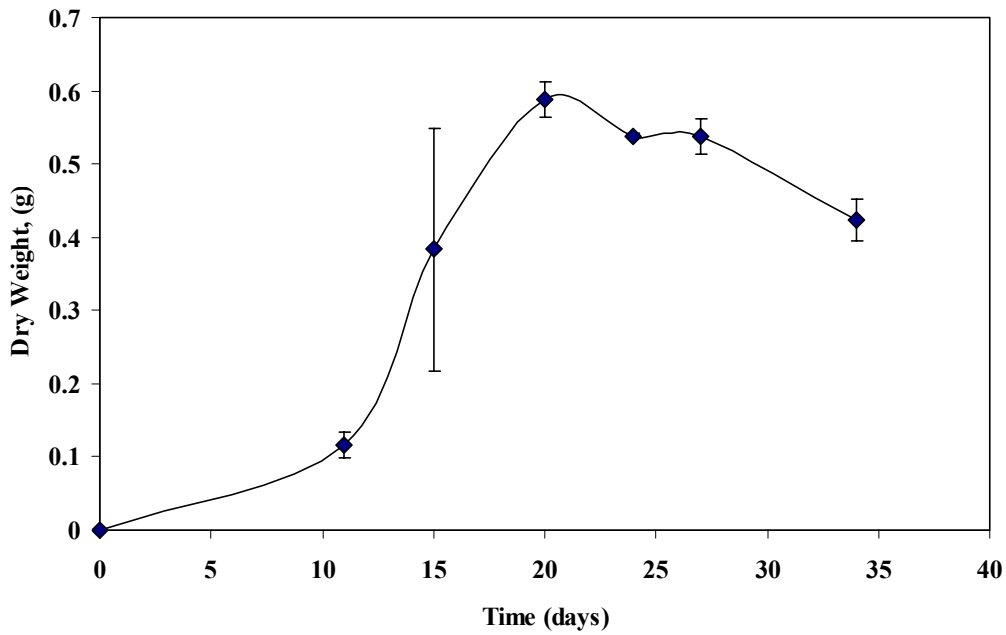


Figure 6.11: Arabidopsis Growth Curve: Growth curve for Arabidopsis seedlings, showing the dry weight of the seedlings at various time steps. High variability in the dry weights is observed during the exponential phase from day 10 to 20, following which deviation between samples falls to below 4%, and biomass levels reach a plateau. The stationary phase is seen to be reached by 20 days.

6.4: Discussion

Given the wide use of Arabidopsis in phytoremediation studies, determination of its pathway is important. Our studies show Arabidopsis to be a robust system, capable of transforming TNT rapidly, but also affected by the toxicity of high levels of TNT. Arabidopsis seedlings, in their exponential phase of growth, removed TNT rapidly from the system, with most of the TNT at low to medium concentrations (up to 58 mg/L, 0.25 mM) being removed within 20 hours. The rate of TNT removal was found to be a very strong function of the growth stage of the seedlings (Figure 6.10). While the removal of TNT by Arabidopsis does not represent a significant finding, the identification of 2 and 4HADNT, and 4,4'-Azoxy are unique findings.

6.4.1: Radiolabeled Mass Balance Studies in Arabidopsis

Uniformly labeled TNT essentially contains random carbon atoms on the benzene ring labeled with a ^{14}C isotope. Hence any permutation of the six carbon atoms on the ring could be labeled with ^{14}C. The radioactive TNT is combined with cold TNT and this sample is fed to the plants. The ratio of cold to hot TNT remains constant throughout the experiment, in all three phases (extracellular, intracellular-extractable and intracellular-bound). Hence, measuring the amount of radioactivity in any phase can be used to calculate the total amount of TNT-derived metabolite present. This enables a complete mass balance of TNT in Arabidopsis and a comparison of all the three phases of metabolites. In the 15 mg/L (0.07 mM) system, greater than 80% of the ^{14}C label was recovered at all time steps, with greater than 90% recovery for four of the time steps (Figure 6.2A). Full label was recovered at the final time step of 168 hours, which proved than TNT did not breakdown into CO_2 but rather got bound to the biomass. In the 50 mg/L system, lower mass balances were observed, with greater than 60% recovery at all time steps, and greater than 70% recovery at 3 time steps (Figure 6.2B). As with the 15 mg/L system, a complete mass balance was obtained at the final time step of 168 hours. The incomplete mass balances in the intermediate steps are probably due to experimental error caused by the saturation of CO_2 during the combustion of the biomass in the bio-oxidizer. In the final time step, biomass fractions were split into two

and analyzed which prevented CO_2 saturation and completed the mass balance. Prohibitive costs of radiolabed TNT prevented a repetition of the experiment.

These experiments also showed the accumulation of the label as the intracellular-bounds in the plant biomass as shown for the 50 mg/L (0.22 mM) system (Figures 6.3A and B). The proportion of these bounds increases to 69% of the label in 7 days, which signifies that 69% of the initial added TNT had been immobilized in the biomass of the plant as a lignin-like polymer, composed of conjugative monomers. The intracellular-bounds are not removable by routine methods of extraction, including sonication, and hence are not bioavailable. When 100% of the TNT reaches this stage of bounds, its phytotransformation is effectively completed. Previous mass balance studies on *C. roseus* roots revealed a total of 30 to 40% of the label as intracellular bounds after 8 days, while the bulk of the label remained as intracellular-extractable (63%) (Bhadra, Wayment *et al.*, 1999)(See Figure 2.10). 10 to 16% of the label remained in the extracellular phase (Bhadra, Wayment *et al.*, 1999). In contrast, in the 15 mg/L TNT fed Arabidopsis, only 3% of the label remained in the extracellular fraction and 10% as intracellular-extractable, while 81% of the label was accumulated as the intracellular-bounds 7 days after TNT addition. The greater levels of the intracellular-bounds in Arabidopsis seem to indicate the complete transformation of TNT to its final fate faster than *C. roseus*. This is also borne out by observation of the rates of removal of TNT between the two systems. For a similar initial concentration of 25 mg/L (0.1 mM), Arabidopsis seedlings removed all the TNT within 20 hours, whereas the *C. roseus* roots took nearly 45 hours to accomplish the same (Bhadra, Wayment *et al.*, 1999). Wang *et al.* report a TNT removal rate constant of 3.6×10^{-4} $Lhr^{-1}P^{-1}$ in *M. aquaticum* for an initial TNT concentration of 25 mg/L (Wang *et al.*, 2003), comparable to values calculated in Arabidopsis (4.2×10^{-4} $Lhr^{-1}g^{-1}$). However, initial biomass and growth stage considerations need to be made to accurately confirm the superiority of either species. As seen in Figure 6.10, minor changes in growth stage results in significant differences in xenobiotic removal efficiency. Hence, comparing two systems, or even comparisons within a system, need to be evaluated critically. Using stationary phase Arabidopsis seedlings, 20 days old, (Figure 6.11) can help standardize the starting conditions and result in meaningful comparisons.

6.4.2: Metabolite Formation in Arabidopsis

Experiments from Chapter 3 and 4 showed the formation of hydroxylamines and their role in the transformation of TNT by Arabidopsis. In addition, a pathway for TNT transformation was suggested (see Figure 3.1) based on the various feeding experiments. This chapter attempts to build on those studies, by delineating the characteristics of the pathway in greater detail and provide a kinetic analysis of TNT uptake. Based on the TNT feeding experiments, it was determined that TNT is initially reduced to the hydroxylamines, which in turn undergo further rapid metabolism. Biotic and abiotic reactions compete for hydroxylamines, resulting in the formation monoamines and azoxies respectively. In addition to these reactions, the hydroxylamines directly conjugate, as proved through the hydroxylamine and monoamine feeding studies (Chapter 4). Hence, the observed HADNTs disappear rapidly from the system, lasting from around 4 hours to 12 hours after TNT addition. 4,4'-Azoxy was observed in the system only at one time step of 4 hours. The azoxy feeding studies showed the rapid uptake of these metabolites; however no metabolites were observed on the transformation of the azoxies. Hence the transformation fate of these metabolites remains unclear, although Wang (2003) has speculated that the azoxies may be conjugated and polymerized, similar to the monoamines.

These monoamines, formed from the reduction of hydroxylamines, were observed through out the course of the experiment until the end, usually 120 hours. Monoamines also have been shown to further conjugate, and the thus formed conjugates polymerize and associate with the biomass. As seen in Figures 6.6 and 6.7, the previously identified conjugates TNT-1 and TNT-2 are formed initially, which are subsequently replaced by the conjugate 4A-1. The conjugate 2A-1 was observed in the intracellular fraction at trace levels. Over time, all the conjugates are incorporated into the bounds. Ion suppression HPLC did not reveal the formation of oxidative metabolites; hence, either no oxidative metabolites are being produced, or they are formed in below detectable concentrations. In Appendix 1, we have shown the identification of oxidative metabolites from TNT transformation studies in *M. aquaticum*.

There also exists a significant percentage of TNT that appears to proceed through unidentified metabolites, probably polar metabolites. This has been shown in our ^{14}C labeled

TNT feeding studies and also has shown to be the case in *C. roseus* (Subramanian and Shanks, 2003) wherein a significant percentage (up to 50%) of the ^{14}C label remained unidentified. Since TNT is a strongly electronegative compound, the initial mode of attack is necessarily via reduction of the nitro groups. This seems to indicate that the hydroxylamines, which are formed by partial TNT reduction, are potentially being transformed to unidentified polar metabolites. Hence, identification of hydroxylamines in the TNT transformation is useful in determining alternative TNT phytotransformation pathways.

In order to determine the relative toxic effects exerted by each metabolite, the fresh weights of the seedlings exposed to that particular metabolite were determined 55 hours after amendment (Figure 6.9). The higher toxicities of the HADNTs is seen from this figure, as seedlings exposed to these metabolites weighed around 8 g, compared to the controls which weighed 12 g. The metabolite 4,4'-Azoxy also showed similar toxic effects on the seedlings. In contrast, ADNTs and TNT, at the concentrations tested, did not show any toxic effects; indeed, the ADNTs appeared to be marginally beneficial to growth as those seedlings had higher biomass weights than the controls. The high toxicity displayed by the hydroxylamines at such low concentrations of 5 mg/L (0.02 mM) indicates their importance in the scheme of TNT phytotransformation.

6.4.3: Characteristics of the TNT Transformation Pathway

Based on the HADNT and ADNT feeding experiments it was found that Arabidopsis seedlings appear to transform 4ADNT more efficiently than 2ADNT. 2ADNT and 4ADNT feeding studies showed the comparatively faster uptake of 4ADNT from the media (Figure 6.8B). In 2HADNT and 4HADNT feeding studies, the ADNTs formed from the reduction of the HADNTS showed the same trend- 2ADNT took longer to form and remained in the system longer than 4ADNT (Figure 6.8A). In addition, very low levels of the 2-subsituted conjugates are formed, in contrast to the high levels of 4-substituted conjugates. Results from the TNT feeding studies revealed that the conjugates derived from hydroxylamines TNT-1 and TNT-2 were formed in the extracellular media from hour 10 to 40, and subsequently disappeared (Figure 6.7). TNT-1 is formed from 2HADNT, while TNT-2 is formed from 4HADNT; hence at this stage of the pathway, the 2 and 4-substituted portions of the pathway

appear well represented. As the pathway proceeds, however, the 2ADNT derived conjugate of 2A-1 is not seen in the extracellular media and is seen at trace levels in the intracellular-extractable fraction. In contrast, the 4ADNT derived conjugate of 4A-1 is seen in both in extracellular and intracellular-extractable fraction of the seedlings. Additionally the extracellular 2ADNT concentration appears to hold steady, while the extracellular 4ADNT concentration appears to decline over time (probably being conjugated to 4A-1). This seems to substantiate the earlier claim that Arabidopsis seedlings transform 4ADNT more readily than 2ADNT, unlike *C. roseus* and *M. aquaticum* which form both 2A-1 and 4A-1 in near equal proportions (Subramanian and Shanks, 2003). This is an important characteristic to be borne in mind during the comparison of Arabidopsis mutants and wild-types. It has been shown that the 2-substitued metabolites are more toxic than their 4-substituted isomers (Tan *et al.*, 1992; Padda *et al.*, 2000), and hence, it appears, Arabidopsis's preference for the 4-subsituted metabolite fortuitously confers it with better resistance to TNT.

In addition to characterizing the pathway, kinetic studies on the TNT transformation by the seedlings were also performed for various initial concentrations (Figure 6.5). A high rate of TNT removal was observed until concentrations of 58 mg/L (0.25 mM), while at concentrations of 110 mg/L (0.48 mM) and above, the rate of TNT transformation was severely reduced. Table 6.1 shows the values of the rate constants, determined by two methods, for all initial concentrations. The first table of values was obtained from a direct exponential plot fit of TNT removal versus time, while the second table of values was determined from a linear fit of ln[TNT] versus time. Both schemes give nearly identical results; it observed that above the concentration of 110 mg/L, the first order rate of TNT removal was an order of magnitude lower than for concentrations below 55 mg/L (0.24 mM). At these high initial concentrations of TNT (above 110 mg/L), the Arabidopsis seedlings displayed visible signs of stress from yellowing to leaves to the collapse of the biomass. However, even at these high concentrations, the seedlings were able to transform TNT to its metabolites although less efficiently (Figure 6.5).

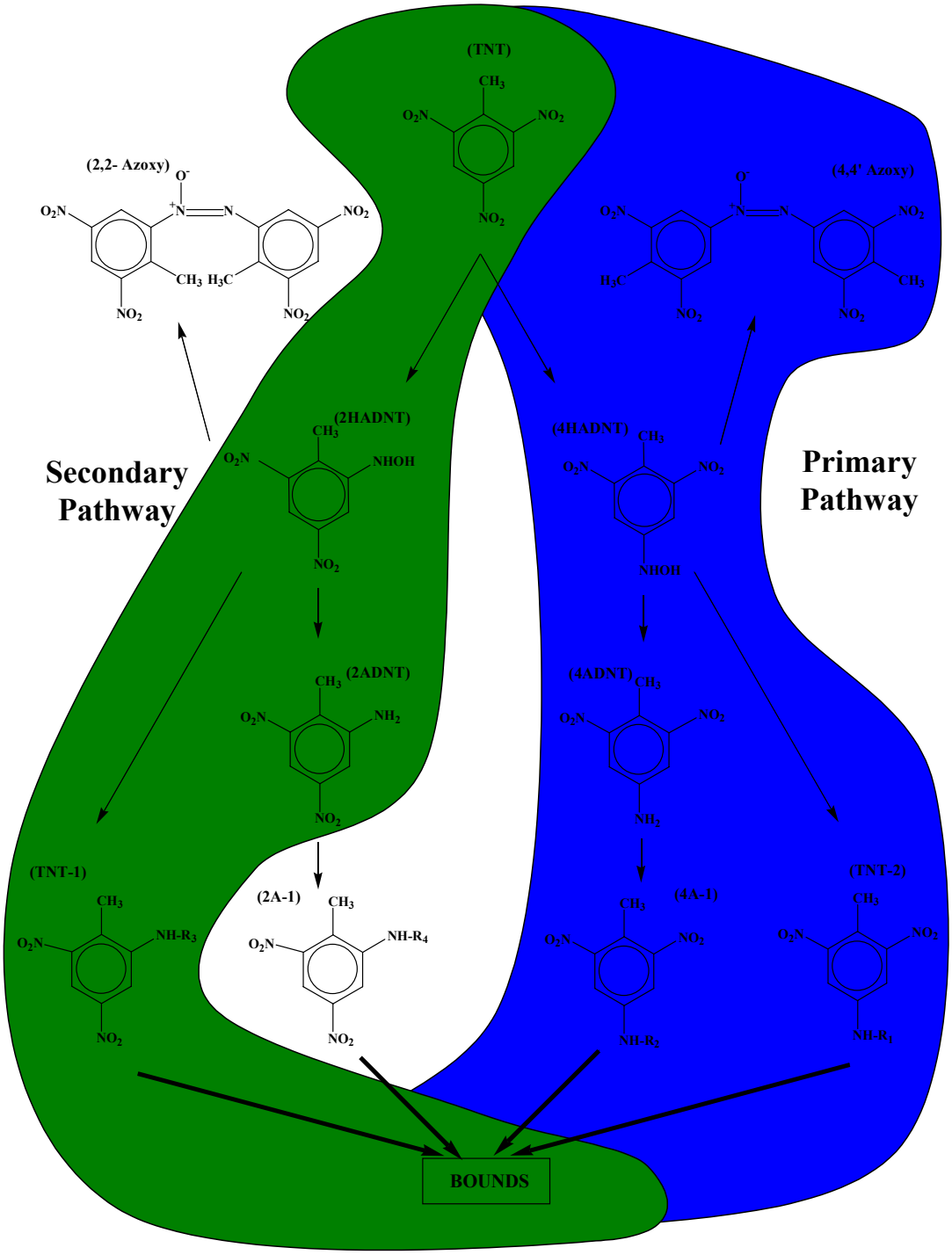
Based on TNT and metabolite feeding studies, a pathway for TNT transformation in Arabidopsis is shown to be as in Figure 6.12. This pathway shows the flow of flux from TNT to the final bounds. The blue shaded area labeled primary pathway shows the portion of the

pathway the Arabidopsis seedlings prefer during TNT transformation; the green shaded area labeled secondary pathway shows the portion of pathway of secondary importance, while the unshaded areas show the least emphasized sections of the pathway. These sections were determined by levels of metabolites observed in those sections, and the relative rates of formation and removal.

6.5: In Conclusion

Completing a TNT transformation mass balance and characterizing the pathway in Arabidopsis was accomplished with work presented in this chapter. Arabidopsis was seen to be a robust system capable of transforming TNT up to high concentrations, although the efficiency of removal was found to be a strong function of the growth phase. A radiolabeled mass balance on Arabidopsis revealed that the bulk of the label entered the bounds phase by the end of the experiment, at which point the compound is not readily bioavailable. The routinely identified metabolites, comprising of the "green liver model" were observed in the system; in addition hydroxylamines and the 2,2',6,6'-tetranitro-4,4'-azoxytoluene (4,4'-Azoxy) were unique metabolites isolated in an axenic phytoremediation system. The lower efficiency of the seedlings in transforming 2ADNT, when compared to 4ADNT, appears to be an innate characteristic of Arabidopsis seedlings. These observations on wild-type Arabidopsis will be used in Chapter 7, in the analysis of mutant Arabidopsis strains for beneficial TNT removal properties.

Figure 6.12: TNT Transformation Pathway in Arabidopsis: The blue shaded portion represents the portion of the pathway that the seedlings use primarily during TNT transformation. The green shaded section of the pathway cordons off metabolites of secondary importance, while the unshaded metabolites were of the least importance. Different species appear to favor unique patterns on the pathway, as shown in *C. roseus* and Arabidopsis studies.


(TNT)
(2,2- Azoxy)
(4,4' Azoxy)
Secondary Pathway
Primary Pathway
(2HADNT)
(4HADNT)
(2ADNT)
(4ADNT)
(TNT-1)
(2A-1)
(4A-1)
(TNT-2)
BOUNDS

Chapter 7: Metabolic Analyses of Arabidopsis Mutants in TNT Transformation

7.1: Introduction

The previous chapters of this dissertation described in some detail the metabolic pathway involved in TNT transformation, and identified some of the metabolites involved. Armed with this knowledge on the pathway, research is now focused on correlating this information with the genetics of TNT remediation. With the knowledge of genes involved in this process, comes information on the enzymes implicated too. Hence, a database on genes, enzymes and metabolites involved in TNT phytotransformation can be developed.

The power of Arabidopsis as a medium to study TNT transformation has been described previously. The completely sequenced genome, the ease of transformation, the ease of culturing, and the availability of gene identification and function information are key reasons for the popularity of this plant. In addition, mutant libraries of Arabidopsis can be generated, that can subsequently be screened to isolate individual mutants with enhanced TNT resistance. These mutants can be analyzed for their metabolic and genetic properties; the enzymes affected by the mutations can also be identified thereby shedding light on the role of that enzyme in the transformation of TNT.

This chapter describes metabolic pathway analyses performed on such isolated mutants. The screening studies and genetic studies described were performed by Dr. Hangsik Moon in Prof. David Oliver's lab in the Department of Genetics, Development and Cell Biology at ISU. These studies have been briefly summarized to present a complete picture of the project.

7.1.1: Basic Premise

Libraries of Arabidopsis mutants can be generated by infecting the plants with *Agrobacterium tumefaciens*. Mutants belonging to these libraries share one common characteristic- an under or over-expression of one or two genes. If the mutated genes play any role in the transformation of TNT, it would be expected to modify their efficiency of TNT transformation. By isolating such mutants from the library through screening studies, the mutated genes can be identified. In addition, metabolic analyses on the mutants could be

used to determine which specific step of the transformation pathway, if any, is affected by the mutation. Hence, knowledge of the specific gene(s) involved in TNT phytotransformation could be obtained. Putative Arabidopsis mutants, under or over-expressing this specific gene can then be obtained from the Arabidopsis genome project (The Arabidopsis Information Resource, TAIR, www.arabidopsis.org), to verify if the gene is indeed involved in TNT transformation. Follow-up metabolic studies can help document the precise advantages conferred to the plant by the mutation, and the concomitant practical advantages and limitations.

7.1.2: Mutant Libraries

Three types of libraries were considered for the screening of mutants- a T-DNA insertion library (T-DNA library), an enhancer trap library (ET library) and an ethylmethane sulfonate library (EMS library). The T-DNA library was generated by transforming the Arabidopsis genome with a Ti plasmid (T-DNA) from *Agrobacterium tumefaciens*. The T-DNA sequence inserts itself randomly into the genome, and inactivates any gene that it lodges within. A library of 300,000 lines of this library was generated using this methodology; the mutants in this library lack function of one or two genes. The T-DNA sequence can also be equipped with an enhancer sequence, and depending on the site of the T-DNA insertion, an increase or decrease in gene expression can result. The T-DNA sequence (with the enhancer) is again randomly inserted via the *Agrobacterium*, and if it lodges within a promoter sequence, the flanking gene is upregulated, whereas if it lodges within the gene itself, the gene function is lost (similar to the T-DNA library). An ET library with over 100,000 mutants has been constructed with this methodology; mutants belonging to this library can either under or over-express specific genes. The third kind of library, the EMS library was generated by mutagenizing Arabidopsis seedlings with EMS. Over 120,000 lines with point mutations comprise this library. Since the mutants in this library are affected by only point mutations, obtaining information on the genes affected by the mutants is challenging. In addition, the point mutations can cause either the activation or suppression of gene-expression; this further complicates efforts to understand the genetics of TNT transformation.

All the three above libraries contain several hundred thousand lines, each under or over-expressing a single or in some cases, two genes. This should, theoretically assure every possible gene disruption (Arabidopsis has only 30,000 genes; hence the number of lines per library are 3 to 4 times the number of genes). With the T-DNA and ET libraries, once a mutant of interest is isolated, backtracking information on the gene affected is simplified by the presence of the T-DNA tag. With the sequence of the T-DNA tag, a primer can be designed that reads the tag and the adjacent gene. One primer would read from the first sequence on the tag and the other would read the sequence at the end of the gene. Using this primer, cloning and amplification of the gene of interest is possible. By comparing the sequence of the thus cloned gene to the Arabidopsis genome database, information on the gene can be obtained. In addition, knock out mutants may be available that do not have the gene of interest. These mutants can be tested for lesser or greater resistance to TNT, which will validate their role in TNT transformation. As an additional means of analysis, transcription and proteomic studies on Arabidopsis seedlings exposed to low concentrations of TNT can help identify mRNA sequences and proteins activated by TNT.

7.1.3: Screening Studies

Isolating mutants from the vast library was performed by Dr. Hangsik Moon, from the Dept of Genetics, Development and Cell Biology at ISU under Prof. David Oliver. This was accomplished by exposing all mutants from a library to a concentration of TNT lethal to wild-type Arabidopsis. Only mutants that could survive the high TNT concentrations were collected and subjected to a second round of TNT exposure. While screening studies were performed on both the T-DNA and ET libraries, mutants from the latter were considered for further analysis since they possessed both under and over-expressing genes.

Approximately 300,000 mutants of the ET library were challenged with 25 mg/L (0.11 mM) of TNT in the growth medium, while being grown on Agar plates. 235 mutants showed enhanced resistance to TNT and were removed from plates and grown on the soil until they reached maturity. 200 seeds from each of these 234 mutants were subsequently used in a secondary screening experiment in which they were challenged with 25 mg/L (0.11 mM) of TNT. 24 lines from this experiment showed better germination than wild-type and were

potted in soil. The T-DNA used in generation of the mutants in the T-DNA and ET libraries encoded a sequence for a *BAR* gene, which conferred the transformed plant resistance to the herbicide Basta. Hence, all the potted mutants were sprayed with Basta; only seedlings with the T-DNA insert comprising the *BAR* gene would survive. Subsequent to the spraying of Basta, the surviving seedlings were used in the tertiary screening, where they were challenged again with 25 mg/L (0.11 mM) of TNT. Twelve lines outperformed the wild-types and they were chosen for further characterization. These lines were designated as ET20, ET25, ET30, ET32, ET40, ET148, ET169, ET183, ET212, ET218, ET226 and ET231.

7.2: Materials and Methods

7.2.1: Plant Materials

Wild-type and mutant Arabidopsis seedlings were grown axenically from seeds. 50 seeds of the plant were surface sterilized with 20% bleach for 15 minutes and subsequently rinsed three times with sterile water. They were next transferred to 50 ml of Arabidopsis growth media (4.2 mg/L of MS media salts, 20 g Sucrose, 1 ml Gamborg B-5 vitamins, MES buffer, Potassium phosphate in 1 L of nanopurified water at a pH of 5.7) in a 250 ml Erlenmeyer flask and shaken at 100 rpm under light. Seedlings 14 days old or 20 days old were used in the TNT and metabolite feeding experiments. These seedlings consisted of both roots and leaves.

7.2.2: Chemicals

Solid TNT for feeding experiments was purchased from ChemServices (West Chester, PA), while liquid HPLC standards of TNT, 2ADNT, 4ADNT, 2HADNT, 4HADNT and 4,4'-azoxy were purchased from AccuStandards (New Haven, CT). All solvents, including 2-propanol, methanol, ethanol, ethyl ether and acetonitrile were purchased from Fi-sher Scientific.

7.2.3: Analytical Methods

Reverse-phase HPLC was used for the separation and identification of metabolites from the phytotransformation studies. A Waters system with a 717 autosampler equipped with a PDA detector was used for these studies. Two types of columns were used, a NovaPak C8 and a NovaPak C18 column. A mobile phase of 82% water and 18% 2-propanol were used with the C8 column to isolate and identify TNT, 2ADNT, 4ADNT and the previously identified conjugates of TNT-1, TNT-2, 2A-1 and 4A-1 (Bhadra *et al.*, 1999).

7.2.4: Transformation Studies

A number of TNT transformation studies were performed on the mutants at varying initial TNT concentrations, with seedlings at different levels of maturity. The mutants were divided into two samples sets for experimental ease. In all these experiments two-week old seedlings were used except in one set of studies where 20-day old stationary roots were used. During the course of all experiments, extracellular media samples were taken at periodic intervals and analyzed for metabolite production. In addition, at the end of the experiment, plant samples were sacrificed and analyzed for intracellular metabolite production. Table 7.1 lists the various experiments performed, with the initial concentration of TNT used, and the mutants tested. In the final experiment, in order to obtain systems with uniform growth and germination, stationary-phase seedlings were used.

7.3: Results

7.3.1: Low and medium concentration TNT feeding experiments

Initial concentrations of 6 mg/L (0.03 mM) and 75 mg/L (0.33 mM) of TNT were added to the Arabidopsis mutants ET-30, ET-40, ET-148 and ET-231, and TNT uptake rates and metabolite formation rates monitored. Initial fresh weight biomass measurements were made for all seedlings and were found to be 12 ± 2g per flask. This ensured that biomass concentrations did not influence the rate of TNT removal. Figures 7.1A and B show the TNT concentrations for these mutants and wild-type seedlings for these concentrations. As seen in

Table 7.1: Experiment and Result Summary: Summary of experiments and the results obtained is presented. The mutants ET-148 and ET-40 appear to be the best in two experiments, while the mutants ET-218 and ET-226 appear to perform the best in another study.

Initial TNT, mg/L	Growth Phase	Arabidopsis Mutants Tested	Fresh Weight, g	Results
6 mg/L	Exponential	ET-30, ET- 40, ET-148, ET- 231	12 ± 2g	Equal Removal Rates
75 mg/L	Exponential	ET-30, ET-40, ET-148, ET-231	12 ± 2g	Equal Removal Rates
100 mg/L	Exponential	ET-25, ET-55, ET-183, ET-212, ET-218, ET-226	12 ± 2g	Equal Removal Rates
120 mg/L	Exponential	ET-30, ET-40, ET-148, ET-231	10 ± 1g	ET-148>ET-40>ET-231>ET-30>WT
140 mg/L	Exponential	ET-25, ET-55, ET-183, ET-212, ET-218, ET-226	12 ± 2g	ET-218>ET-226=ET-183>ET-212>ET-25=ET-55>WT
140 mg/L	Stationary	ET-25, ET-30, ET-32, ET-40, ET-55, ET-148	15 ± 2g	Equal Removal Rates
170 mg/L	Exponential	ET-30, ET- 40, ET-148, ET-231	13 ± 2g	ET-40>ET-148>ET-231=ET-30>WT

the figures, there does not appear to be a significant difference in TNT removal rates between the wild-type and mutants at these concentrations. In the case of the 6 mg/L system, all the TNT disappears within 4 to 8 hours after amendment, while in the 75 mg/L system, 8 to 12 hours are required for complete TNT removal. Figure 7.2A and B show the formation of monoamines in the 75 mg/L system; as observed from the figures, there appears to be no significant difference in the rates and amounts of 2ADNT and 4ADNT formation. The amounts of 4ADNT formed are higher than that of 2ADNT; about 10% of the initial TNT goes towards 4ADNT formation, while less than 4% is goes through 2ADNT. At both these concentrations, the seedlings did not show visible signs of stress and appeared green and healthy at the end of the experiment.

7.3.2: High Level TNT Feed Experiments

Initial concentrations of 100 mg/L (0.44 mM) of TNT were added to two-week old wild-type seedlings and the mutants ET-25, ET-55, ET-183, ET-212, ET-218 and ET-226. These were a different set of mutants from those tested in the previous experiment. A higher concentration of TNT was used to elicit differences in the performance of the wild-type and the mutant seedlings. Initial fresh weight biomass measurements were made for all seedlings and were found to be 12 ± 2g which ensured that biomass concentrations did not influence the rate of TNT removal. Figure 7.3 shows the extracellular TNT concentrations for the wild-types and the mutants. There does not appear to be a difference in the rate of TNT removal between wild-type and the mutants. Hence, even at a high concentration of 100 mg/L, the mutants do not appear to possess any specific advantages in TNT removal. All the seedlings, however, showed severe signs of stress due to the high levels of TNT; browning, collapsing of the biomass and loss of form were observed in all the mutants and the wild-type seedlings. Despite the severe phytotoxic effects, the seedlings removed all the TNT within 25 hours.

Previously tested mutants ET-30, ET-40, ET-148, ET-231 and wild-type seedlings were again challenged with 120 mg/L (0.53 mM) of TNT in a separate experiment. Two-week old axenic seedlings were used in this experiment; initial fresh weight measurements revealed 10 ± 1g of fresh (wet) biomass per system. Figure 7.4 shows the extracellular TNT concentrations in the wild-type and mutant seedlings; as observed from this figure, at this

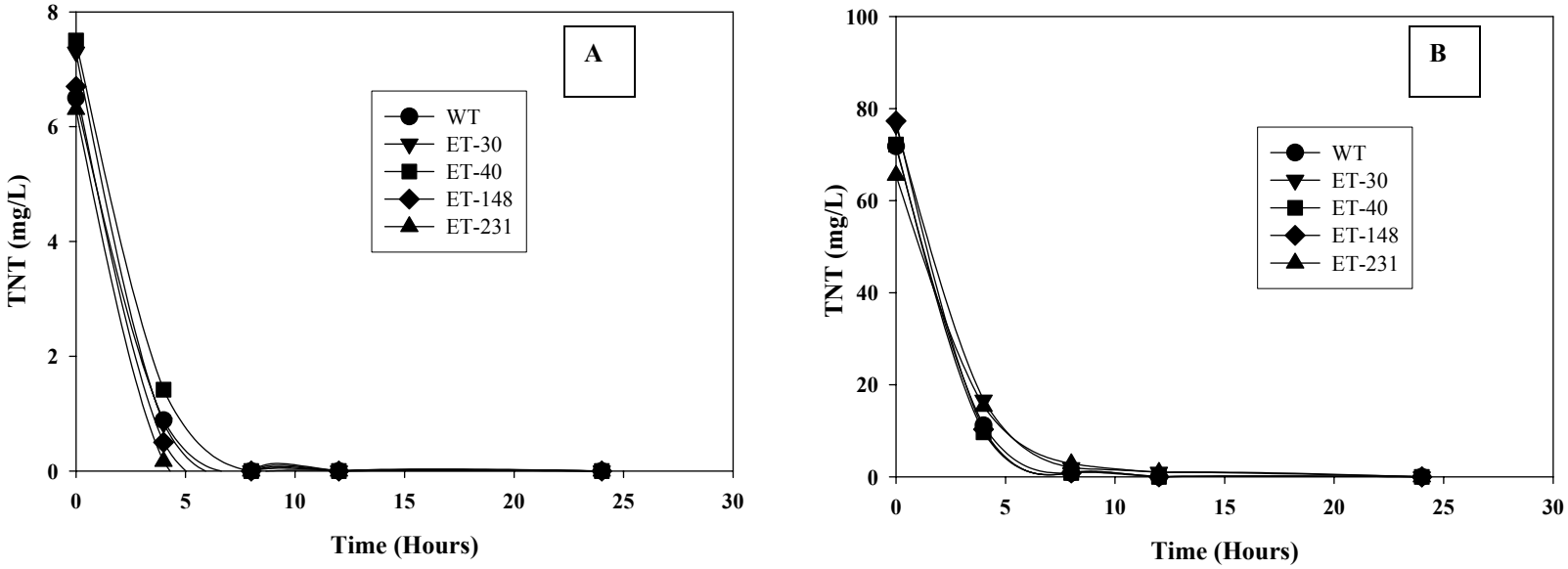


Figure 7.1: Mutant Study at 6 mg/L and 75 mg/L TNT: Extracellular TNT concentrations in wild-type and mutant Arabidopsis seedlings for an initial concentration of 6 mg/L (0.03 mM) (A) and 75 mg/L (0.33 mM) (B). At these concentrations, the mutants appear to have no advantage over the wild-types in TNT removal and health.

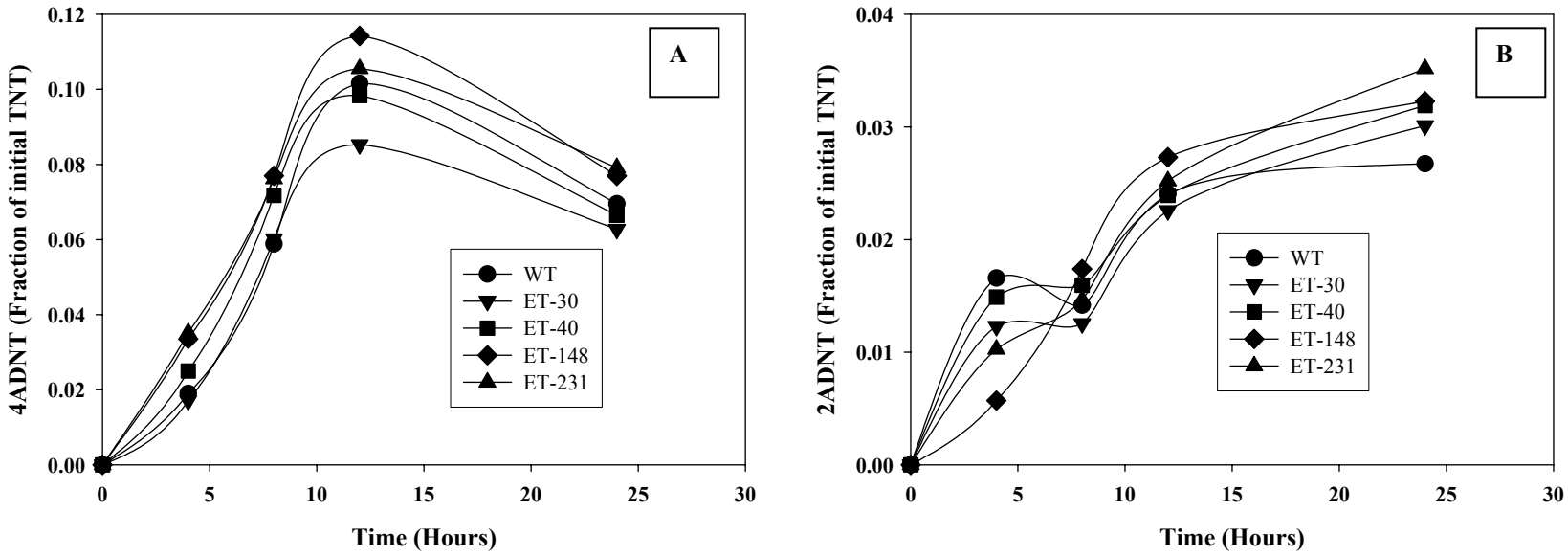


Figure 7.2: Monoamine Comparison at 6 mg/L and 75 mg/L: Extracellular 4ADNT (A) and 2ADNT (B) concentrations formed in the extracellular and wild-type and mutant Arabidopsis seedlings exposed to 75 mg/L (0.33 mM) of TNT. 4ADNT is formed about two times the level of 2ADNT; there does not appear to be a difference in the levels or trends of formation of the monoamines between the wild-type and mutants.

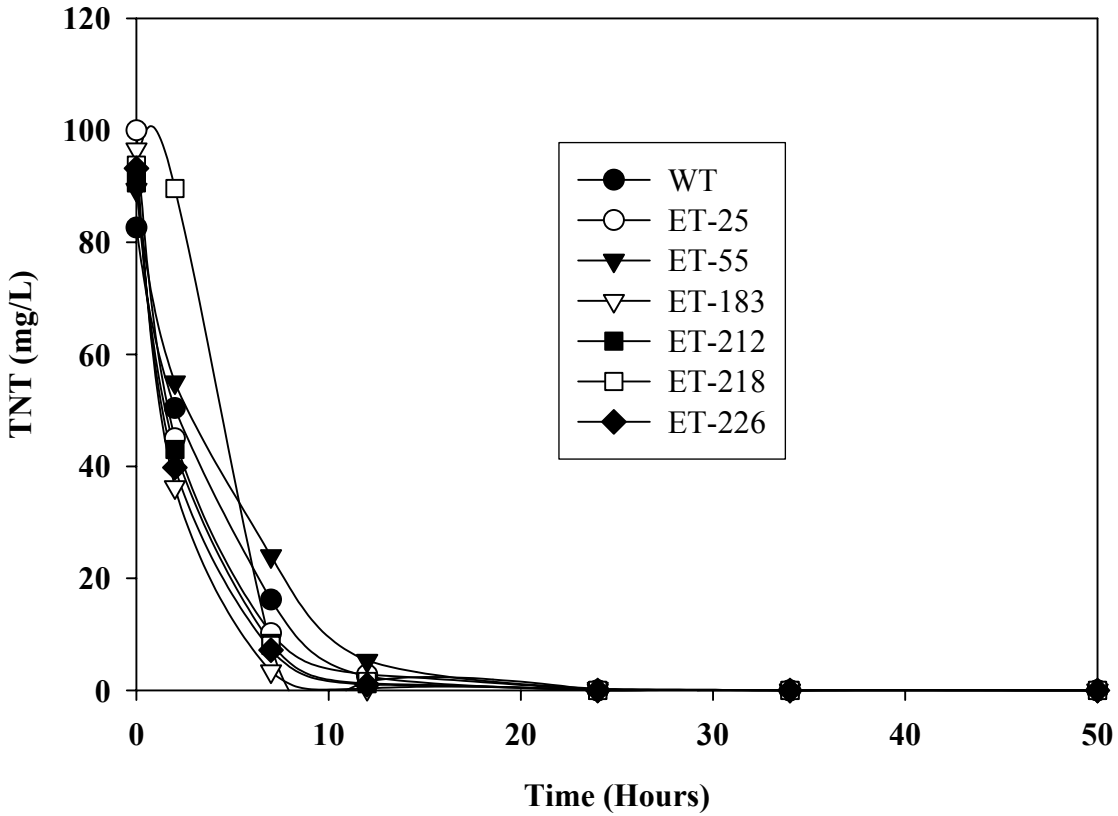


Figure 7.3: Mutant Study at 100 mg/L: Extracellular TNT concentrations in wild-type and mutant Arabidopsis seedlings, exposed to approximately 100 mg/L (0.44 mM) of TNT. No significant differences in TNT removal capacities is seen between the wild-type and mutant seedlings.

concentration, there is a difference in the uptake characteristics of the wild-type and mutants. While the wild-type seedlings take 120 hours to remove around 95% of the TNT, it takes the ET-148 mutant only 50 hours to do so. The mutant ET-40 takes around 70 hours to eliminate all TNT from the media, while the mutant ET-231 takes just less than 100 hours for the same. The mutant ET-30 performs marginally better than the wild-type with complete TNT removal within 120 hours. Figures 7.5A and B show the monoamine concentrations for the wild-types and mutants for this concentration of TNT. 4ADNT is formed in twice the amount as 2ADNT in all the systems; no significant differences were observed between the wild-type and the mutants.

7.3.3: Very High Level TNT Experiments

In order to probe the response of wild-type and mutant seedlings to very high levels of TNT, 140 mg/L (0.62 mM) of TNT was added to the mutants ET-25, ET-55, ET-183, ET-212, ET-218, ET-226 and wild-type seedlings. Figure 7.6 shows the extracellular TNT concentrations in the wild-type and the mutants. As seen in this figure, the mutants outperform the wild-type in TNT removal; the mutants ET-183, ET-218 and ET-226 remove all the TNT from the media in about 12 hours from this high an initial concentration, while mutants ET-25 took 25 hours for the same. The mutants ET-55 and ET-212 also showed better TNT removal capacities that the wild-type by removing all the TNT from the media within 35 hours. In contrast, it took the wild-type seedlings 50 hours to remove 80% of the TNT. Figures 7.7A and B show the extracellular monoamine concentrations for the wild-type and mutants; no notable differences between the two systems are observed. The health of the seedlings, both wild-type and mutants, were acutely affected by the high levels of the TNT. The seedlings browned, the leaves and shoots drooped, while the roots also showed brownish coloration. The wild-type seedling appeared to be dead by the end of the experiment, while the mutants were in comparatively better shape. The photos depicted in Figure 7.8 shows the comparison in biomass health between the wild-type and the various mutants. The wild-type biomass is completely submerged in the media, while the mutants appear to be standing. Hence, the mutants, although in bad health, appear to be more resistant to the TNT than the wild-type.

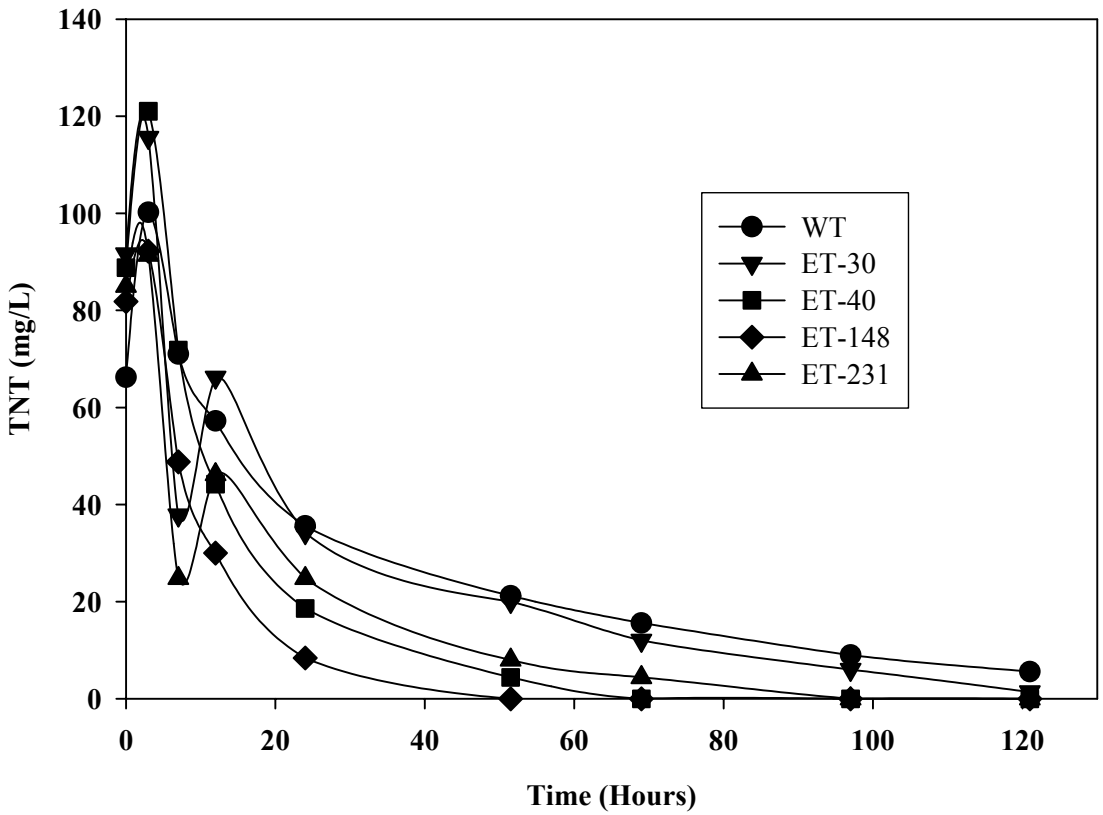


Figure 7.4: 120 mg/L Mutant Study: Extracellular TNT concentrations in wild-type and mutant Arabidopsis seedlings, exposed to approximately 120 mg/L (0.53 mM) of TNT. At this concentration, differences between the wild-type and mutants in the uptake of TNT are seen. While the mutant ET-148 removes all the TNT from the medium within 50 hours, it takes the wild-type 120 hours to remove 95% of the TNT. All the mutants perform better than the wild-type in TNT transformation.

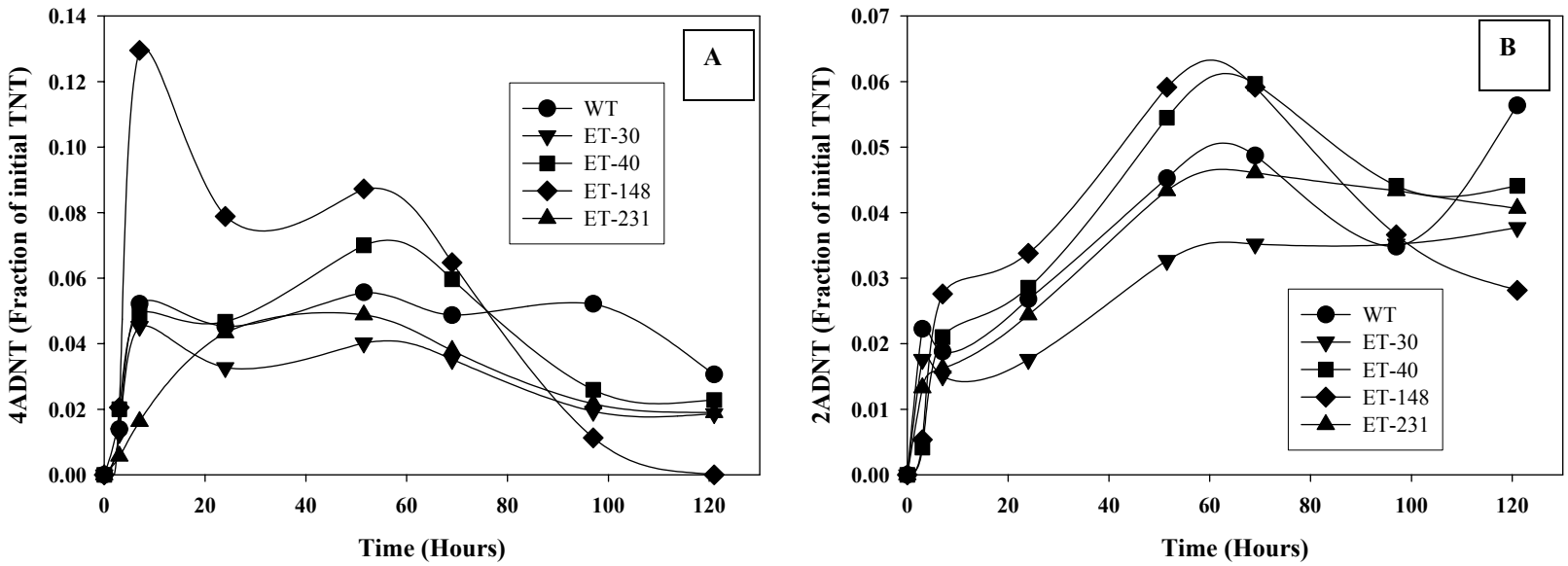


Figure 7.5: Monoamine Comparison at 120 mg/L TNT: Extracellular 4ADNT (A) and 2ADNT (B) concentrations formed in the extracellular and wild-type and mutant Arabidopsis seedlings exposed to 120 mg/L (0.53 mM) of TNT. 4ADNT is formed about two times the level of 2ADNT. The mutant ET-148 appears to convert more TNT to 4ADNT during the first 10 hours after amendment, as seen by a sharp increase in the 4ADNT concentration. Apart from this feature, no significant differences are seen between the wild-type seedlings and mutants.

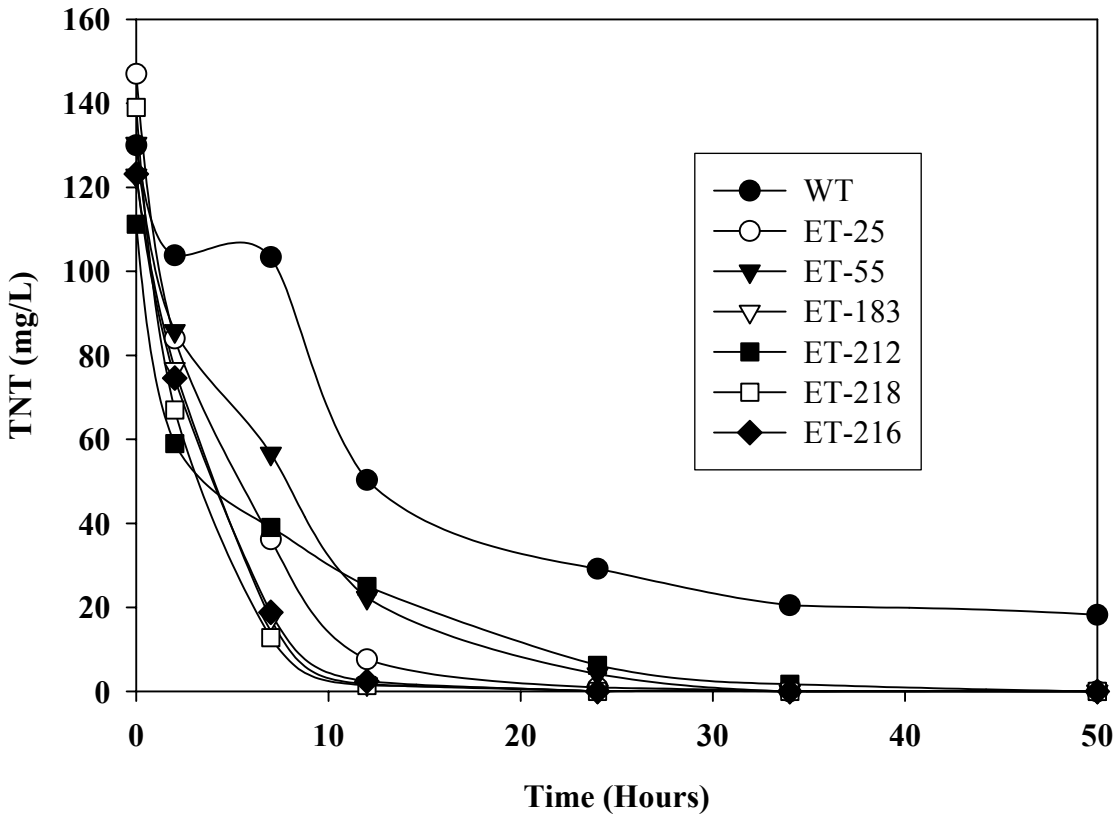
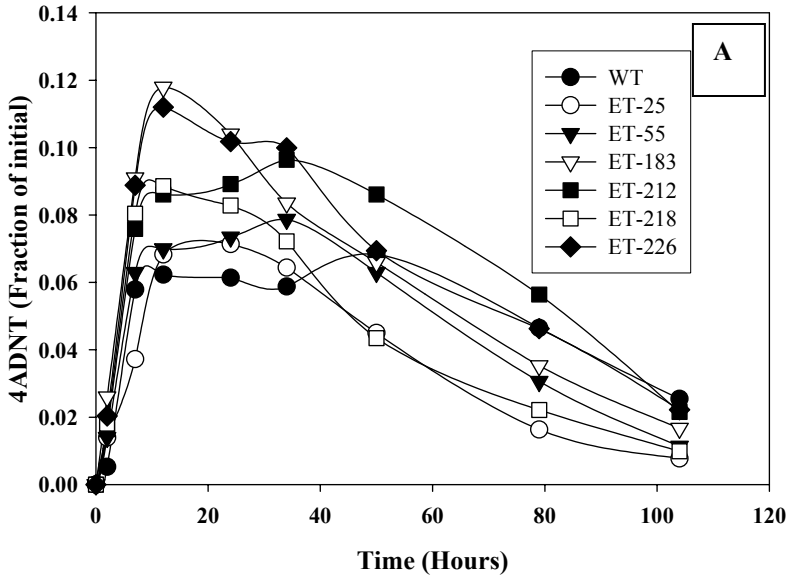


Figure 7.6: 140 mg/L Mutant Study: Extracellular TNT concentrations in wild-type and mutant Arabidopsis seedlings, exposed to approximately 140 mg/L (0.61 mM) of TNT. At this concentration, differences between the wild-type and mutants in the uptake of TNT are seen. The mutants ET-183, ET-218 and ET-226 perform the best, while the mutants ET-25, ET-55 and ET-212 also perform better than the wild-type.



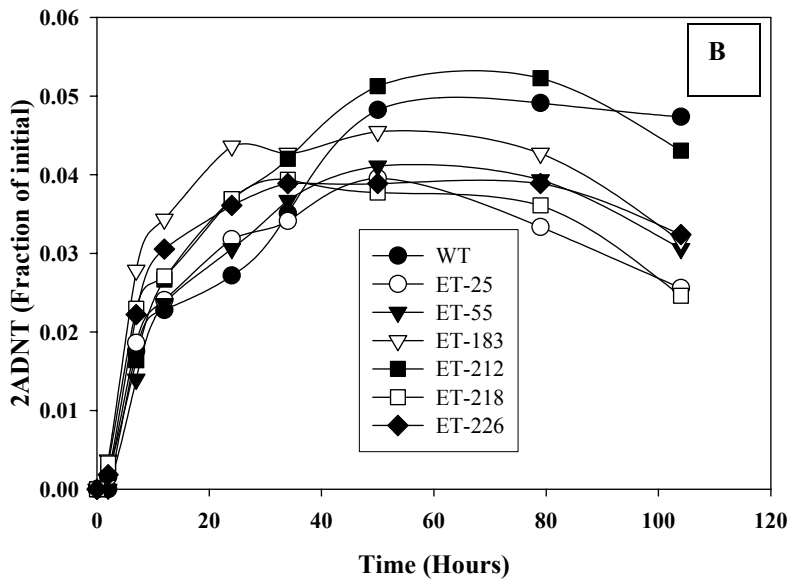


Figure 7.7: Monoamine Comparison at 140 mg/L TNT: Extracellular 4ADNT (7.7A) and 2ADNT (7.7B) concentrations formed in the extracellular and wild-type and mutant Arabidopsis seedlings exposed to approximately 140 mg/L (0.61 mM) of TNT. 4ADNT is formed in about two times the level of 2ADNT; there does not appear to be a difference in the levels or trends of formation of the monoamines between the wild-type and mutants.

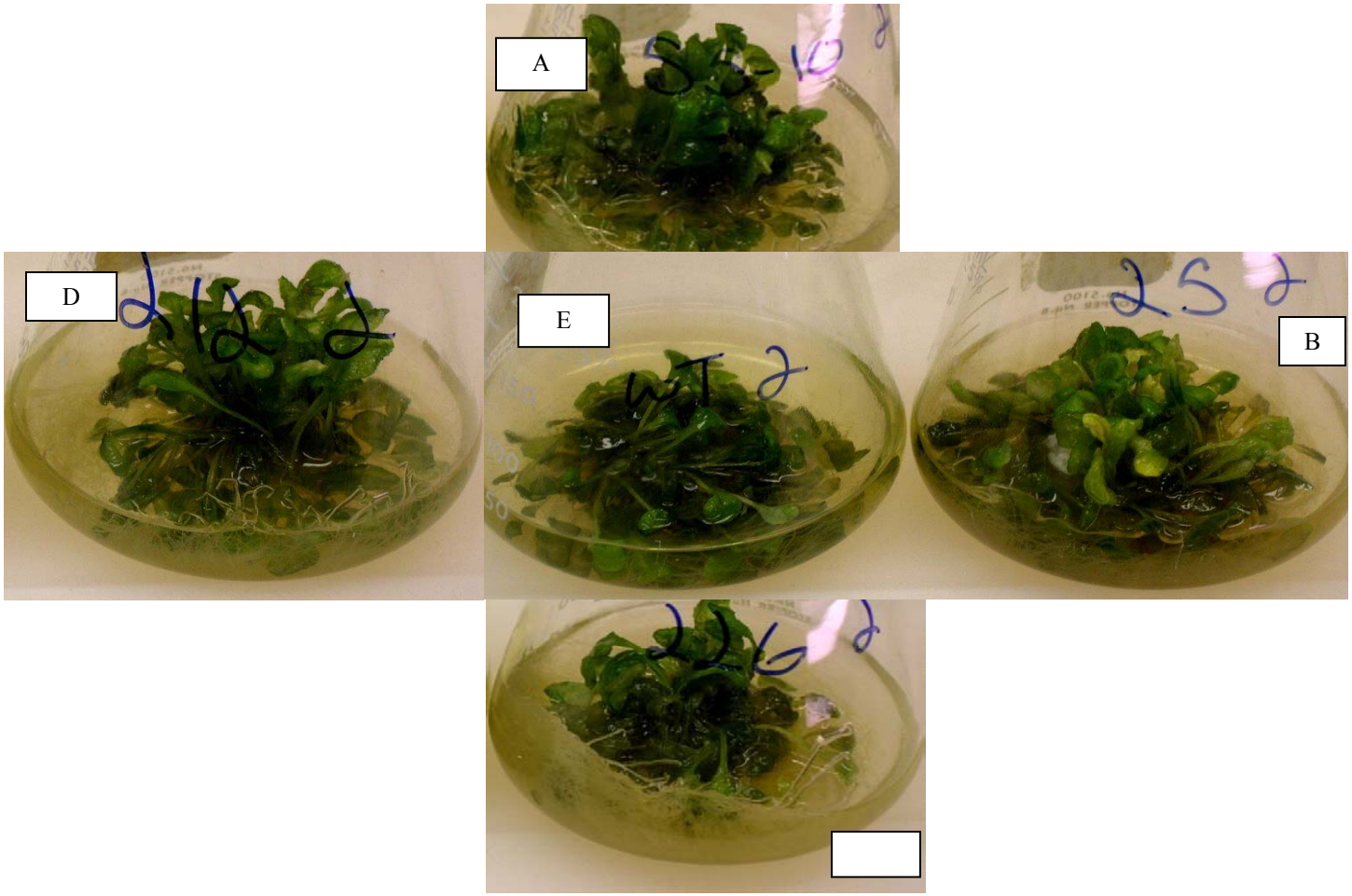


Figure 7.8: Mutant Health Comparison: Health of mutant seedlings ET-55 (A), ET-25 (B), ET-226 (C), ET-212 (D) and wild-type (E) 106 hours after the addition of 160 mg/L of TNT. The wild-type seedlings appear completely dead and submerged, while the mutant seedling show better health. The mutants ET-55 and ET-212 appear to be in best health.

A separate set of axenic two-week old mutants were exposed to an initial concentration of 170 mg/L (0.75 mM) of TNT. The mutants exposed to the concentration were ET-30, ET- 40, ET-148 and ET-231 along with wild-type seedlings. Figure 7.9 shows the extracellular TNT concentrations in all these mutants and the wild-type. As seen in this figure, the mutants ET-40 and ET-148 appear to perform better than the mutants ET-30 and ET-231 which in turn perform better than the wild-types. These four mutants appeared to remove 40 to 50% of the TNT from the media in 120 hours, in contrast to the wild-type seedlings which managed to only eliminate 20% of the TNT after 120 hours. However, there does not appear to be any difference in the levels and trends in the monoamines formed between the wild-type and mutants (Figures 7.10A and B).

A final set of experiments with 140 mg/L (0.61 mM) of TNT were performed on 20-day old axenic Arabidopsis seedlings in stationary phase. These stationary phase seedlings were used to minimize variability between samples due to non-uniformity in growth and germination. The mutants tested in this experiment were ET-25, ET-30, ET-32, ET-40, ET-55, ET-148; they were tested in duplicates or triplicates. Figure 7.11 shows the extracellular TNT profile in these mutants and the wild-type; as seen from this figure, there are no notable differences in the rate of TNT removal between the wild-type and the mutants. The high amount of biomass which was built up by day 20 by the Arabidopsis seedlings, seemed to have blunted the toxic effects of TNT and allowed its easy removal by all seedlings. Hence, no difference in the removal rate of the mutants and wild-type was noted.

7.4: Discussion

The various transformation experiments performed were designed to elicit differences between mutants and the wild-type in the uptake and transformation of TNT. These mutants had, in previous screening studies, demonstrated their superiority over wild-type seedlings in resisting 25 mg/L of TNT. However, the screening studies were performed with seeds being exposed to TNT; hence the superiority of the mutants was with regards to germination rather than growth. In addition, apart from a visual assessment of health, no metabolite measurements were performed. With the metabolism studies performed, as described in this chapter, with 14 and 20 day old seedlings, differences were expected in the rate of TNT

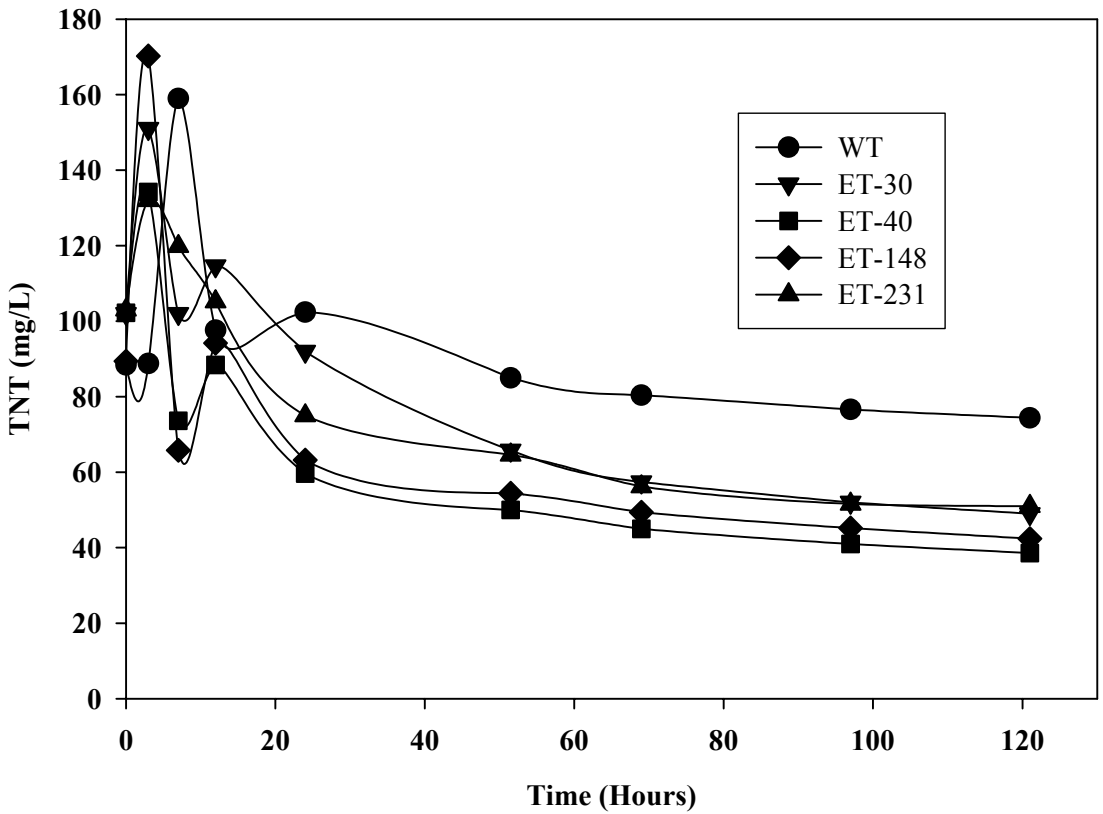
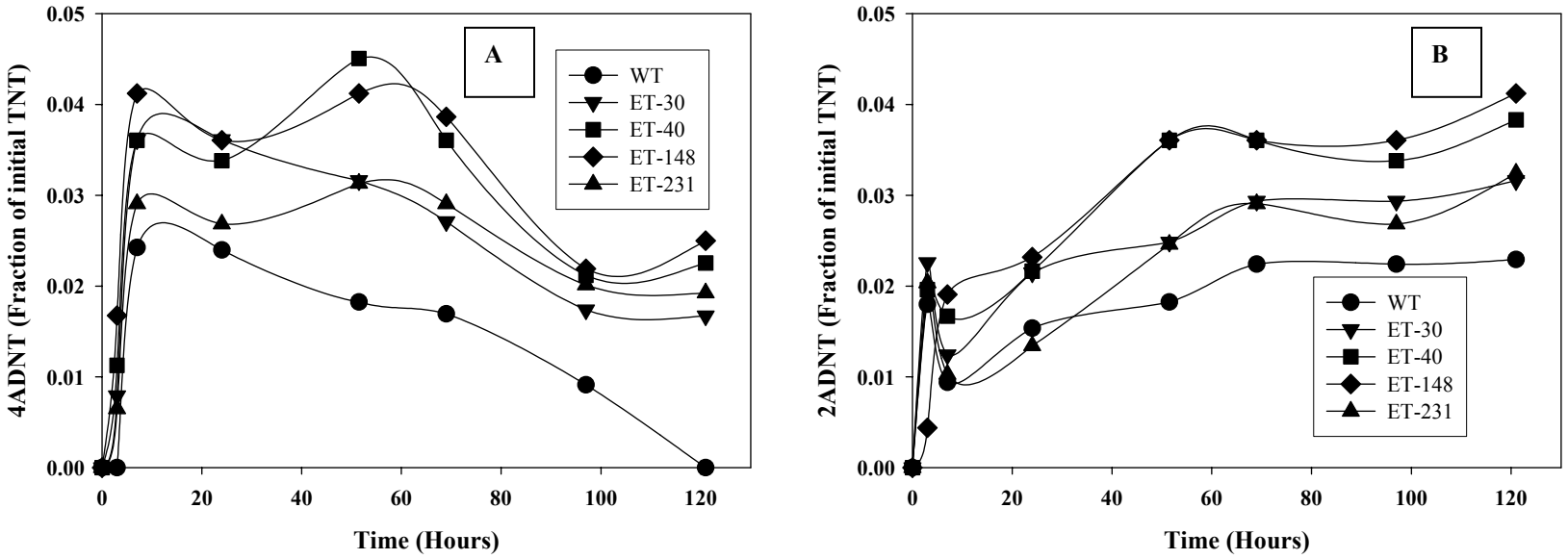


Figure 7.9: 170 mg/L Mutant Study: Extracellular TNT concentrations in wild-type and mutant Arabidopsis seedlings, exposed to approximately 170 mg/L (0.75 mM) of TNT. At this concentration, differences between the wild-type and mutants in the uptake of TNT are seen. The mutants ET-40 and ET-148 appear to perform better than the mutants ET-30 and ET-231, which in turn appear more efficient in TNT uptake than the wild-types. None of the mutants, however, appear capable of completely removing TNT from the system.



Figures 7.10A and B: Monoamine Comparison at 170 mg/L TNT: Extracellular 4ADNT (A) and 2ADNT (B) concentrations formed in the extracellular and wild-type and mutant Arabidopsis seedlings exposed to 170 mg/L (0.75 mM) of TNT. 4ADNT is formed about two times the level of 2ADNT; there does not appear to be a difference in the levels or trends of formation of the monoamines between the wild-type and mutants.

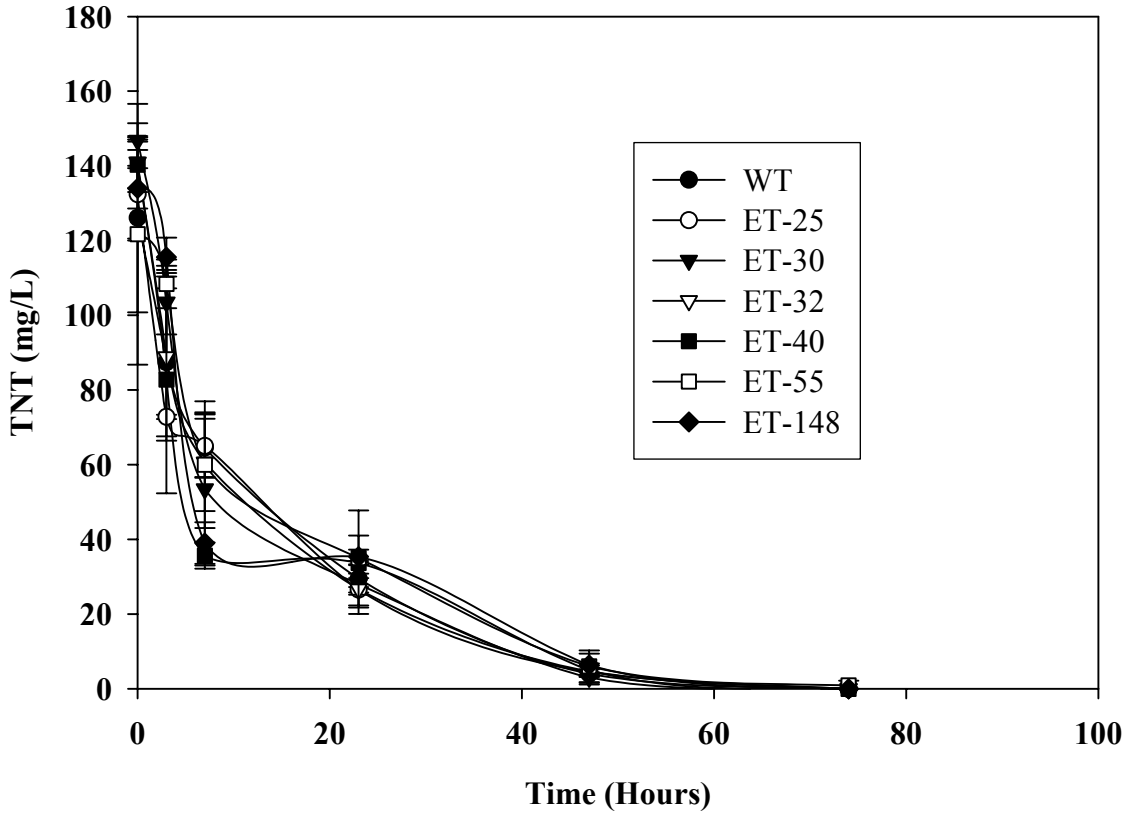


Figure 7.11: Mutant Study at 140 mg/L: Extracellular TNT concentrations in stationary phase, 20-day old axenic Arabidopsis wild-type and mutant seedlings, exposed to approximately 140 mg/L (0.61 mM) of TNT. The values for the wild-type, ET-25, ET-30 and ET-55 are averages and standard deviations of duplicates, while the values for the ET-32, ET-40 and ET-148 are averages and standard deviations of triplicates. The efficiency of TNT removal appears to be approximately the same for all mutants and the wild-type.

uptake and the metabolite profile. These results, in conjunction with genetic studies on the mutants, would help identify the mutations conferring the seedling with extra resistance to TNT. This would also lead to identification of the specific phenotypical differences between the mutants and the wild-type, with specific regards to TNT transformation.

7.4.1: TNT Transformation Experiments: TNT Solubility

The various TNT transformation experiments were performed at all levels of initial TNT, from 6 mg/L (0.03 mM) to 170 mg/L (0.75 mM). As higher concentrations were approached, however, saturation of TNT in the media was an issue. As observed in the high concentration TNT studies (Figures 7.4 and 7.9), there appears a spike in the TNT concentration at around 3 hours after TNT addition. This initial jump in TNT concentration is followed by a regular decrease in concentration. It has been suggested that the saturation of TNT occurs around 140 mg/L (0.61 mM) (Lynch *et al.*, 2001) in water at 25 °C; however this is apt to change depending on ionic salts and sugars present in the media. We found that up to 175 mg/L (0.77 mM) of TNT stayed in media solution at room temperature (25 °C), although, when left standing for days crystallization of TNT was observed. In our transformation experiments, it was observed that when high concentrations about 150 mg/L (0.66 mM) of TNT were added to seedlings, its solubility was suppressed. However, in about 3 hours, all the TNT appears to be back in solution, which leads to an increase in its concentration. When TNT dissolved in methanol is initially added to the media, it probably does not instantly enter into solution. A two-phased partition (water and methanol) could exist, which over a matter of hours with shaking at 100 rpm, is broken and all the TNT enters the aqueous phase. Hence, the TNT concentration, in the very high level TNT systems, appears to reach its peak in about 3 hours after TNT addition. It should be noted that all systems compared in a single plot were exposed to the same amount of TNT, although due to solubility issues with TNT, the concentrations are reflected differently. For example, in Figure 7.9, the apparent initial concentrations of TNT are different, probably due to the different trends in resolubilization of the TNT. However, since the wild-type and all the mutants were exposed to a uniform amount of TNT they are compared on a single plot. The actual initial TNT concentration is probably closest to the highest concentration seen in the

system. The absolute initial concentration cannot be accurately determined since in addition to the solubility effect of TNT, the biomass absorbs TNT in the initial hours, hence altering the concentration.

In contrast to the low and high concentration TNT studies, the very high level exposure studies revealed differences between wild-types and mutants. Up to concentrations of 100 mg/L (0.44 mM), the wild-type and mutants removed TNT at the same rapid rate from the media. At the concentration of 120 mg/L (0.53 mM) and above, a difference in the rate of TNT removal was observed. The only exception to this was to a single study at 140 mg/L (0.61 mM) with stationary phased 20-day old roots. Uniform TNT removal was observed between all the mutants in this study, which was probably due to the very high biomass built up by all the seedlings. When a sufficiently high biomass concentration is reached, TNT removal efficiency is very high; hence, even very high levels of TNT do not slow down the metabolism of the seedlings. Therefore, while a difference in TNT removal was expected at 140 mg/L, the detrimental phytotoxic effect of TNT was offset by the biomass of the seedlings. Table 7.1 summarizes the performance of the various mutants in eliminating TNT from the system; as seen the mutants ET-148 and ET-40 outperform wild-types in two studies, while the mutants ET-183, ET-218 and ET-226 outperform wild-types in another study. In addition, the mutants ET-30 and ET-231 do slightly better than the wild-types in two studies, while the mutants ET-212 and ET-25 do slightly better than wild-types in one study. Hence, the most promising mutants to emerge from these studies were ET-40, ET-148, ET-183, ET-218 and ET-226.

7.4.2: Monoamine and Conjugate Trends

Along with determination of relative efficiency in TNT removal, trying to understand a specific manifestation of the mutation was also attempted by measuring the concentration of the various metabolites, such as hydroxylamines, monoamines and conjugates. This approach was similar to that adopted in Chapter 5, where attempts were made to understand the effect of the bacterial nitroreductase in transgenic tobacco. Only low concentrations of hydroxylamines were detected in the very high level TNT studies, in both mutants and wild-types (data not shown). The hydroxylamine-derived conjugates of TNT-1 and TNT-2 were

also detected in low levels at the early stages of the experiment (data not shown). However, a few more trends were observed with the monoamine levels and conjugate formations. In all the studies, 4ADNT was formed in twice the amount 2ADNT was produced; this was a trend uniform to both wild-types and mutants (Figures 7.2, 7.5, 7.7, 7.10). Another noticeable trend amongst all systems was the declining nature of 4ADNT concentrations at the end of the experiment (120 hours) in contrast to the 2ADNT concentrations, which showed a flatter profile. This seems to indicate that 4ADNT is being transported into the cell and conjugated at a faster rate than 2ADNT. The 2ADNT-derived conjugate 2A-1 was observed at lower levels than the 4ADNT-derived conjugate of 4A-1 (data not shown). Tables 7.2 and 7.3 show the list of conjugates detected in the extracellular medium in all the studies. All these findings are in conformity to the results presented in the previous chapter (Section 6.3.3), wherein it was found the Arabidopsis seedling appear to favor 4ADNT over 2ADNT, and additionally favor the formation of the 4ADNT derived conjugate 4A-1.

It was observed, from Figure 7.10 that the mutants producing the highest amount of monoamines also removed TNT from the media the earliest. This trend was found to be consistent amongst all mutants. This indicated that the TNT was not only being taken off the media by the mutants, but they were also being transformed into the monoamines. In Figure 7.5A, the best performing mutant ET-148 shows an early peak of 4ADNT, which is much higher than the wild-type and other mutants. However, subsequent to that initial peak, 4ADNT concentrations are similar to the other mutants and the wild-type. In addition, in ET-148, the 4ADNT concentration reaches zero by hour 120, whereas none of the other systems show that behavior. This seems to indicate that 4ADNT has been completely transported to the intracellular fraction, and has probably been conjugated. The presence of 4ADNT in the other systems seems to indicate that its active transformation is still occurring. In Figure 7.5B, in the 2ADNT transient profile, ET-148 shows a sharp declining trend in 2ADNT levels at 120 hours, in contrast to all other systems. An examination of conjugate formation in this system reveals the early formation of the conjugate 2A-1, when compared to the other wild-types and mutants (data not shown). Since 2A-1 is formed from 2ADNT, its early detection implies significant progress along the TNT transformation pathway (see pathway

Table 7.2: Conjugate Formation, Set 1: List of extracellular conjugates identified from the mutant metabolic analysis studies. There appear to be no stark differences in the conjugate formation characteristics between the mutants the wild-type seedlings.

2-substituted Conjugates (A)

Initial TNT	ET-30	ET-40	ET-183	ET-212	ET-218	ET-225	WT
100 mg/L	TNT-1	TNT-1	TNT-1	TNT-1	TNT-1	TNT-1	TNT-1
140 mg/L	TNT-1	TNT-1	TNT-1 2A-1	TNT-1	TNT-1 2A-1	TNT-1	TNT-1

4-substituted Conjugates (B)

Initial TNT	ET-30	ET-40	ET-183	ET-212	ET-218	ET-225	WT
100 mg/L	TNT-2 4A-1	TNT-2 4A-1	TNT-2 4A-1	TNT-2 4A-1	TNT-2 4A-1	TNT-2 4A-1	TNT-2 4A-1
140 mg/L	TNT-2 4A-1	TNT-2 4A-1	TNT-2 4A-1	TNT-2 4A-1	TNT-2 4A-1	TNT-2 4A-1	TNT-2 4A-1

Table 7.3: Conjugate Formation, Set 2: List of extracellular conjugates identified from the mutant metabolic analysis studies. There appear to be no stark differences in the conjugate formation characteristics between the mutants the wild-type seedlings.

2-substituted Conjugates (A)

Initial TNT	ET-25	ET-55	ET-148	ET-231	WT
7 mg/L	None	None	None	None	None
75 mg/L	TNT-1 2A-1	TNT-1	TNT-1 2A-1	TNT-1	TNT-1
120 mg/L	TNT-1 2A-1	TNT-1 2A-1	TNT-1 2A-1	TNT-1 2A-1	TNT-1 2A-1
170 mg/L	TNT-1	TNT-1	TNT-1	TNT-1	TNT-1

4-substituted Conjugates (B)

Initial TNT	ET-30	ET-40	ET-148	ET-231	WT
7 mg/L	TNT-2 4A-1	TNT-2 4A-1	TNT-2 4A-1	TNT-2 4A-1	TNT-2 4A-1
75 mg/L	TNT-2 4A-1	TNT-2 4A-1	TNT-2 4A-1	TNT-2 4A-1	TNT-2 4A-1
120 mg/L	TNT-2 4A-1	TNT-2 4A-1	TNT-2 4A-1	TNT-2 4A-1	TNT-2 4A-1
170 mg/L	TNT-2 4A-1	TNT-2 4A-1	TNT-2 4A-1	TNT-2 4A-1	TNT-2 4A-1

Figure 2.7), and is further evidence for the enhanced metabolism in ET-148. Hence, ET-148 not only removes TNT the fastest from the system, it also accelerates the entire pathway.

7.4.3: Genetic Studies Summary

Along with the metabolic studies, genetic characterization of the mutants was also performed; these studies were conducted by Dr. Hangsik Moon, from the Dept of Genetics, Development and Cell Biology at ISU under Prof. David Oliver. Table 7.4 summarizes the location of the T-DNA inserts in the various mutants, while Table 7.5 correlates this genetic information to function. The genes upregulated or downregulated by the T-DNA inserts are identified by their loci. In some cases, more than one insertion was located in the genome, and in one mutant, three insertion events were identified. Once the locus of a certain gene is obtained by tracking the T-DNA insertion in the Arabidopsis genome, this information can be used to determine if this gene has been characterized and if gene function information is available. This data is available from The Arabidopsis Information Resource (TAIR) (www.arabidopsis.org). As seen in Table 7.5, a number of the gene functions are unknown, while a significant other number of genes code for transcription factors. Some genes appear to code for DNA replication and recombination while others are involved in routine metabolism tasks like GTP binding. The mutant ET-218, one of the strong performing mutants, has a gene mutation on the gene At4g28720. This gene affects the activities of monooxygenase, oxidoreductase and other enzymes; these enzymes can potentially be involved in TNT transformation. A recent paper shows, through SAGE transcription studies, the involvement of reductases and cytochrome P450 monooxygenases in TNT transformation (Ekman *et al.*, 2003). Section 2.10 analyzes in some details the findings of this paper and other related genetic studies in TNT phytotransformation. A number of the identified genes from our study encode transcription factors; these proteins control the expression levels of large number of enzymes. Hence mutants in which these factors have been mutated, a wide number of genes may be affected. Further information on the specific genes controlled by each transcription factor will be required to understand the effect of the mutations. While no genes explicitly involved in the TNT biochemical pathway were identified, a number of other genes possibly involved in plant defense have been identified. This appears to be in

Table 7.4: Genes Identified in ET-Mutants: Genes flanked by T-DNA inserts in the enhancer trap mutants isolated from the screening studies. Table was kindly provided by Dr. Hangsik Moon. The insertion site entry also serves as a name of the gene.

Mutant lines	Number of insertions revealed by TAIL-PCR	Insertion sites
ET25	1	Between At4g24750 and At4g24760
ET30	2	Between At4g35940 and At4g35950 Between At4g12890 and At4g12900
ET32	2	Between At5g28590 and At5g28610 Between At4g35940 and At4g35950
ET40	1	In the 3rd exon of a pseudo gene, At5g38192
ET148	1	Between At3g61630 and At3g61640
ET183	1	Between At4g24880 and At4g24890
ET212	3	Between At1g09940 and At1g09950 In the 4th exon of At1g49560 Between At1g25330 and At1g25340
ET218	2	Between At4g28720 and At4g28730 In the 2nd intron of At5g41780
ET226	1	Between At3g04570 and At3g04580
ET231	2	In the 3rd intron of At5g53740 Between At3g60930 and At3g60940

Table 7.5: Gene Functions: Summary of genes upstream or downstream of the T-DNA insertions in the enhancer trap mutants, with the function of the gene, as determined from The Arabidopsis Information Resource website (www.arabidopsis.org). Transcription factors appear to be the most widely affected genes.

Gene Name/ Locus	Mutant	Function
At4g24750	ET-25	Unknown
At4g24760	ET-25	Unknown
At4g35940	ET-30	Unknown
At4g35950	ET-30	small GTPase mediated signal transduction, GTP binding
At4g12890	ET-30	Unknown, catalytic activity
At4g12900	ET-30	proteolysis and peptidolysis, carboxypeptidase A activity
At5g28590	ET-32	Unknown
At5g28610	ET-32	Unknown
At4g35940	ET-32	Unknown
At4g35950	ET-32	small GTPase mediated signal transduction, GTP binding
At5g38192	ET-40	DNA recombination, RNA-dependent DNA replication
At3g61630	ET-148	regulation of transcription, DNA-dependent, transcription factor activity, DNA binding
At3g61640	ET-148	Unknown
At4g24880	ET-183	Unknown
At4g24890	ET-183	protein serine/threonine phosphatase activity
At1g09940	ET-212	porphyrin biosynthesis, glutamyl-tRNA reductase activity
At1g09950	ET-212	Unknown
At1g49560	ET-212	regulation of transcription, DNA binding
At1g25330	ET-212	regulation of transcription, DNA binding
At1g25340	ET-212	regulation of transcription, DNA binding
At4g28720	ET-218	electron transport, removal of superoxide radicals, dimethylaniline monooxygenase (N-oxide-forming) activity, disulfide oxidoreductase activity, ferredoxin hydrogenase activity, monooxygenase activity, oxidoreductase activity, thioredoxin-disulfide reductase activity
At4g28730	ET-218	electron transporter activity, thiol-disulfide exchange intermediate activity
At5g41780	ET-218	copper ion transport, ATP binding, copper-exporting ATPase activity
At3g04570	ET-226	regulation of transcription, DNA-dependent, DNA binding
At3g04580	ET-226	signal transduction, regulation of transcription,
At5g53740	ET-231	Unknown
At3g60930	ET-231	phenylalanyl-tRNA aminoacylation, ATP binding, phenylalanine-tRNA ligase activity
At3g60940	ET-231	Unknown

conformity with the metabolism studies, where an overt difference in rates of TNT removal was not observed; rather a greater resistance to the toxic effects of TNT at high concentrations was observed by the mutants.

7.4.4: Toxicity Resistance of Mutants

The difference in TNT removal capacity between the wild-type and the mutants at only very high TNT concentrations coupled with the fact that the mutant plants appeared healthier than the wild-type plants at these levels is an interesting observation. This seems to suggest that the mutants do not possess any specific advantages in transforming TNT; for example, they do not possess upregulated genes such as nitroreductases that are directly involved in TNT reduction. Instead, the mutants appear to be more resistant to the phytotoxic effects of TNT, which explains their better health. Since they are less affected by TNT than the wild-types, this manifests in a better rate of TNT transformation. At low and high concentrations, the wild-type seedlings were not significantly affected by the toxic effects of TNT; hence mutants and wild-types removed TNT with equal efficiency. At very high concentrations, while the wild-type seedling appeared dead, the mutant seedlings appeared to be in slightly better shape (Figure 7.8); hence the mutants transformed TNT more efficiently than the wild-types. In addition, the genetic studies did not reveal any specific enzymes such as nitroreductases or cytochrome P450s which could be involved in TNT transformation. Genes regulating general transcription factors and metabolism controllers were seen to be affected in the mutants. Only, the mutant ET-218 appears to have mutated genes involved in direct TNT transformation. This situation, wherein the greater resistance to TNT by the mutants is not due to a specific over-expression of an enzyme directly involved in TNT transformation, is more difficult to analyze. Drawing out the precise nature of the mutation, and the phenotypical difference between the mutant and the wild-types upon exposure to TNT will require significantly more studies, beginning with plant physiology studies, and probably metabolomics of the system. The next chapter (Chapter 8, section 8.3.3) goes through in greater details the kind of experiments that can be performed to elucidate the specific advantage the mutations have conferred on the Arabidopsis seedlings.

7.5: Conclusions

Arabidopsis mutants have been tested with TNT and those resistant to TNT have been identified. These include the ET mutants ET-40, ET-148, ET-183, ET-218, ET-226, and ET-231. It should be emphasized, however, that these studies are subject to further refinement since attaining uniformity of biomass samples has been a challenge thus far. Hence, variations in germination rates between various systems could bias the TNT uptake rates. Improved experiments devised to attain uniformity between independent samples are needed to corroborate these results. Such an experiment is described in Chapter 8 (section 8.3.3.1), and is currently being attempted. The mutants which demonstrated a greater TNT removal efficiency did so only at very high concentrations of 140 mg/L (0.61 mM) and higher. This seems to indicate their resistance to TNT is due to mutations that protect them from TNT, but does not confer any specific advantages in removal rates. Hence, these mutants would be useful in situations requiring the cleanup of high levels of TNT wastes, in army lands and waters, where these mutants would be able to resist toxic effects of TNT better than the wild-types. Further experiments, including monitoring the health of the plant by measuring respiration rates, will help confirm if indeed these mutants are more resistant to the toxic effects of TNT. Genetic studies appear to corroborate these findings, since none of the genes identified appear to directly impact TNT metabolism, but rather are involved in the general housekeeping of the plant. The regulation of these genes probably provided the mutants with better health when exposed to toxic levels of TNT.

Chapter 8, section 8.3.3 lays out in greater detail the future proposed experiments in uniform analysis of these mutants, and isolating more mutants from screening studies. Experiments to measure toxic responses of the seedlings are also planned. Studies to further identify the specific role these enzymes and transcription factors play are also required. When the functionality of these genes is discovered in the future, it will help in better understanding of TNT transformation.

Chapter 8: Conclusions and Future Directions

Phytoremediation of pollutants has evolved from a concept to a practice over the last 10 years. With advantages such as low cost and ecological sustainability, this technology has the potential for widespread industrial application. Many factors, however, hold back the progress of phytoremediation, key amongst which are the toxicity of the pollutant, and the slow rate of pollutant removal. Both these issues are unique to each pollutant and circumstance, but can be understood better by studying the underlying processes governing cleanup. In the case of TNT, this would lie in understanding the transformation pathway followed by plants. While, a basic knowledge of metabolites and pathway were known, some key variables and metabolites were missing from the knowledge-base.

8.1: Inferences

A basic knowledge of the nature of hydroxylamines in the transformation pathway has been developed in this dissertation; this has been demonstrated in two axenic systems. While hydroxylamines were hypothesized to be present in TNT phytotransformation studies, their reliable identification and quantification has posed challenges. With the new analytical methods and optimized derivatization schemes developed, their detection is more likely. Hydroxylamines are probably the first metabolites formed in TNT transformation, and their concentration varies widely depending on the plant and the concentration of TNT. Efficient sample handling is necessary for the identification of these unstable metabolites. The role of hydroxylamines in the pathway- specifically in the formation of conjugates was also delineated. This suggests alternative schemes for TNT transformation which would be more efficient that the wild-type metabolism. This is precisely the kind of suggestions expected upon the confirmation of the presence of hydroxylamines in the pathway. However, implementation of these schemes would require much more knowledge on the enzymes and genes involved in TNT transformation.

This leads directly to the second phase of the project, that of identifying the genes and thereby the enzymes, involved in TNT phytotransformation. Arabidopsis was chosen as the system of interest, given its ease of axenic culturing in the lab, the completely sequenced and readily accessible genome, the correlation of genes to gene function for many of its genes,

and the ready availability of putative mutants. The TNT transformation pathway in wild-type Arabidopsis was characterized and there was found to be an affinity for the 4ADNT over 2ADNT. ^{14}C-TNT mass balances were also performed on Arabidopsis seedlings, to determine the contribution of various sections of the pathway towards TNT removal. It was demonstrated that by the end of the experiment, most of the TNT was in a bound form, immobilized in the biomass. Libraries of Arabidopsis mutants were screened and resistant lines isolated and analyzed. Metabolic and genetic analysis of mutants revealed the strongest mutants did not appear to possess any specific TNT transformation boosting enzymes; rather the mutant seedlings appeared to be more resistant to the toxic effects of TNT. A number of genes involved in TNT toxicity resistance have been identified from this project; however, determining the function of these genes is still a subject of future research.

In addition to Arabidopsis mutants, two transgenic tobacco strains were also characterized for their metabolite production. These two strains have different bacterial nitroreductases inserted in them that conferred them with enhanced TNT removal capacities. It was found that one of the strains, the NR 3-2, formed large amounts of 4HADNT and the corresponding conjugate of TNT-2. This was subsequently shown to be due to the inserted nitroreductase. The other transgenic, PETN, did not show such a remarkable split in metabolite profiles, but did show low levels of both hydroxylamine formations. It was inferred, from these studies, that the single most influential mutation with regards to TNT cleanup, is probably the one governing TNT to HADNT. Once the hydroxylamine is formed, it rapidly disappears from the system, thus moving the pathway forward and rapidly rendering the contaminant less bioavailable.

8.2: Overall Conclusions

The importance of the TNT transformation pathway in improving phytoremediation efficiencies has been demonstrated. Its analysis and understanding can lead to the development of transgenic or native systems better equipped to handle remediate TNT. It also is involved in elucidating the toxic response of TNT to plants, and will be able to suggest ways in which these effects can be minimized. It can suggest schemes for more efficient removal as well as resistance to higher concentrations of TNT than would have normally been

possible. In Chapters 3 and 4, whence the role of hydroxylamines was clarified, the obvious rate limiting steps of in the TNT transformation pathway, as currently understood, were also made clear. It was found that TNT to hydroxylamine and conjugation of monoamines are steps that slow down the process of TNT phytotransformation. These two chapters laid the foundation for the analysis of genetically modified plant systems as described in Chapters 5 and 7. In Chapter 5, where transgenic analysis of tobacco was analyzed, the value of a single step over-regulation was experimentally observed. The NR 3-2 seedlings produced large amounts of 4HADNT which disappeared rapidly from the system. In addition to transforming TNT rapidly, these seedlings also had the best health amongst all the tobacco systems tested, thereby correlating a faster rate of TNT removal with superior resistance to TNT. In Chapter 7, wherein Arabidopsis mutants were tested, a reverse effect was noted. Better health enjoyed by the mutants empowered them to transform TNT more efficiently than wild-type seedlings. Hence, the toxicity response appeared independent of the TNT pathway, as we currently know it. However, it is possible that currently undiscovered sections of the pathway could be playing a role in the toxicity response.

8.3: Future Experiments

8.3.1: TNT Pathway Experiments- Polar Metabolite Identification

While considerable progress has been made on elucidating the TNT transformation pathway, there remain portions of the pathway that have not been identified. These probably consist of metabolites in a polar section of the pathway. TNT is a strongly electronegative molecule due to the three nitro-groups present on the ring; hence, the first step of its transformation is necessarily reduction of the nitro groups. This leads to the formation of nitroso and hydroxylamino metabolites, which can be further transformed in many directions because of their instability. In radiolabeled TNT studies, a significant fraction of the radioactivity was observed to be flushed out in the early part of the run; these were possibly polar metabolites, not separated by the routine methods of HPLC analysis. In order to complete identification of these metabolites, it is necessary to separate and purify them. We

have developed a scheme for the same, which is expected to yield enough dry samples for MS, NMR and IR spectroscopic identification.

This scheme essentially consists of running multiple 2 ml media samples from plants exposed to [ring-U ^{14}C] TNT for 60 hours, in a Nova-Pak (non-polar) C8 column and collecting the eluent from 0 to 5 minutes. The sample thus collected will be frozen in liquid nitrogen and immediately placed in a freeze dryer to concentrate the sample. Samples will be freeze dried to dryness in 12 hours and subsequently dissolved in 1 ml of water. At this stage, it was found that ether extraction of the sample did not extract any of the radioactivity. Hence, all the metabolites of interest were still present in the aqueous phase, and further separation focused on this fraction. The aqueous phase can be separated by a Synergi-Polar-RP (From Phenomenex, California) column. It was found, using the PDA and FloScin 515 detectors, that the metabolites of interest eluted between the times 5.7 to 10 minutes, 10 to 18 minutes and 24 to 27.7 minutes. Hence, these fractions can be collected, frozen with liquid nitrogen and subsequently freeze-dried for 24 hours to eliminate all moisture from the system. The samples obtained from this processing will be ready for chemical structure analysis. Small amounts of such sample have been isolated and preliminary NMR has been performed on them. Larger volumes of initial sample are necessary for greater metabolite recovery.

8.3.2: Ancillary Pathway Studies

The role of azoxies in the TNT transformation pathway has not been fully understood. These metabolites appear to be formed abiotically from hydroxylamines and are subsequently rapidly taken up by the seedlings. The eventual fate of these metabolites is not known, although it has been speculated that they are conjugates and assimilated into the plant biomass (Wang *et al.*, 2003). Our studies also showed their formation in TNT transformation studies and their rapid uptake in azoxy feeding studies. No metabolites were observed to be formed in the azoxy feeding studies. Hence, repeating this experiment with higher initial levels of the azoxy and rigorously monitoring the formation of metabolites is necessary. Different analytical identification schemes, including different columns, may be needed for

this experiment. Delineating the role of azoxies in the TNT transformation pathway may help shed further light on toxicity mechanisms of TNT and pathway characteristics.

Using ^{15}N labeled TNT is another tool to understand the TNT transformation pathway to a greater extent. While the TNT ring is preserved through its transformation (not cleaved due to ring stability) the nitro groups are subject to splitting. Hence radiolabeled nitro groups on TNT can lead to the identification of different metabolites. Potential bottlenecks in this project include the high cost of obtaining such a radiolabeled substrate.

8.3.3: Arabidopsis Mutant Experiments

8.3.3.1: Mass Balances on Uniform Hydroponic Samples. The various metabolic analysis experiments on the Arabidopsis mutants revealed a difference between the mutant and wild-type transformation at very high levels of initial TNT. However, since the Arabidopsis seedlings used were at an exponential phase of growth, variability could exist between samples due to the difference in germination characteristics. Hence, these variablities could have biased the results obtained from the experiments. In an effort to obtain uniform samples, 20-day old stationary phased seedlings were used; however the biomass attained by these seedlings was high enough to blunt the toxicity of TNT. Results from a 140 mg/L initial TNT study with stationary phase seedlings showed a rapid TNT removal rate by all seedlings, including the wild-types. Hence, these set of experiments were not well suited to eliciting differences between wild-types and mutants.

To overcome these shortcomings, a new experimental design is suggested to ensure lower biomass and uniform number of seedlings across all system. This experiment involves growing a large number of seedlings in a large flask, and transferring an equal number of seedlings to each system. Around 300 seeds will be added to 250 ml of media in a 2L conical flask and at 150 rpm under light at 25 °C. At the end of 7 days, 10 seedlings will be selected and transferred to a 50 ml sterile centrifuge tube. This will be repeated for all systems, including wild-types and mutants. This will ensure equal number of viable seedlings in each system, which will minimize variability. The seedlings will be allowed to acclimatize for 24 hours in their new environment, following which radiolabeled [ring-U ^{14}C] TNT will be added. Hence, the seedlings will be 8 days old, in their early exponential phase, but an equal

number of seedlings per system will help maintain uniformity of germination. Low biomass of the seedlings will allow the use of low concentrations of TNT. Since radiolabeled TNT is used, complete transformation characteristics, including the formation of bounds, can be obtained.

8.3.3.3: Determination of Effect of Mutation. Genetic and metabolic evidence of the ET- mutants suggest that the mutations conferring TNT resistance to the mutants may not be directly related to the TNT transformation biochemical pathway. Rather they seem to confer the mutants the ability to resist the toxic effects of TNT. Thus far, only visual evidence for the health of the mutants has been observed. Physiological experiments, such as those measuring rates of production of chemicals, metabolites or oxygen (from photosynthesis) may help in quantitative monitoring the health of the seedlings. These metabolites or chemicals could be enzymes or other routinely produced indicators that signal health or signs of stress in the plant. When exposed to TNT, the levels of these chemicals can vary depending on the extent of the stress response. This would be a quantitative indicator of the stress experienced by each mutant to TNT, and provide evidence if they indeed are more resistant to TNT toxicity. In addition to these experiments, completing a metabolomic and proteomic study of the wild types and the mutants when exposed to TNT may help identify differences in their response. This response will not be confined to the TNT transformation pathway, but rather encompass the entire metabolism of the plant, and hence can help draw out the differences.

8.3.3.4: Analysis of Knock-Out or Over-Expressing Arabidopsis Mutants. With the mushrooming use of Arabidopsis in basic research in plant molecular biology, there exist a number of available knock-out or over-expressing mutants that can be tested for TNT resistance. An example of such mutants include the OPR (Oxyphytodienoate reductase) mutant, which either under or over-expresses this enzyme. This enzyme may be implicated in TNT transformation (Ekman *et al.*, 2003), hence testing upregulated and downregulated phenotypes of these mutants can decisively reveal its role in TNT transformation. Many such putative mutants with different expression levels of various enzymes involved in TNT transformation, such as glutathione transferase, cytochrome P450 monooxygenases, nitrite

reductases and ATP binding cassettes are available. Testing of these individual mutants can unequivocally determine the role of the enzyme in TNT transformation.

Appendix: Oxidative Metabolite Formation in *Myriophyllum aquaticum*

1: Introduction

While reduced and conjugated metabolites are produced by TNT transformation in a number of plant species, oxidized metabolites have been identified, thus far, only in *Myriophyllum aquaticum* (parrot feather) (Bhadra *et al.*, 1999). In addition, diazoxy compounds have been identified in this aquatic plant too. Precursor feeding studies on *M. aquaticum* also indicated that all the oxidative products and diazoxy products were formed from the hydroxylamine metabolites. A wide array of metabolites and a well elucidated pathway structure makes *M. aquaticum* an ideal candidate for hydroxylamine feeding studies, high concentration TNT studies, and enzymatic analysis. Given that the *M. aquaticum* has widely different means of transforming TNT from terrestrial plant understanding these differences using an enzymatic and genetic framework can prove useful. For these reasons, TNT transformation studies were done on *M. aquaticum*, in an effort to duplicate previously obtained data, and identify oxidized metabolites.

2: Materials and Methods

2.1: Plant Material: *M. aquaticum* was purchased from a local nursery and grown under fluorescent light at 26°C, submerged partially in water. The plant was allowed to acclimatize to the lab conditions prior to the start of the experiment. The healthy plant was green colored, while the sick part showed yellowing. Sterile conditions were not maintained.

2.2: Chemicals: Solid TNT (minimum 35% water), 2ADNT and 4ADNT were obtained from ChemService, while all the liquid standards (TNT, 2ADNT, 4ADNT, 2HADNT and 4HADNT) were obtained from Accustandards. Previously isolated and purified samples of oxidative metabolites were used for the identification of oxidation products.

2.3: TNT Transformation Studies: Three flasks of *M. aquaticum* were used for the experiment: a 35 mg/L TNT concentration with 400 ml of media, a 75 mg/L TNT concentration with 250 ml media, and a TNT free control, with 250 ml media. The biomass concentrations were 13 g/flask. The *M. aquaticum* was initially acclimatized to its new environment by being allowed to grow in the flasks. The *M. aquaticum* cultures were not

axenic, but were soil free. Sterility was never maintained during the course of the experiment. Samples were taken periodically through out the experiment, which was allowed to continue for 144 hours. Samples were analyzed through the HPLC for reduced and oxidized metabolites.

3: Results and Discussion

The primary purpose of this experiment was to duplicate earlier published results (Bhadra *et al.*, 1999), and establish a procedure for identification of oxidative metabolites. *M. aquaticum* efficiently transformed TNT, within 300 hours of initial amendment, for both concentrations. Low levels of monoamines were seen to be produced in the system (Bhadra *et al.*, 1999). A previous identified oxidative metabolite, labeled as F-5, was also observed in the system. While, this metabolite's structure was not fully elucidated, presence of an oxidative moiety had been confirmed. No other previously identified oxidative products were detected. Possible reasons for this could include the non-aeration of the system using stir bars. Oxygen is a substrate for the cytochrome P450s which are probably the enzymes that carry out the oxidative steps in the pathway, and the non-aeration probably resulted in a shortage of substrate to the enzyme; hence, the resultant absence of majority of oxidative products. In addition, insufficient biomass could have resulted in a reduced uptake of TNT, and hence lower production levels of metabolites. The methanol present in the TNT addition may have in addition led to toxicity, although no visible effects of this were apparent.

This experiment was conducted primarily to identify oxidative metabolites, and use the analytical schemes developed here in other systems.

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Phytotransformation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX): A study of the biochemical pathway in *Arabidopsis thaliana*

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has met the thesis requirements of Iowa State University

Major Professor

For the Major Program

Dedication

This thesis is dedicated to my husband, Derrick Rollo, and my parents, Brian and Susan Frank. I would not have been able to complete this without their support. I would like to thank Derrick for his never-ending motivation, encouragement, and assistance. I am grateful for all of the time he has spent helping me, in lab and at home, and making sure that I do my best. Several hours of his time went into this research and this thesis. I would also like to thank my parents for their guidance, inspiration, and support throughout all of my life.

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Abstract

RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) is deposited on soil at ammunition manufacturing and testing sites, contaminating the surface soil and groundwater. The mobility of RDX in the groundwater has made it difficult to remediate the contaminant using traditional soil treatments. RDX is toxic to many living organisms, but it can be taken up by plants to reduce contamination of the groundwater. Phytoremediation has the potential to be used for sustained military range clean-up, due to its ability to remediate the wide-spread contamination in the surface soil and groundwater, while enabling training activities to continue on the military reserves. Research is needed to determine how plants remove RDX from soil and groundwater, as well as to identify the environmental fate of RDX in plants.

In order to make the phytoremediation process more effective, a knowledge base of biochemical reaction pathways must be formed. This work focuses on developing an understanding of the phytotransformation pathway of RDX in *Arabidopsis thaliana*. ^{14}C-labeled RDX was used to determine what products were formed by phytotransformation in *Arabidopsis*. ^{14}C-mass balances were also conducted to identify the metabolic fate of RDX in the plant cultures. RDX was removed from the media by the *Arabidopsis* cultures and significant mineralization to carbon dioxide was observed. The rates of mineralization were comparable to other plant and bacteria remediation systems in the literature. Documenting the fate of RDX in an axenic *Arabidopsis* plant culture is an important step in understanding the process of phytotransformation.

Since *Arabidopsis thaliana* has been completely genetically sequenced, using *Arabidopsis* in screening studies may lead to additional insight into the RDX pathway that would not be possible with other plants. Utilizing the fact that energetic materials are toxic to plants, varying concentrations of RDX were used to screen *Arabidopsis* mutant libraries. Germination and root length screening was conducted to identify important genetic sequences involved in the RDX pathway. Three mutants were selected for further analysis using the root length screen. These mutated genetic sequences could be used to help identify the RDX pathway, as well as to screen native plants or develop transgenic plants with enhanced transformation pathways to use for phytoremediation of contaminated sites.

Chapter 1 - Introduction

Phytoremediation is an innovative technique that can be used to reduce contamination in soil and groundwater. Plants are able to take up pollutants and store the contaminants in the leaves or break them down to detoxify them. The use of phytoremediation is especially promising for sustained military range clean-up, due to the wide-spread contamination in the surface soil. Phytoremediation has the potential to remediate this soil, while enabling training activities to continue on military reserves.

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is especially problematic at contaminated military sites. The mobility of RDX, with a log K_{ow} of 0.81 to 0.87, causes it to form RDX plumes in the groundwater. These plumes are especially concerning at the Massachusetts Military Range due to their close proximity to the drinking water supply. The mobility of RDX has made it difficult to remediate RDX by traditional soil treatments, including surface soil removal followed by incineration. Phytoremediation allows plants to be used to clean up contamination in surface soil and wetlands and could be very useful in reducing the concentration of RDX present in the plumes. Research is needed to determine how plants remove RDX from soil and water, as well as to identify the environmental fate of RDX in plants.

In order to make the phytoremediation process more effective, a knowledge base of biochemical reaction pathways must be formed. This work focuses on developing an understanding of the phytotransformation pathway of RDX in *Arabidopsis thaliana*. ^{14}C-labeled RDX was used to determine what products are formed by phytotransformation in *Arabidopsis*. ^{14}C- labeled RDX also allows identification of the metabolic fate of the metabolites by following the location of the radioactivity throughout the culture. This enables a carbon mass balance to be conducted, which can be used to predict what metabolites will be present in the field and where the metabolites will end up in the environment. Documenting the fate of RDX is an important step in understanding the process of phytotransformation.

Since *Arabidopsis* has been completely genetically sequenced, using *Arabidopsis* in screening studies may lead to additional insight into the RDX pathway that would not be possible with other plants. Genetic screening can be conducted to identify important genetic

sequences that are involved in the RDX pathway. These sequences could then be used to help identify the RDX pathway, as well as to screen native plants or develop transgenic plants with enhanced transformation pathways.

Chapter 2 gives a review of the background literature for phytoremediation and RDX research. A summary of phytoremediation is outlined, followed by a discussion of the toxicity and chemical properties of RDX. An overview of the current literature on the bioremediation and phytoremediation of RDX is also given.

Chapter 3 discusses the ^{14}C-labeled RDX mass balance experiments. The results from the mass balance, along with a discussion of how this work impacts the current understanding of the RDX pathway, are presented. The results of the growth curves and RDX uptake curves are also summarized.

Chapter 4 describes the toxicity studies of RDX in solid media. Germination screening methods and root screening methods are given. Results for both screening methods are also discussed.

Chapter 5 highlights the main conclusions that can be drawn from this research. Future work is also summarized in this chapter.

The appendices contain additional figures and tables from the mass balance and screening studies. A glossary and additional chemical information are also available in the appendices.

Chapter 2 - Literature Review

Phytoremediation

Phytoremediation is an *in situ* technology that uses plants to actively remove contamination from polluted soil or water (1-3). Although plants have been used for remediation for years, the term phytoremediation is fairly new (2). Phytoremediation has been used to prevent hazardous waste from leaking out of landfills and to clean up wastewater in artificial, man-made wetlands. Phytoremediation has been used to clean up heavy metals, radioisotopes, hydrocarbons, phenols, and nitro aromatic explosives. The goal of phytoremediation is to metabolize and chemically degrade the xenobiotics to less toxic compounds, ideally, carbon dioxide (CO_2), nitrate (NO_2), or ammonia (NH_3). Plants have developed a wide range of strategies to protect themselves from toxic compounds present in the environment (Table 2.1). The major advantage to using phytoremediation is its low cost, which can be up to 40 times cheaper than traditional remediation methods (2). Operating costs usually range from $0.02 to $1.00 per cubic meter (3). Another advantage is that excavation is not required, which has helped to gain public acceptance of this new technology (2,3). Plants are capable of continually removing contaminants, which makes phytoremediation an ideal technology for industrial pollution and other sites with repeated contamination (2). Phytoremediation is limited by toxic effects of many of the contaminants. Phytoremediation can also be a slow process, continuing over several weeks to years, partly due to retarded plant growth in the presence of the toxic chemicals and a reduction in plant activity during cooler seasons (2,3). Phytoremediation is the most effective when the contamination remains near the soil's surface and when contamination levels remain low (2.5 to 100 mg/kg) (3). Since phytoremediation may not fully remove the pollutant, monitoring may be needed to determine if the plants have successfully lowered contamination. Since implementation of this technology is fairly recent, there remains a large amount that is unknown about the phytoremediation process in the field (2,3). Laboratory studies can help shed light on what happens to the contaminants inside the plants and what environmental effects may be caused by phytoremediation (2).

Table 2.1. Phytoremediation mechanisms and their definitions (2, 3).

Mechanisms	**Definitions**
Blastofiltration	To remove pollutants from water using seedlings
Hydraulic pumping	To lower the water level preventing pollutant migration and enhancing volatilization organic compounds from the soil
Phytoaccumulation	To accumulate contaminants into plant tissues
Phytodegradation	To metabolically break down toxins into harmless compounds
Phytoextraction	To absorb contaminants through the roots and accumulate them into plant tissues
Phytomining	To take up metals from the soil and store them in the plant tissues from which they may be retrieved by further processing
Phytosorption	To sorb contaminants to either living or dead plant tissues
Phytostabilization	To immobilize contaminants in the soil by incorporating them into either plant cell walls (lignification) or into the soil humus (humification)
Phytostimulation	To promote growth of microorganisms in the rhizosphere
Phytotransformation	To absorb and convert toxic pollutants into innocuous compounds by activating plant metabolic systems
Phytovolatilization	To take up the contaminants and release volatile compounds into the atmosphere through transpiration
Rhizodegradation	To mineralize contaminants in the soil by microbial activity in the rhizosphere
Rhizofiltration	To absorb, adsorb, or precipitate pollutants from aqueous environments and concentrate them into plant roots

Chemical Properties of RDX

Numerous papers have documented the chemical properties of RDX. Table 2.2, below, summarizes some of these characteristics. Soil studies have shown that sorption of RDX to soil is minimal, but RDX can be difficult to remove from soil once it is bound (4). Since solubility in water and sorption to organic matter is minimal, RDX is very mobile and is frequently found in plumes at contaminated sites (5-8). It is possible for phytoremediation to effectively removed RDX from the water because RDX is hydrophobic and has a log K_{ow} value between 0.81 and 0.87. Additional factors, including plant uptake rate, transpiration rate, and contaminant concentration, may affect phytoextraction by the plant (3).

Table 2.2. RDX Chemical Properties

Property	**Value**	**Reference**
Chemical Name	Hexahydro-1,3,5-trinitro-1,3,5-triazine	
Common Name	1,3,5-Trinitrohexahydro-*s*-triazine 1,3,5-Trinitro-1,3,5-triazacyclohexane Cyclonite Cyclotrimethylenetrinitramine Hexogen Hexolite RDX Research Department Explosive Royal Demolition Explosive Trimethylenetrinitramine Trinitrocyclotrimethylene	5
Chemical Abstract Service Registration Number	121-82-4	6
Molecular Formula	$C_3H_6N_6O_6$	6
Molecular Weight	222.12	6
Density	1.82×10^{20} g/cm^3	6
Crystal Form	White orthorhombic crystals	6
Melting Point	202 – 205.5 °C	7
Ignition Temperature	229 °C	7
Activation Energy	47.08 kcal/mol	7
Specific Heat	0.30 cal/(g * °C)	7
Heat of Combustion	2285 kcal/kg	7
Heat of Detonation	1324 kcal/kg	7
Vapor Pressure (25 °C)	4.6×10^{-9} Torr	7
Vapor Pressure (202 °C)	0.09 Torr	7
Solubility in Water (25 °C)	60 mg/L	7
Solubility in Acetonitrile (25 °C)	860 g/L	7
Solubility in Acetone (25 °C)	6430 g/L	7
Solubility in Media (pH 5.7, 25 °C)	47.5 mg/L	8
Henry's Constant	1.2×10^{-5} atm*m^3/mol	5
Log K_{ow}	0.81 - 0.87	5

Toxicity of RDX

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) has been documented as toxic to a broad range of organisms (9-12). According to the Chemical Hazard Evaluation Group at the Oak Ridge National Laboratory, the primary toxic effect of RDX was on the central nervous

system (CNS) in humans. Chronic exposure to RDX can cause convulsions, headaches, nausea, vomiting, and unconsciousness. Acute exposure to explosive mixtures containing over 90% RDX may result in the same symptoms (12).

Several toxicity studies for RDX have been conducted with rats and mice. Rats fed 40mg/kg/day developed numerous toxic effect symptoms, including weight loss, CNS effects, and mortality. Mice exposed to 100 mg/kg/day had increased kidney and heart weights. Decreased fertility and decreased birth weights were also seen in female rats exposed to an oral dose of RDX (12). RDX was not found to be carcinogenic to rats, but an increase in cancer incidence was observed in exposed rats. Due to the increase in liver carcinomas in mice, the EPA has assigned RDX as a possible human carcinogen (12).

Chronic exposure was characterized as an oral dose of 0.003 mg/kg/day by the EPA (12). RDX was recently placed on the Drinking Water Contaminant Candidate List (CCL), published in February 2005 by the EPA (13). The EPA Integrated Risk Information System (IRIS) lists RDX as a suspected carcinogen (14). RDX is listed as a gastrointestinal or liver toxicant by the National Institute for Occupational Safety and Health in the Registry of the Toxic Effects of Chemical Substances (RTECS) database (15). The Agency for Toxic Substances and Disease Registry (ATSDR) lists RDX as a neurotoxicant and a reproductive toxicant on the Minimal Risk Levels (MRLs) for Hazardous Substances List (16).

Several studies have documented the phytotoxicity of RDX (Table 2.3). The results from these studies show that the toxicity of RDX varies depending on plant species and type of environment (17-21). RDX is the least toxic in soil studies and the most toxic in studies with emergent aquatic plants (19,20). Currently no information on the toxicity of RDX on *Arabidopsis thaliana* exists in the literature.

Energetic Materials Contamination

Energetic materials have contaminated groundwater and surface soil at several ammunition manufacturing, packing, testing, and training sites (22). Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), 2,4,6-trinitrotoluene (TNT), 2,4-dinitrotoluene and 2,6-dinitrotoluene (DNTs) are all examples of chemicals that have been identified in contaminated areas. Contamination can also be found at ammunition manufacturing and testing sites that are no longer in use (22,23). This has created a real potential for

environmental damage, including ecosystem effects and groundwater contamination, in these areas.

Camp Edwards at the Massachusetts Military Range has several contaminated testing and training sites (24). In 1997, the Environmental Protection Agency (EPA) issued an order requiring certain training activities to be suspended on the range. In January 2000, the EPA issued another order requiring that action be taken to decrease the threat to the public water supply due to contamination from training activities. The Cape Cod Aquifer, which supplies the drinking water for western Cape Cod, was located directly underneath the training ranges, allowing explosives and other organic chemicals to be deposited into the public water supply over time (24). Soil and groundwater sampling taken from Camp Edwards between October 2002 and August 2003 indicated the presence of several ammunition chemicals. 30.4% of the soil contamination was due to aminodinitrotoluenes, 12.1% was perchlorate, 11.6% was dinitrotoluenes, and 10.1% was from RDX. Groundwater sampling indicated that 45.9% of the contamination was from perchlorate and 30.9% was due to RDX. Several RDX plumes are also present at the Massachusetts Military Range. Two small RDX plumes have even migrated outside of the military range border, increasing the need for remediation. Additional military sites also have documented contamination. In August 1996, water samples were taken at the Milan Army Ammunition Plant in Milan, Tennessee. The samples were taken from well M-146 and were analyzed by Best *et al.* (1999). The water samples contained over 1975 µg/L RDX and over 1350 µg/L TNT (19). In order to take the appropriate action to remedy this situation, the Department of Defense (DoD) has funded several research and development efforts (24).

The Strategic Environmental Research and Development Program (SERDP) was established in 1990 to help address the environmental issues facing the DoD (25). SERDP is a partnership between the Department of Defense (DoD), the Department of Energy (DoE), and the Environmental Protection Agency (EPA) (24). SERDP funds environmental research and development in four areas: cleanup, compliance, conservation, and pollution prevention (25,26). Since it was founded in 1990, SERDP has supported a broad assortment of research projects related to range sustainment (24). This research project was conducted as a response to SERDP's statement of need indicating that the energetic materials (RDX, HMX, TNT, and DNT) are possible sources of groundwater and surface soil (<1 ft) contamination on DoD

training and testing sites. The purpose of this research is to study phytoremediation of energetics, in order to develop an innovative technology capable of sustained prevention of migration of surface and near surface contamination by energetic materials.

Table 2.3. Phytotoxicity of RDX

Plant	**Study**	**Toxic Level**	**Time**	**Reference**
Maize	Hydroponic	Toxic at 21 mg/L	30 days	17
Wheat	Hydroponic	Toxic at 21 mg/L	30 days	17
Sorghum	Hydroponic	Not toxic at 21 mg/L	30 days	17
Soybean	Hydroponic	Not toxic at 21 mg/L	30 days	17
Poplar	Hydroponic	Not toxic at 21 mg/L	14 days	18
Parrot feather	Water (emergent)	Toxic at 1.5 mg/L		19
Sweet flag	Water (emergent)	Toxic at 1.5 mg/L		19
Wool grass	Water (emergent)	Toxic at 1.5 mg/L		19
Reed canary grass	Water (emergent)	Not toxic at 1.5 mg/L		19
Elodea	Water (submersed)	Not toxic at 1.5 mg/L		19
Pondweed	Water (submersed)	Not toxic at 1.5 mg/L		19
Water stargrass	Water (submersed)	Not toxic at 1.5 mg/L		19
Alfalfa	Sassafras sandy loam soil	Not toxic at 10,000 mg/kg	16 to 19 days	20
Japanese millet	Sassafras sandy loam soil	Not toxic at 10,000 mg/kg	16 to 19 days	20
Ryegrass	Sassafras sandy loam soil	Not toxic at 10,000 mg/kg	16 to 19 days	20

Remediation of Energetics

RDX concentrations has been documented as high as 74,000 mg/kg in the soil on military sites, and is also present in the groundwater beneath many sites (5). Photolysis is the most successful transformation mechanism currently reducing the level of RDX on contaminated sites. Studies have shown that RDX is not removed well by microbial degradation and hydrolysis compared to other energetic materials, but it is unclear if this is due to low solubility or other factors (5). Total removal of surface soil has been effective at removing all contamination in the soil, but this does not remove all of the RDX, since the majority of the RDX is in the groundwater (19). The fact that RDX is mobile has prevented

other traditional methods of range decontamination, such as controlled range burns, from being successful. Development of an inexpensive and effective process to remove RDX and other energetics from soil and groundwater could reduce the environmental and economic burden caused by this contamination. Significant research has been done to develop new methods to remove energetic materials from soil and groundwater.

Bioremediation of Energetics

Microbial degradation of RDX readily occurs under aerobic and anaerobic conditions (4, 27-29). As with TNT, examination of RDX transformation in bacteria can be used as the first basis to examine behavior in plants. Hexahydro-1,3-dinitro-5-nitroso-1,3,5-triazine (MNX), hexahydro-1-nitro-3,5-dinitroso-1,3,5-triazine (DNX), hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX), methylene-dinitramine (MEDINA), and 4-nitro-2,4-diazabutanal (NDAB) are all documented metabolites of RDX. These metabolites can be seen below in Figure 2.1. It is believed that formaldehyde (CH_2O), nitrogen (N_2), nitrous oxide (N_2O), methane (CH_4), and carbon dioxide (CO_2) are all formed from the breakdown of the RDX metabolites.

Hawari and their collaborators have published several papers about the degradation of RDX with bacteria. In 2004, Fournier, *et al*., reported that 30% of RDX was mineralized to CO_2 by *Rhodococcus* sp. strain DN22 under aerobic conditions, with another 64% of the RDX was converted to NDAB (27). Almost 50% of the RDX was converted to CO_2 by microcosms in cold marine soil under anaerobic conditions, while autoclaved soil degraded less than 1% to CO_2. MNX was also detected in the soil, but DNX and TNX were not found (28). These studies support the aerobic and anaerobic RDX degradation pathways derived by Hawari (Figures 2.2 and 2.3) (29).

Phytoremediation of Energetics

Phytoremediation is an emerging technique that can be used with minimal cost to continually reduce the level of energetic materials from soil and water following the “green liver” model (22,30,31). Phytoremediation research has focused on pesticides and industrial pollutants, but phytoremediation studies have also shown that the use of plants is a potential method to help reduce energetics contamination (18,22,32). Since significant contamination

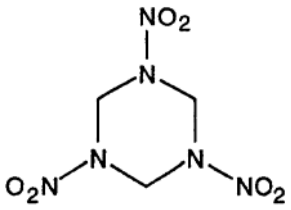
occurs in surface soil at military training sites, phytoremediation may become an ideal treatment for energetic contamination (18,30,33). Considerably more bacterial transformation pathways are available in the literature and can be used as foundation for generating phytoremediation pathways.

Many plant species have been shown to transform TNT (18,32,34-39). A basic understanding of the TNT transformation pathway has also been developed (18,30,32-40). TNT can be transformed by several plants and we have developed a good understanding of the transformation process. Much less is known about the degradation and fate of RDX, which makes it especially important to study this energetic material in depth. RDX can be taken up by plants and has also been shown to bioaccumulate in plant tissue as a parent compound, but little else has been known about the phytoremediation pathway until recently (19,23,30,31,33,41-45).

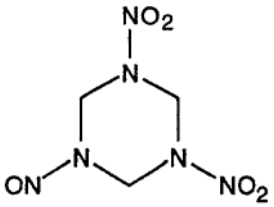
Very few plant metabolism studies have been performed for RDX, but the details of the transformation pathway of RDX are beginning to emerge. The proposed pathway for RDX in reed canary grass (Just and Schnoor, 2004) and hybrid poplar (VanAken, *et al.*, 2004) is shown in Figure 2.4 (41,42).

Phytophotolysis of RDX has been hypothesized by Just and Schnoor to be responsible for the first step in the degradation of RDX. Nitrous oxide and NDAB were observed as metabolites in the reed canary grass. Media controls exposed to light degraded RDX into NDAB, nitrous oxide (N_2O), nitrite (NO_2^-) and formaldehyde (CH_2O). Since no plant activity was involved in breaking down the RDX in the controls, the RDX must break down in a similar process with or without plants. Phytophotolysis occurred within the plants to degrade the RDX, while photolysis transformed RDX in the controls. Mineralization to CO_2 was not observed in this study (41).

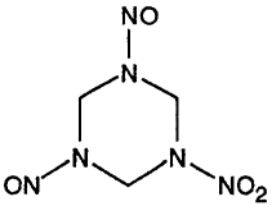
In poplar tissue cultures studied by VanAken, *et al.*, (2004) MNX and DNX were identified as metabolites of RDX. MNX and DNX were then transformed to formaldehyde and methanol, if the poplar tissue cultures were exposed to light. The cultures could also mineralize formaldehyde (CH_2O) and methanol (CH_3OH) to carbon dioxide, regardless of light exposure. After 60 days, the RDX had almost completely disappeared and significant mineralization (17%) of RDX to CO_2 was reported in the plant tissue cultures. Heat deactivated controls showed some RDX transformation due to light exposure (35%), but the



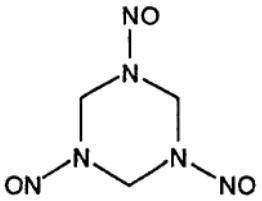
Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)



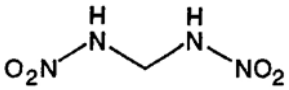
Hexahydro-1,3-dinitro-5-nitroso-1,3,5-triazine (MNX)



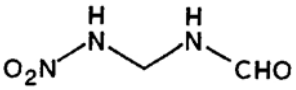
Hexahydro-1-nitro-3,5-dinitroso-1,3,5-triazine (DNX)



Hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX)



Methylene-dinitramine (MEDINA)



4-nitro-2,4-diazabutanal (NDAB)

Figure 2.1. The structures of RDX and its metabolites.

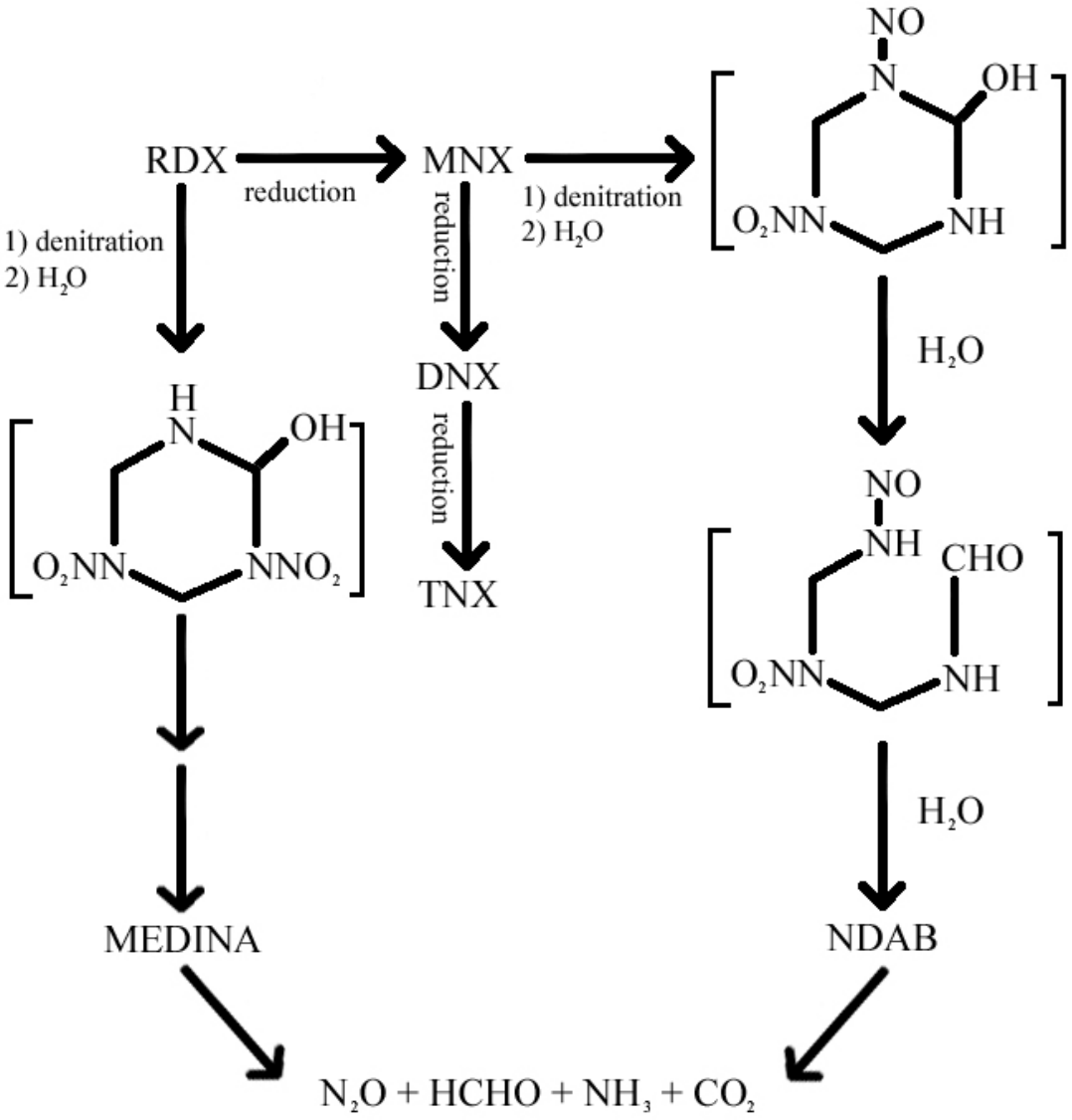


Figure 2.2. The anaerobic degradation pathway for RDX in bacteria. (29)

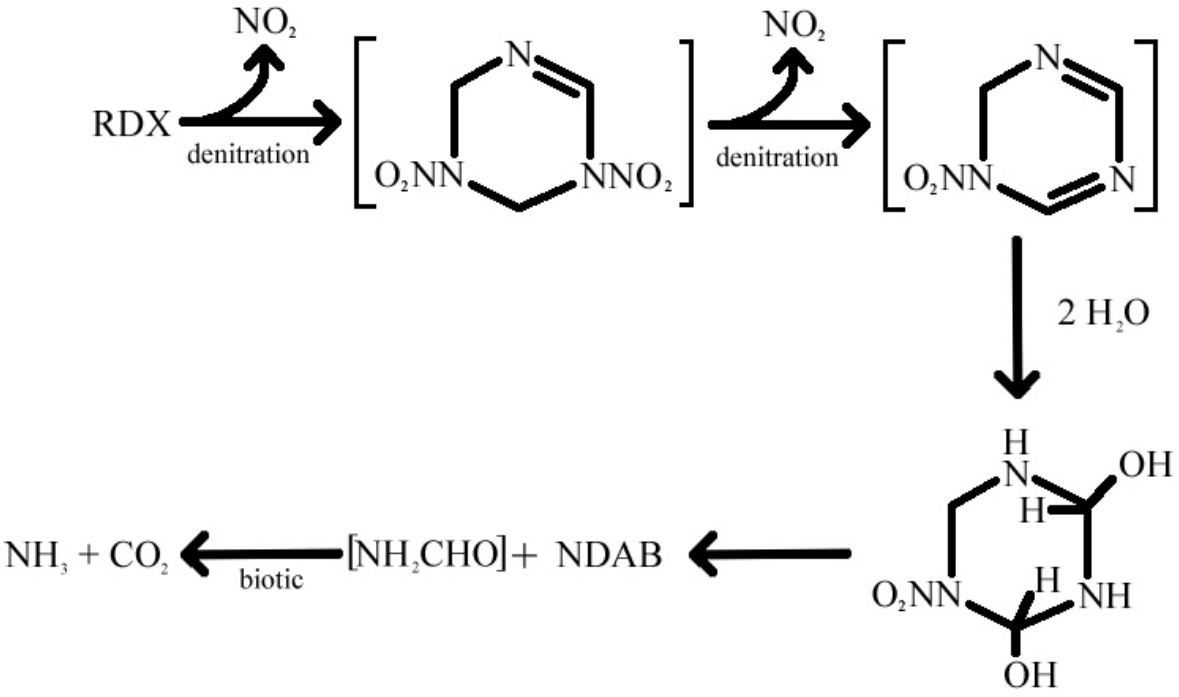


Figure 2.3. The aerobic degradation pathway for RDX in bacteria. (29)

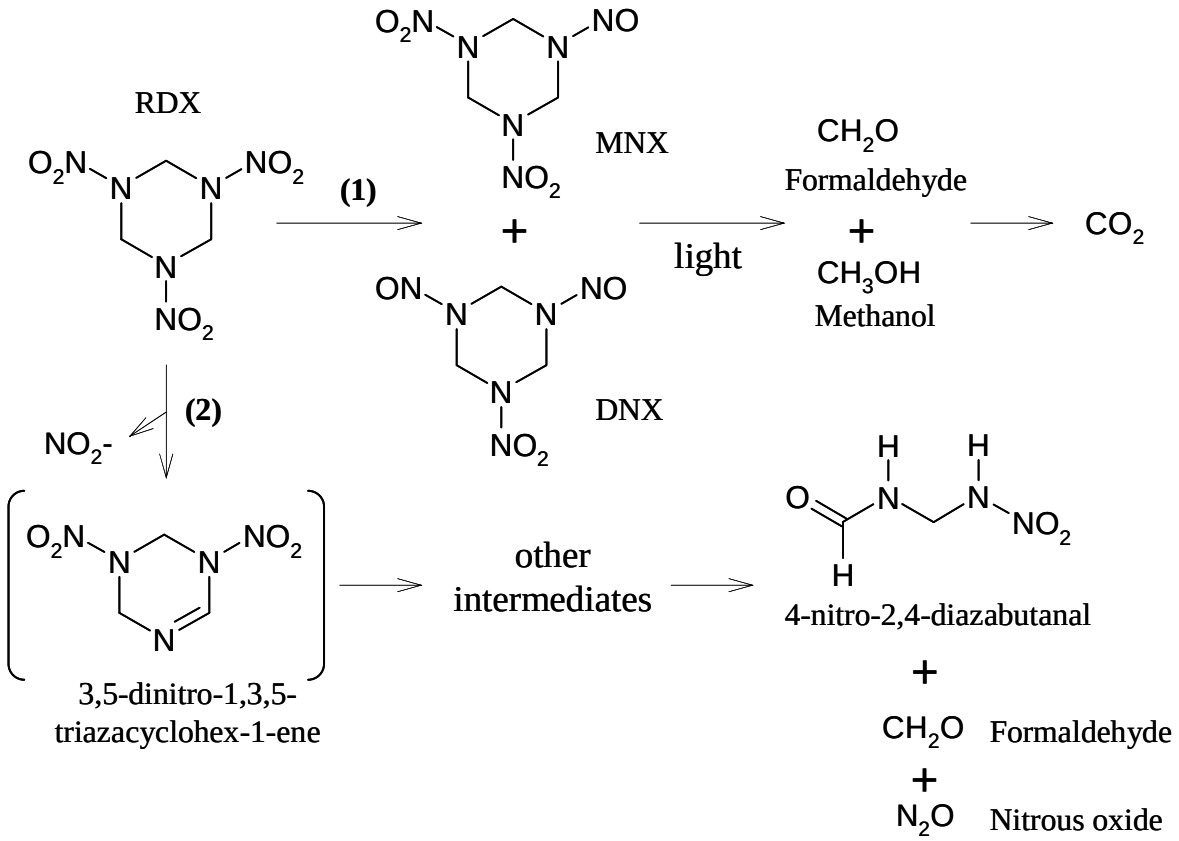


Figure 2.4. RDX degredation pathways in plants. Pathway 1 is from poplar tissue cultures studied by VanAken, *et al.*, 2004 (42). Pathway 2 was observed in reed canary grass by Just and Schnoor, 2004 (41).

lower levels showed that plants accelerated the RDX degradation. MNX (14%) and DNX (4%) were observed near the beginning of the 60 day culture (42).

Tissue cultures in the light removed practically all of the initial RDX from the media, while cultures kept in the dark only removed 49% after 60 days. The majority (53%) of the radioactivity was in the plant extracts and nonextractables at the end of the cultures, while the media controls had most (89%) of the radioactivity remaining in the media. Poplar leaf crude extracts verified that intact cells were needed for the first and final steps in the conversion of RDX to CO_2 (42). While this paper showed the first significant mineralization to CO_2 by plant tissue cultures, it should be noted that endophytic bacteria were isolated from the poplar tissues, that are capable of mineralization (46). The conversion of RDX to CO_2 may be due to the plant activity, but axenic plant studies are needed to verify mineralization in plants. The mass balance and pathway outlined by this study will be used as a foundation for the RDX phytotransformation in future phytoremediation research.

Conclusions

Before phytoremediation technology can be applied, there must be a general understanding of how the process works. Phytoremediation is a slow process and may be limited by the toxicity of energetic materials to plants. Current knowledge of the toxicity of RDX to plants and its degradation products is limited. A well-defined phytoremediation pathway would also help identify the environmental fate of these compounds. The final products of RDX need to be distinguished in order to learn the environmental impact that phytoremediation of contaminated areas may have. A complete transformation pathway for RDX must be known in order to fully understand the phytoremediation process.

Certain genes may be responsible for the phytoremediation of TNT and RDX. Important genetic sequences need to be identified to help understand more about the pathway in plants. With understanding of these transformation pathways, native plants could be selected that would improve the transformation of RDX. From knowledge of how genetic manipulation effects remediation, mutant plants could be generated to enhance transformation. The identification of these genetic sequences could also uncover the metabolic pathway in plants. Additional research is needed to construct a genetic and

biochemical knowledge base for RDX detoxification and the transformation reaction pathways.

This research will use ^{14}C-labeled RDX to perform a carbon mass balance to identify the fate of RDX in *Arabidopsis thaliana*. Toxicity in liquid culture and in solid media will be addressed. Screening studies will be used to help identify important genes that are involved in the RDX pathway. The results of this work will be used to help generate an RDX phytotransformation pathway for *Arabidopsis*. Continual work by several groups will lead to an understanding of the chemical pathway and environmental fate of RDX in plants.

Chapter 3 - RDX Transformation in *Arabidopsis thaliana*

Introduction

Phytoremediation is a slow process and may be limited by the toxicity of energetic materials to plants. Knowledge of the toxicity of RDX and the plant degradation products is incomplete. Gathering basic information about phytoremediation is an important step in order to be able to implement phytoremediation. A complete transformation pathway for RDX must be known in order to fully understand the process. A well-defined pathway would also help identify the end metabolites formed from RDX. The environmental fate of RDX and its transformation products, as well as their toxicity, must be known before phytoremediation can be completely successful. Additional research is needed to construct a genetic and biochemical knowledge base for RDX detoxification and transformation reaction pathways.

In order to develop a biochemical knowledge base about the RDX pathway in *Arabidopsis*, mass balances and metabolite analysis were performed. Wild-type plants were grown in the presence of several concentrations of RDX (0 to 35 mg/L). Plant and media samples were taken at several time steps throughout the experiment to obtain growth curves and RDX uptake curves. Since RDX transformation products are not known, ^{14}C-labeled experiments, along with mass balances, were used to help identify these RDX transformation products and their fate. Intracellular and extracellular samples from ^{14}C-labeled experiments were taken periodically and analyzed with HPLC to identify the RDX degradation. This information will help build an understanding of the RDX pathway. The completed growth curves, RDX uptake curves, and ^{14}C mass balances are shown and discussed.

Materials and Methods

Chemicals

Bulk RDX in acetone (50 mg/ml) was purchased from AccuStandard (New Haven, CT). RDX standards (1mg/ml RDX in acetonitrile) were purchased from ChemService (West Chester, PA). Standards of MNX, DNX, TNX, MEDINA, and NDAB were purchased in powder form from Ron Spanggord at Stanford Research Institute (Menlo Park, CA).

Uniformly radio-labeled ring 14-C(U) RDX (5.0 mCi at 41.3 mCi/mmol, 97.5% purity) was purchased in solid form from Perkin-Elmer Life Sciences (Boston, MA).

Half-strength Murashige and Skoog (MS) medium was used for all experiments. Medium consisted of 2.2 g of MS salt mixture, 3 mL of a 6% potassium phosphate monobasic (KH_2PO_4) solution, 1 mL of Gamborg's vitamin B_5 solution, 20 g of sucrose, and 0.5 g of 99% MES (2-morpholinoethanesulfonic acid) hydrate added to 1 L of water. The pH was adjusted to 5.8 with NaOH and then the media was autoclaved. The nitrogen content of the half-strength MS media is about 30 mg/L (47).

The MS salt mixture was purchased from Gibco BRL Life Technologies (Carlsbad, CA). Potassium phosphate mono-basic was obtained in crystal form from Fisher Scientific (Hampton, NH). Gamborg's vitamin B_5 solution came from Sigma-Aldrich (St. Louis, MO). Sucrose was purchased from MP Biomedicals (Aurora, OH). 99% MES hydrate was obtained from Acros Organics (Morris Plains, NJ). Potassium hydroxide was also purchased from Fisher Scientific.

All solvents, including acetonitrile, acetone, ethanol, and methanol, were purchased from Fisher Scientific. All scintillation cocktails, including Ultima-Flo M, Ultima Gold MV, Insta-Fluor Plus, PermaFluor E^+, CarboSorb E and Hionic-Fluor, were purchased from Packard Instruments (Boston, MA).

Plants

Arabidopsis thaliana was grown axenically from seeds. Fifty seeds were placed in a microtube and were sterilized with 70% ethanol for 5 minutes and then were rinsed with sterile water. The seeds were then sterilized with 50% Clorox bleach for 20 minutes and then were rinsed five times with sterile water. The seeds were then transferred to 50 mL of half-strength MS media in 250 mL Erlenmeter flasks which were sealed with foam plugs for the growth curve studies (47). Since a carbon-dioxide trap was used for the mass balance studies, the flasks were sealed with a unique rubber stopper designed for these experiments (48). The flasks were shaken at 100 rpm under continuous light and maintained at 23 °C for all experiments (47). For growth curve studies, RDX was added to the flasks when the seedlings were seven days old. Seedlings were ten days old seedlings when RDX was added for mass balance experiments.

Carbon-dioxide traps were used for the mass balance studies, requiring a specialized rubber stopper to be developed for these experiments. Figure 3.1 shows a schematic diagram for the apparatus used (48). Rubber stoppers were purchased and sent to a glass shop to have two vertical holes drilled through them. Test tubes were also sent to a glass shop to have holes drilled in the sides of them. The test tubes were inserted into the large hole in the rubber stoppers in order to suspend them from the top of each flask. The test tubes were filled with 1.5 mL of 1.0 M potassium hydroxide and sealed with serum stoppers. The holes in the side of the test tubes allowed air to exchange between the test tube and the flask. This allowed any carbon dioxide volatilized by the plant to be absorbed by the potassium hydroxide in the test tube. Small glass columns were inserted into the second hole in the rubber stoppers. These glass tubes were also sealed with serum stoppers and were used for sampling the medium throughout the experiment. Vacuum grease was used to help insert the test tube and the glass tube into the rubber stopper. This also helped to maintain an air-tight seal. Stainless-steel 20 gauge needles with a deflected serum point from Popper and Sons (New Hyde Park, NY) were used to sample the potassium hydroxide and the media through the serum stoppers. Needles remained in the flasks throughout the experiment to help reduce contamination that may occur from repeated needle injections. The tops of the needles were sealed with Parafilm when they were in the shaker to prevent contamination from entering through the needle bore. Flasks were transferred to a sterile hood for sampling. Sterile, disposable 3 ml syringes were used to sample the media and remove the potassium hydroxide. The flasks were also left open to allow ventilation for 20 minutes in the sterile hood each time they were sampled. An actual flask used in the experiment can be seen below in Figure 3.2. This setup allowed samples to be taken without opening the flasks, maintained an air-tight environment throughout the experiment, and ensured that a more complete mass balance was performed.

Analytical Methods

Reverse-phase HPLC was used for the analysis of liquid extracellular samples. A Waters HPLC system, consisting of a 717 autosampler and a PDA detector, was used for all experiments. A Nova-Pak C8 column from Waters (Milford, MA) was used for the growth

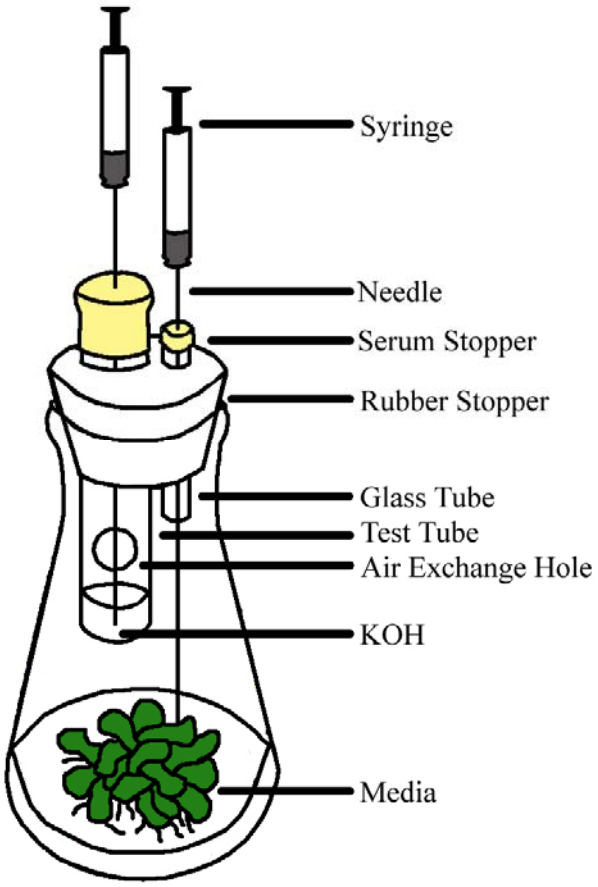


Figure 3.1. Schematic of the air-tight flask apparatus.

curve studies. A mobile phase of 82% water and 18% methanol at a flow rate of 1 mL per minute was used for the growth curve analysis. A Synergi 4µ Polar-RP column (80A, 150 x 4.6 mm) from Phenomenex (Torrance, CA) was used for the mass balance studies to allow for better analysis of metabolites. A mobile phase of 65% water and 35% methanol with a flow rate of 0.7 mL per minute was used for the mass balance studies. Identification and quantification was based on retention time and spectra comparison between samples and standards. Liquid samples were filtered with Xpertek nylon syringe filters (13 mm, 0.2 µm) from P.J. Cobert Associates (St. Louis, MO) prior to HPLC analysis to remove any plant debris.

Figure 3.2. A flask used in the experiment. The rubber stopper apparatus sealed with Parafilm can be seen. Potassium hydroxide is also visible in the test tube.

For the mass balance experiments, quantification of radioactivity during HPLC analysis was done using a Packard 505 Flow Scintillation counter in series with the PDA detector. A ratio of 1:3 of mobile phase to Ultima-Flo M scintillation cocktail was used. The combination of these two methods helped to identify which peaks were involved in the breakdown of RDX. A Packard 2900 TR Scintillation counter was used to quantify the radioactivity in extracellular, intracellular-extractable, and potassium hydroxide samples. A ratio of 1:5 of liquid sample to cocktail was used for all samples. Ultima Gold MV cocktail was used for aqueous extracellular samples, Insta-Fluor Plus was used for organic

intracellular-extractable samples, and Hionic-Fluor was used for concentrated potassium hydroxide samples. Radioactivity in the plant biomass was measured by combusting a portion of the dried biomass in an OX700 Harvey Biological Oxidizer. Oxygen was added to the bio-oxidizer to complete combustion, and nitrogen was added to flush out the residual radioactivity. Fifteen mL of CarboSorb E was used to collect the radioactive carbon-dioxide from the combustion chamber. A portion of this cocktail was combined with PermaFluor E^+ in a ratio of 1:5 and analyzed with the scintillation counter.

Arabidopsis Growth Curve Study

The first growth curve experiment was conducted with *Arabidopsis thaliana* seeds that were cultured for one week, and then 0 mg/L, 15 mg/L, and 30 mg/L RDX were added to the cultures. The flasks were shaken at 100 rpm under continuous light and maintained at 23 °C during the experiment. One seedling was sacrificed at 0 days to estimate the initial dry biomass. Triplicate seedlings were sacrificed at 4 and 10 days when possible. Contamination resulted in duplicate seedlings being sacrificed at 10 days for 0 mg/L and 30 mg/L RDX samples. The dry weight of each plant sample was acquired by freeze drying for 48 hours before weighing the sample. Sacrificed plants were immediately stored in the -80 °C freezer until freeze-dried. Extracellular liquid samples were taken at 0, 10 and 14 days and analyzed with HPLC. Liquid samples were stored in a -80 °C freezer until analyzed to assure chemical compound stabilization.

A second growth curve experiment was conducted on seedlings amended with RDX. Seeds were cultured for one week and then 0 mg/L and 35 mg/L RDX were added to the cultures. The flasks were shaken at 100 rpm under continuous light and maintained at 23 °C during the experiment. Three seedlings were sacrificed at 0 days to calculate the initial dry biomass weight. Triplicate seedlings were sacrificed at 7, 10, 14 and 21 days and the dried biomass was weighed, unless noted. Contamination resulted in duplicate seedlings being sacrificed at 10 days for 0 mg/L RDX samples, as well as at 7, 10, and 14 days for 35 mg/L RDX samples. The dry weight of each plant sample was acquired by freeze drying for 48 hours before weighing the sample. Sacrificed plants were placed in the -80 °C freezer until being placed in the freeze-dryer. A single extracellular liquid sample was taken at 0 days and analyzed with HPLC to evaluate the initial concentration of RDX present. Triplicate

extracellular liquid samples were taken at 7, 10, 14 and 21 days and analyzed with HPLC. Liquid samples were stored in a -80 degree Celsius freezer until analysis.

RDX Mass Balance Studies in *Arabidopsis*

Sterile, ten day old *Arabidopsis* seedlings, grown in liquid half-strength MS media, were amended with 35 mg/L (40 µCi/L) of ^{14}C-labeled RDX. Radio-labeled RDX was combined with cold RDX to get the final concentration and activity. Liquid media samples were taken immediately after adding RDX in order to obtain initial concentrations of RDX and radioactivity for each flask. Complete mass balances were performed at each time step. Media samples were taken and volumes were measured to correct the extracellular radioactivity for volume change due to evaporation. Media samples were analyzed with HPLC for metabolite concentrations and the radioactivity was measured with the scintillation counter.

Radio-labeled RDX allowed for volatilized carbon dioxide to be measured and accounted for. Carbon dioxide traps made of 1.5 mL of 1.0 M potassium hydroxide were used, allowing the carbon dioxide to dissolve in the potassium hydroxide. All of the potassium hydroxide was also removed and analyzed for radioactivity with the scintillation counter at each time step. Fresh potassium hydroxide was then added to the carbon dioxide traps. At each time step, seedlings were sacrificed and intracellular metabolites were extracted using the method described below. The methanol from the extraction procedure was used to determine the concentrations of the intracellular-extractable metabolites. The remainder of the biomass was used to determine the intracellular-bound radioactivity. Whole plant samples were taken before the extraction and were used to verify the total intracellular radioactivity. For the first experiment, liquid media samples, plant samples, and carbon dioxide trap samples were taken 4, 7, 11, and 16 days after the RDX was added. For the second experiment, liquid media samples, plant samples, and carbon dioxide trap samples were taken 3, 7, 14, 20, 27, and 35 days after the RDX was added.

Several controls were used in these experiments. Plant controls, consisting of live plants in flasks with no RDX, were sampled to determine which compounds were only due to normal plant function, and not due to RDX toxicity or metabolism. Heat kill controls, consisting of plants that were autoclaved to ensure that there was no enzymatic activity, were

used to account for adsorption of RDX to plant tissue. Light controls, consisting of media with no plants, were used to account for photodegradation of RDX. Dark controls, consisting of a flask covered in aluminum foil, containing only sterile growth media, were used to account for evaporation of water from the media. These controls were used to help complete the mass balances and verify the fate of the RDX. Figure 3.3, below, shows a sample flask, a heat-killed control flask, and a light control flask.

Figure 3.3. A sample flask, a heat-killed control flask and a light control flask. Figure was taken when plants were 19 days old and 9 days after RDX was added to the flasks.

Extraction of Intracellular Metabolites

Identification and quantification of intracellular metabolites was completed using an organic extraction procedure. Approximately half of the total biomass from the sacrificed seedlings was freeze-dried, ground, suspended in methanol and then sonicated. The average quantity of biomass used for each plant extraction sample was 0.15 grams. The seedlings were first frozen at -80 °C and then were freeze-dried for 48 hours. The dried biomass was then ground into a powder. Twenty-five mL of methanol was added to the ground samples and then the samples were sonicated at 15 °C for 48 hours. The samples were then centrifuged and the methanol extract was decanted. An additional 25 ml of methanol was added to the plant samples, which were sonicated for an additional 48 hours. The samples were centrifuged again and the extract was decanted. A portion of the extracts was then reduced by evaporating off the methanol. A sample of both the dilute and the concentrated extracts were then analyzed using HPLC and the scintillation counter. The remaining plant sample with the bound intracellular metabolites was dried and then analyzed with the bio-oxidizer, following the procedure described above in analytical methods.

Results and Discussion

Arabidopsis Growth Curve Study

The results of the growth curve studies were used to observe the RDX toxicity and rate of RDX uptake by the plants. Varying amounts of RDX were added to one week old seedlings. Biomass was collected at several time points, and then the biomass was dried and weighed following the methods described above. The plant cultures were maintained under sterile conditions. No significant effect was observed on the growth of the seedlings at these concentrations when compared to controls. RDX was not toxic at these levels and/or the RDX was not toxic in the amount of time observed by the study. Growth curves from the experiments can be seen below in Figure 3.4. Since the growth rate does not vary with concentration, RDX does not appear to have a toxic effect on the growth of the seedlings, at the concentrations studied.

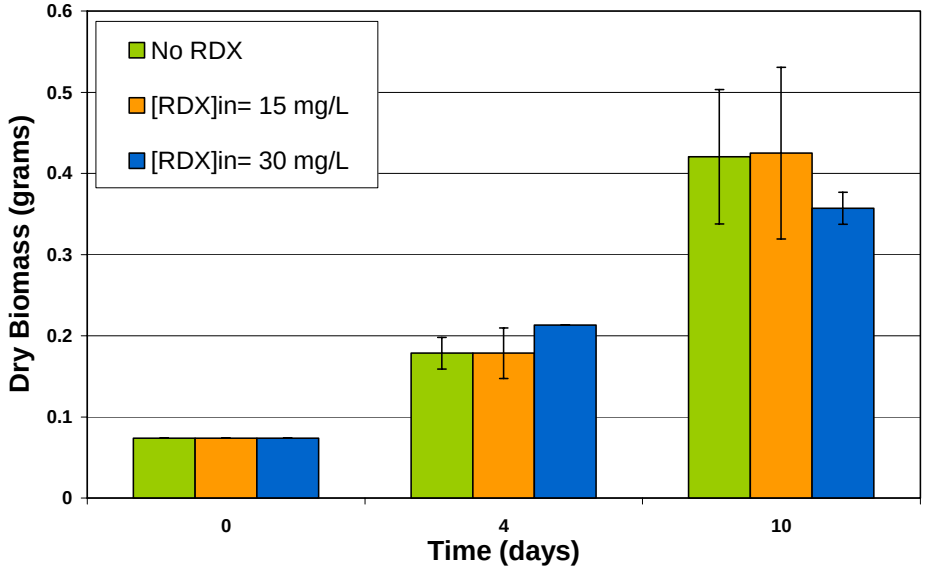


Figure 3.4a.

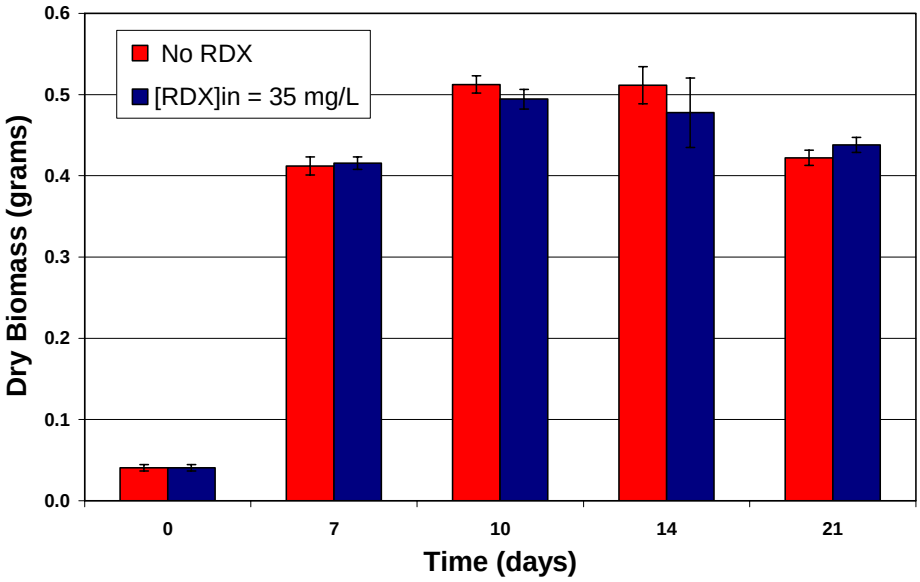


Figure 3.4b.

Figure 3.4. The figures above show the effect of RDX on growth of *Arabidopsis* seedlings during both growth curve experiments. The first experiment is shown in Figure 3.4a, and the second experiment is shown in Figure 3.4b. RDX was added to one week old seedlings and the dry biomass was measured at several time steps. The system was maintained under sterile conditions. RDX does not appear to have a significant effect on the growth of the seedlings. Duplicate or triplicate culture flasks were used for each measurement.

RDX Uptake Study

Initial uptake rates for RDX can be seen from these growth curve studies (Figure 3.5). Liquid samples were analyzed with HPLC to determine the concentration of RDX remaining in the media. The percentage of RDX was taken up by the plants at all concentrations. After 21 days, the plants reduced the RDX concentration from 35 mg/L to 10 mg/L. At 35 mg/L RDX, 70% of the RDX was removed from the system after 21 days. After 14 days, 80% of the 15 mg/L RDX was removed, with 60% removal observed at 30 mg/L RDX. The fraction of RDX removed by these systems can be seen below in Figure 3.5. Although the rate of uptake does not vary significantly, the plants were able to remove the RDX more quickly at lower concentrations. This demonstrates that *Arabidopsis* has the ability to remove RDX from contaminated water and soil. It also shows that it is possible to achieve lower levels of RDX contamination by allowing plants to take up RDX.

RDX Mass Balance Studies in *Arabidopsis*

Liquid media samples, plant samples and carbon dioxide trap samples were taken during the two experiments. These samples were analyzed for RDX and RDX metabolite concentrations, as well as carbon dioxide concentrations. This data shows that *Arabidopsis thaliana* plants removed the majority of the radio-labeled RDX from the media and completely converted over 11% of the initial RDX to carbon dioxide.

The total radioactivity in the media decreased in all flasks during the experiments. Figure 3.6 indicates that living plants removed radioactivity from the media at a faster rate than all of the controls (heat-killed controls, light media controls, and dark media controls). The living plants also removed more total radioactivity from the media than the controls after 35 days. Over 58% of the initial radioactivity was removed from the media by living plants after 35 days, while the controls removed less than 20%.

The media samples were also analyzed with HPLC to determine if there were any known metabolites (MNX, DNX, TNX, NDAB, and MEDINA) present in the media. The media samples did not appear to have any of these metabolites. The media was analyzed by HPLC using 100 µL samples from the 50 mL of media in each culture. It is likely that if the metabolites were present, the concentrations were too low for detection. All of the radioactivity in the media can be attributed to the presence of RDX in the media (Figure 3.7).

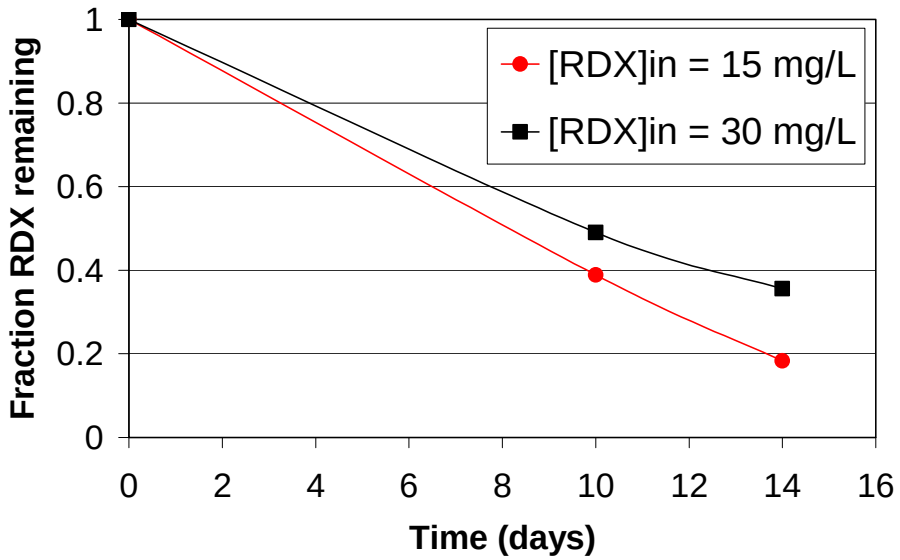


Figure 3.5a.

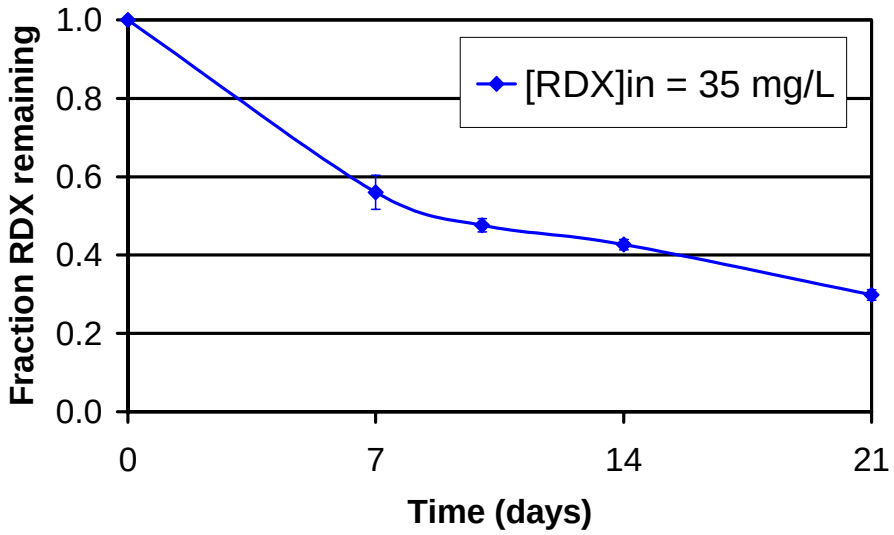


Figure 3.5b.

Figure 3.5. The figures above show RDX removal from *Arabidopsis* seedlings for both growth curve experiments. The first experiment is shown in Figure 3.5a, and the second experiment is shown in Figure 3.5b. Extracellular RDX is shown to be taken up by gradually by *Arabidopsis* seedlings, under sterile conditions. The plants were one week old when RDX was added to them. Lower concentrations of RDX were removed more quickly by seedlings. Concentrations were determined through reverse-phased HPLC, with a polar mobile phase and a Nova-Pak C8 column. Single culture flasks were sampled for the data in Figure 3.5a. Triplicate culture flasks were sampled for data in Figure 3.5b, except for the initial concentration where data from a single culture flask was used.

The radiographs for media samples are shown in Figures 3.8 and 3.9. Figure 3.8 illustrates the radioactivity present in the media from a living plant culture flask. The RDX peak (23 minutes) can be seen decreasing over the 35 day culture. The peak at 19 minutes may be MNX, but this cannot be verified by HPLC due to the low concentration, however the HPLC retention time for the MNX control was 18.8 minutes. The peak at 3 minutes is a stable contaminant from the radioactive RDX. The peak appears in all of the cultures and controls and it does not appear to change in concentration over the course of the experiment. Additional data on this peak is provided in Appendix A. Figure 3.9 shows the radiograph for a heat-killed control flask. The RDX peak does not reduce as much as in the living plant culture sample. This shows that the RDX is being removed by plant metabolism. Also, there is no MNX peak present at 19 minutes in the heat-killed control flask sample.

The total radioactivity in the carbon dioxide traps increased in all flasks during the experiments. The living plants mineralized RDX to form significant amounts of radioactive carbon dioxide, which can be seen in Figure 3.10. Flasks containing living plants converted RDX to carbon dioxide much quicker than controls, with over 10% of the initial radioactivity after 35 days. This is significantly more mineralization than the 0.5% that was observed in all of the control flasks. This would suggest that the ^{14}C-labeled carbon dioxide is only being produced by metabolism of RDX by living plants. The results of the mineralization of RDX in Arabidopsis are repeatable. Results from a prior experiment are shown in Appendix A.

The total radioactivity in the media decreased quicker and more ^{14}C-labeled carbon dioxide was produced in flasks with living plants. Over 58% of the radioactivity was removed from the media by the living plants after 35 days, while over 10% of the initial radioactivity was converted to carbon dioxide. This is significantly more mineralization than the 0.5% that was observed in the heat kill control flasks. The relationship between the decrease in radioactivity in the media and the increase in radioactivity in the carbon dioxide traps is illustrated in Figure 3.11.

In a prior RDX remediation experiment with *Arabidopsis*, one set of living plant culture flasks was sampled throughout the experiment and the potassium hydroxide was replaced with fresh potassium hydroxide at every time step. Other living plant culture flasks were used only to sacrifice for plant samples and the potassium hydroxide was never changed

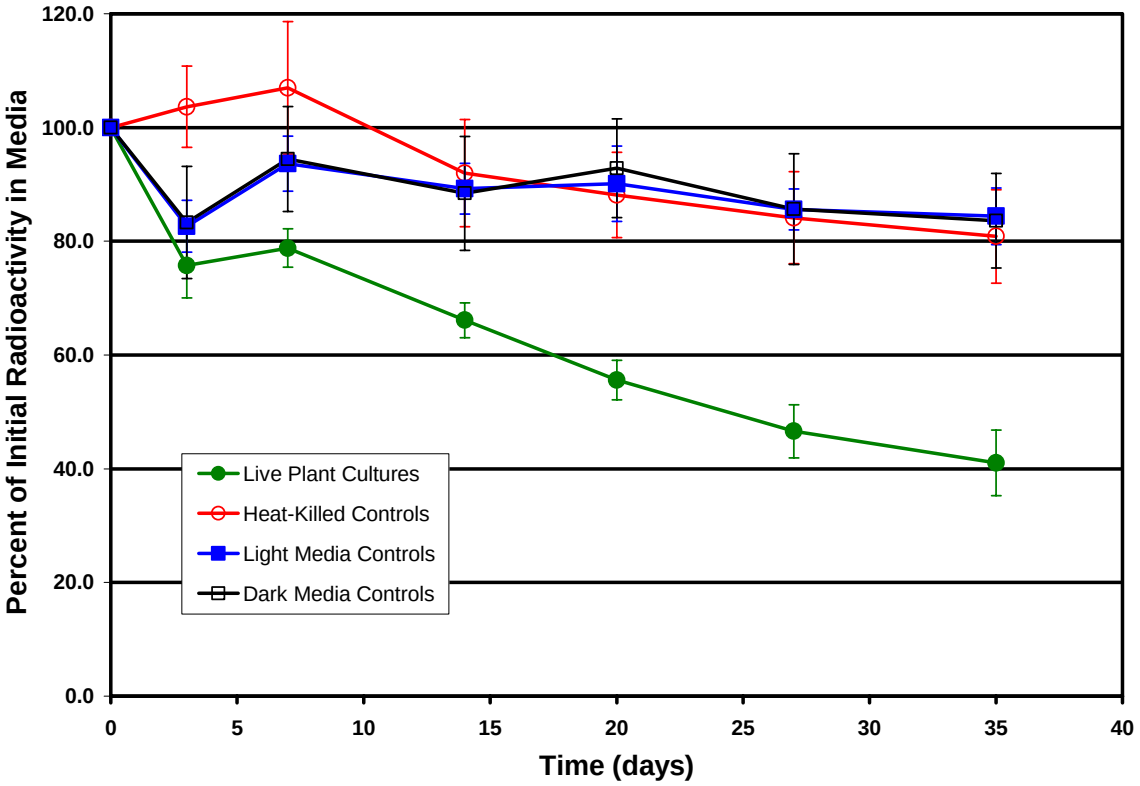


Figure 3.6. The figure above shows that the percent of total initial radioactivity in the media decreases in all flasks during the radio-labeled experiment. Radioactivity decreases much more rapidly in flasks with living plants. Over 58% of the radioactivity was removed from the media by the living plants after 35 days, while all of the controls removed less than 20%. Triplicate axenic *Arabidopsis thaliana* cultures were used for the living plant cultures and the heat-killed controls. Triplicate culture flasks were sampled for the light media controls and the dark media controls.

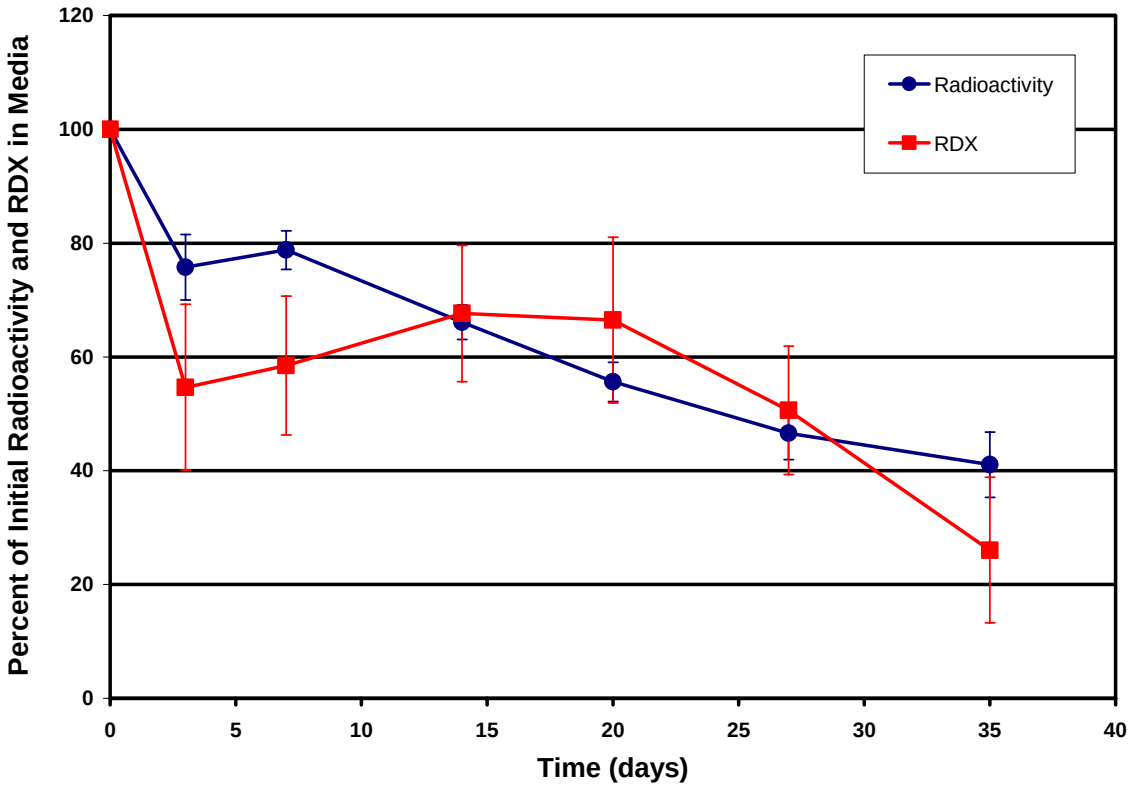


Figure 3.7. The figure above shows the percent of total initial radioactivity and the percent of the initial RDX. Radioactivity decreases at the same rate of RDX uptake. This indicated that the radioactivity present in the media is from RDX. Triplicate axenic *Arabidopsis thaliana* cultures were used for the living plant cultures and the heat-killed controls. Triplicate culture flasks were sampled for the light media controls and the dark media controls.

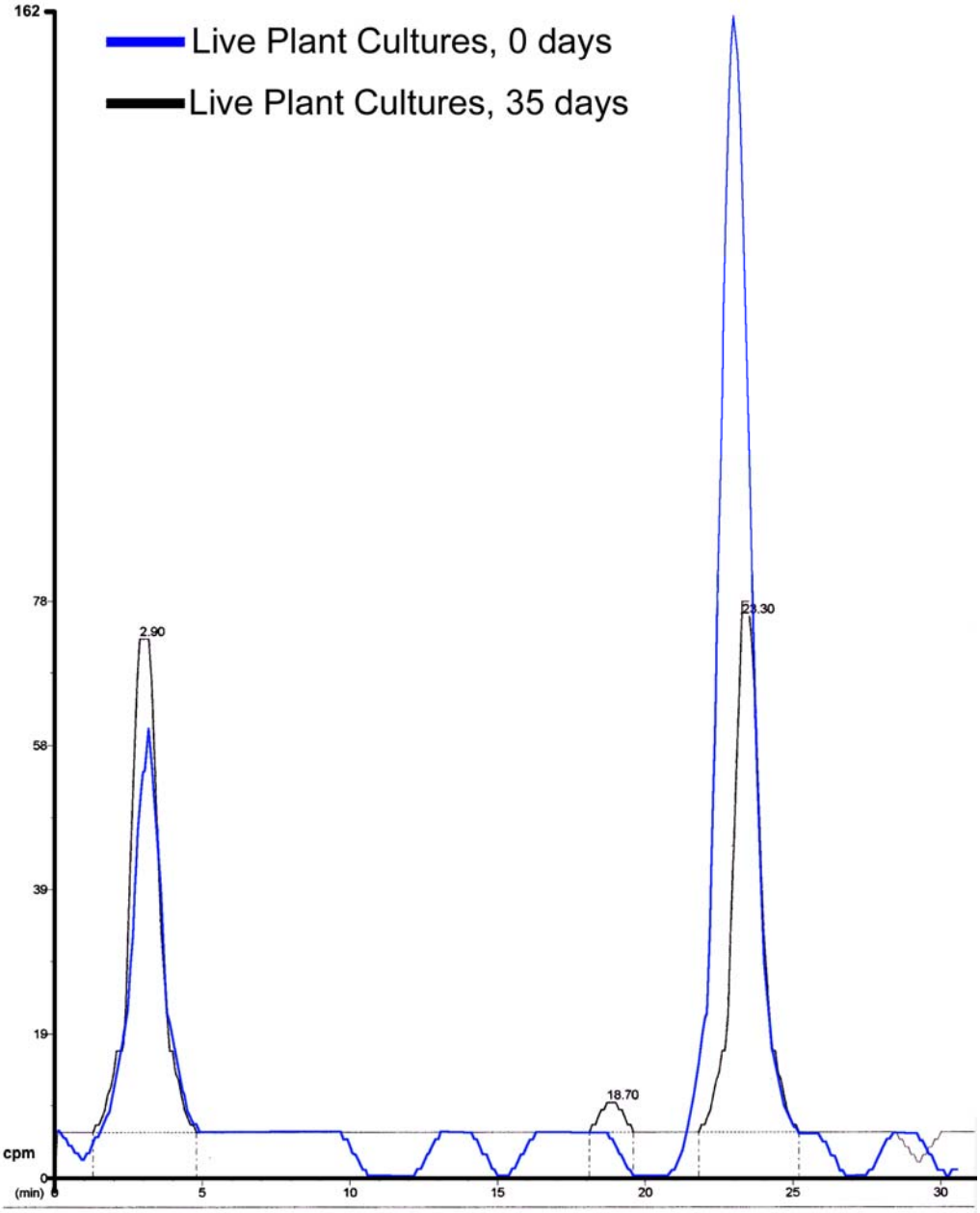


Figure 3.8. The radiograph for media samples from live Arabidopsis cultures is shown. The RDX peak at 23 minutes is decreasing during the experiment. The peak at 19 minutes could be MNX. The retention time is correct for MNX, but it was not detected with HPLC to allow verification. A peak from a ^{14}C-contaminant is visible at 3 minutes.

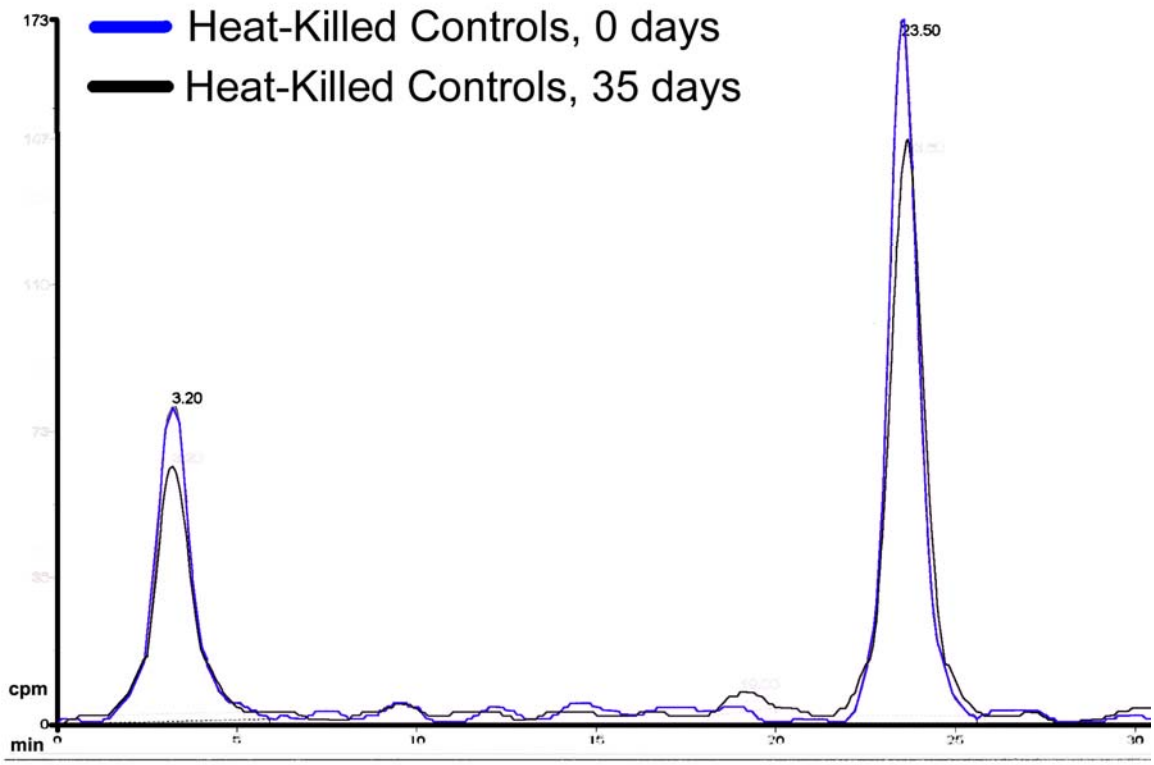


Figure 3.9. The radiograph for media samples from heat-killed controls is shown. The RDX peak at 23 minutes decreases during the experiment. The heat-killed controls did not break down as much of the RDX as the live plant cultures, shown in Figure 3.10.

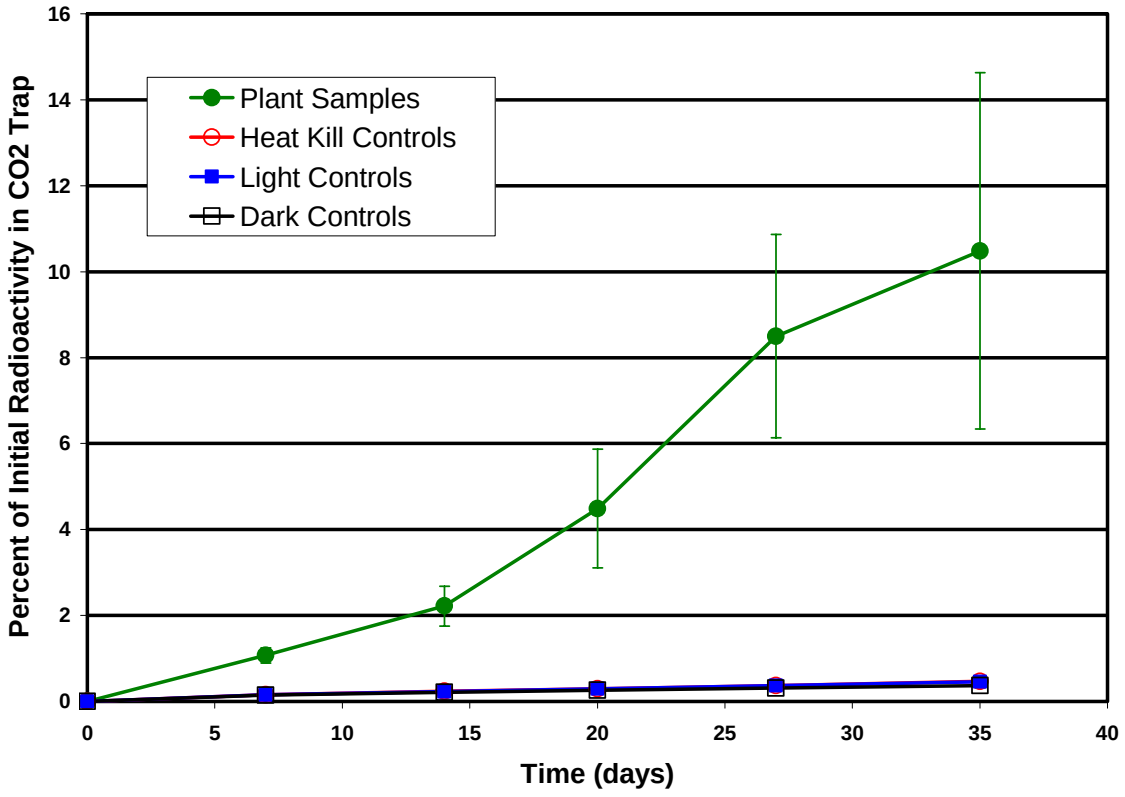


Figure 3.10. The figure above shows the percent of initial radioactivity in the carbon dioxide traps increase in all flasks during the radio-labeled experiment. Flasks containing living plants convert RDX to carbon dioxide much quicker than controls, with over 10% of the initial radioactivity converted after 35 days. This is significantly more mineralization than the 0.5% that was observed in all of the control flasks. Triplicate axenic *Arabidopsis thaliana* cultures were used for the living plant cultures and the heat-killed controls. Triplicate culture flasks were sampled for the light media controls and the dark media controls.

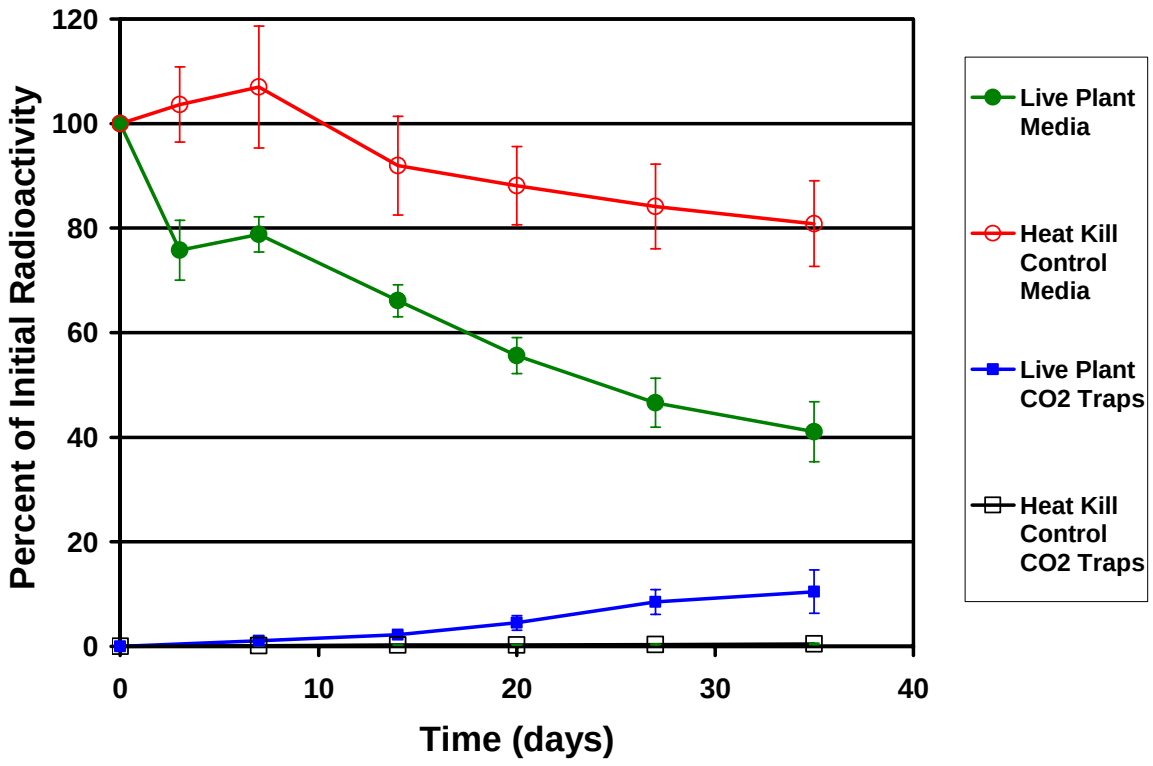


Figure 3.11. The figure above shows that the percent of initial radioactivity in the media decreases faster and the ^{14}C-labeled carbon dioxide is being produced more rapidly in flasks with living plants during the radio-labeled experiment. This shows that plant enzymatic activity is being used to metabolize the RDX to CO_2. Triplicate axenic *Arabidopsis thaliana* cultures were used for the living plant cultures and the heat-killed controls.

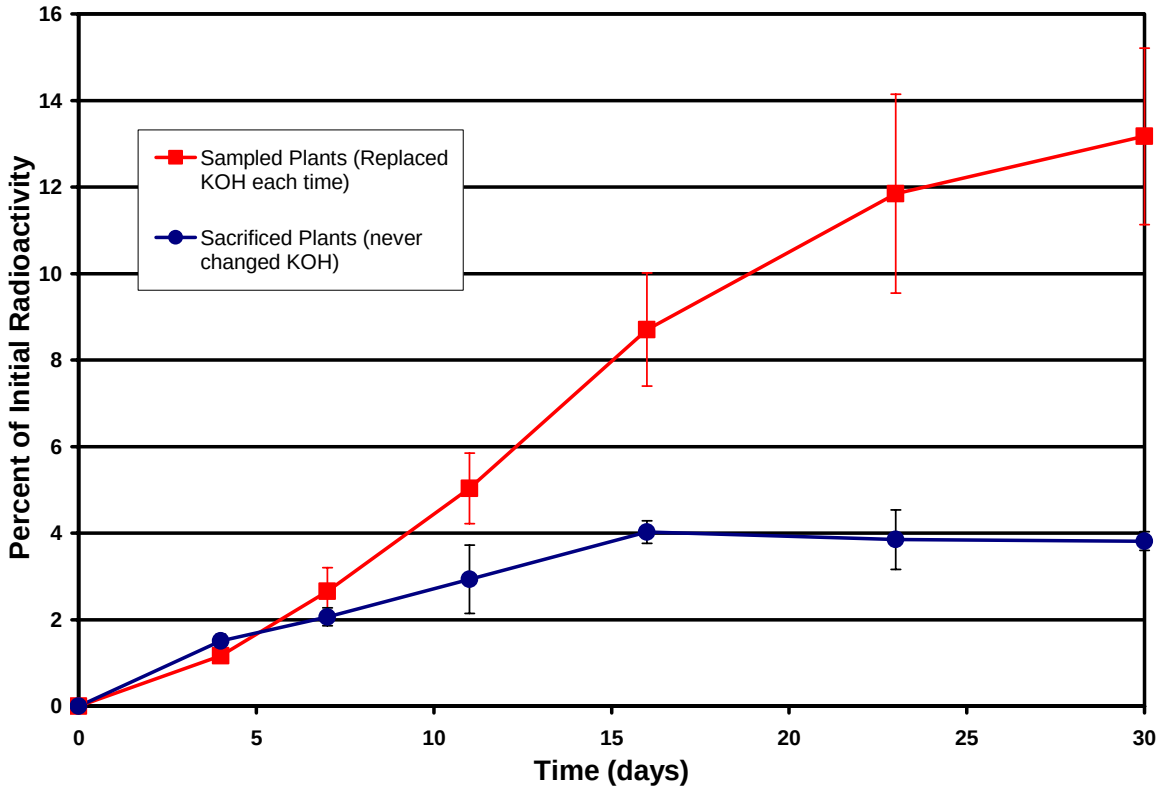


Figure 3.12. Potassium hydroxide was only changed in half of the flasks in the first experiment. The potassium hydroxide in the sacrificed flasks was saturated after 10 days. This shows the importance of replacing the potassium hydroxide at each time step. Triplicate axenic *Arabidopsis thaliana* cultures were used for the living plant cultures.

in these flasks. The sacrificed flasks absorbed significantly less carbon dioxide than the sampled flask, since the potassium hydroxide was saturated after 10 days (Figure 3.12). This shows the significance of replacing the potassium hydroxide at each time step. When this experiment was repeated, the potassium hydroxide was replaced in all flasks at each time step. The remainder of the results from this experiment can be found in Appendix A.

Radioactivity was present in the extracted plant portions for both the living plants and the heat kill controls. The living plants had significantly more radioactivity stored in the plant than the heat kill controls. At times during the experiment, the living plant cultures have over 20% of the initial radioactivity in the extractable portion of the biomass. The extract radioactivity data is shown in Figure 3.13.

The extract samples were also analyzed for RDX metabolites, but no metabolites were detected in the samples. RDX was identified as being present in the plant extract samples. The concentrations of RDX in the extracts are much smaller than the amount of radioactivity detected in the plant extracts (Figure 3.14). Additional radioactivity in the plant extracts must be from metabolites that were not detected with HPLC. Further analysis is needed to determine what compounds are present in the plant extracts.

Radioactivity bound to plant matter was measured by preparing dry extracted plant biomass and analyzing with the bio-oxidizer. The plant biomass was first dried by letting the remaining methanol evaporate completely. The plant biomass was then reground and placed in the bio-oxidizer. The plant samples were combusted and the carbon dioxide generated was trapped in CarboSorb E cocktail. A portion of these samples were then mixed with PermaFluor E^+ cocktail and analyzed with the liquid scintillation counter. Radioactivity bound to plant matter is illustrated below in Figure 3.15. The plant cultures are incorporating a portion of the initial RDX into their biomass.

Mass balances were performed by totaling the amount of radioactivity in the media, the carbon dioxide trap, the plant extracts and the portion bound to plant biomass. This sum represents all of the radioactivity that is accounted for in the experiment. The completed mass balance is shown in Figure 3.16. By reviewing the mass balance, it is possible to see how the radioactivity is distributed throughout the plant system over time. The RDX begins in the media, and then is converted to carbon dioxide or stored in the plant. Since no metabolites were detected with HPLC in the media or in the plant extracts, it is possible that

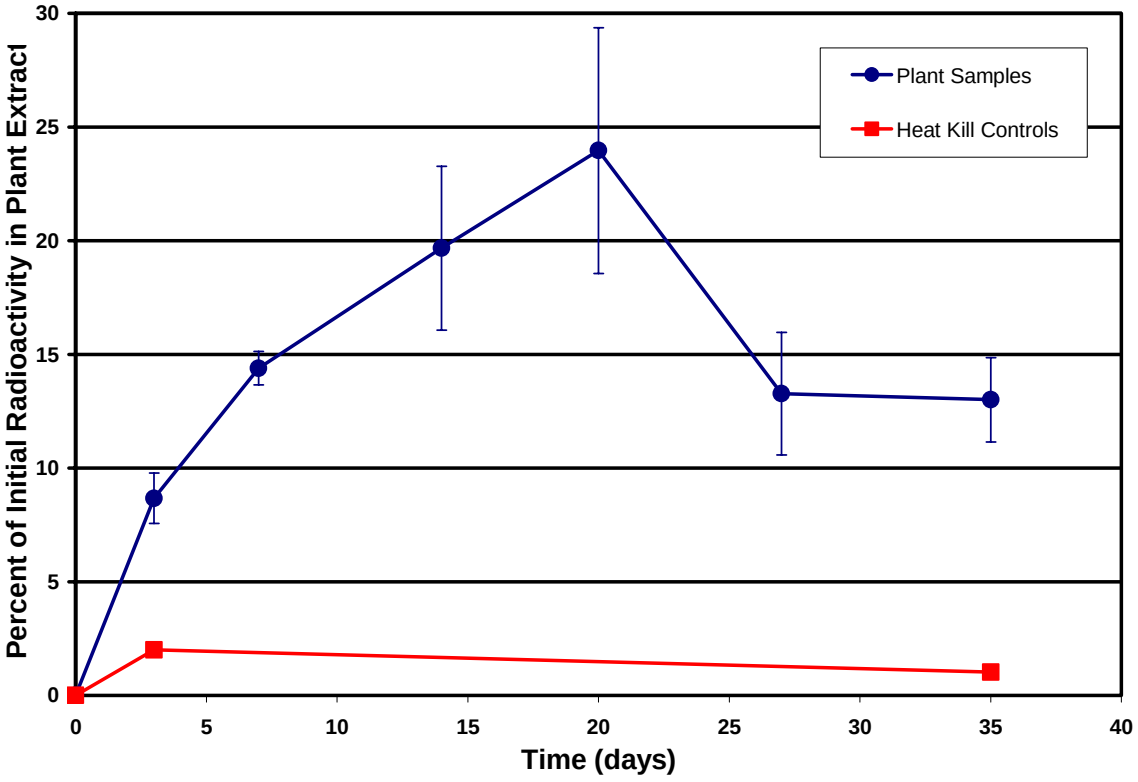


Figure 3.13. The figure above shows the percent of initial radioactivity in the plant extracts increases over time in flasks with living plants during the radio-labeled experiment. This shows that the living plant is taking up the RDX. Triplicate axenic *Arabidopsis thaliana* cultures were used for the living plant cultures and the heat-killed controls.

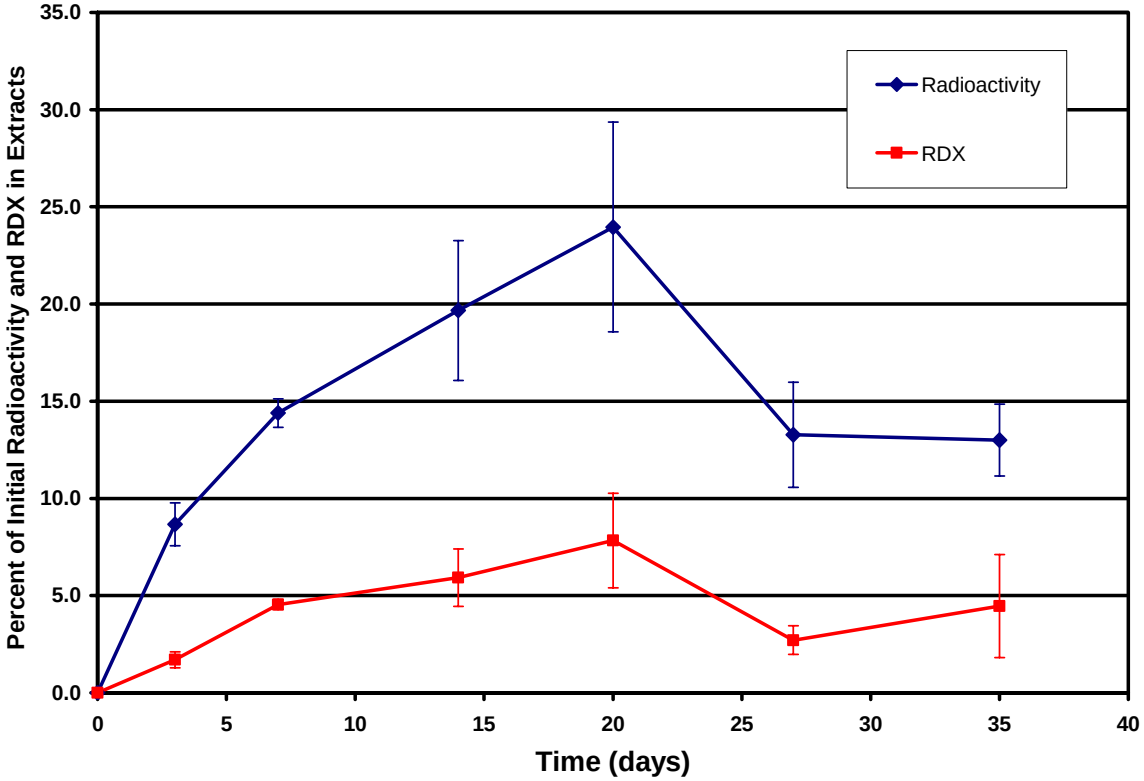


Figure 3.14. The figure above shows that the percent of initial radioactivity in the plant extracts increases quicker than the percent of initial RDX. This shows that the plants are converting RDX to metabolites and the metabolites are present in the plant extracts. The difference between the two lines represents the amount of metabolites present in the plant extracts. Further analysis is needed to determine what metabolites are present in the plant extracts. Triplicate axenic *Arabidopsis thaliana* cultures were used for the living plant cultures.

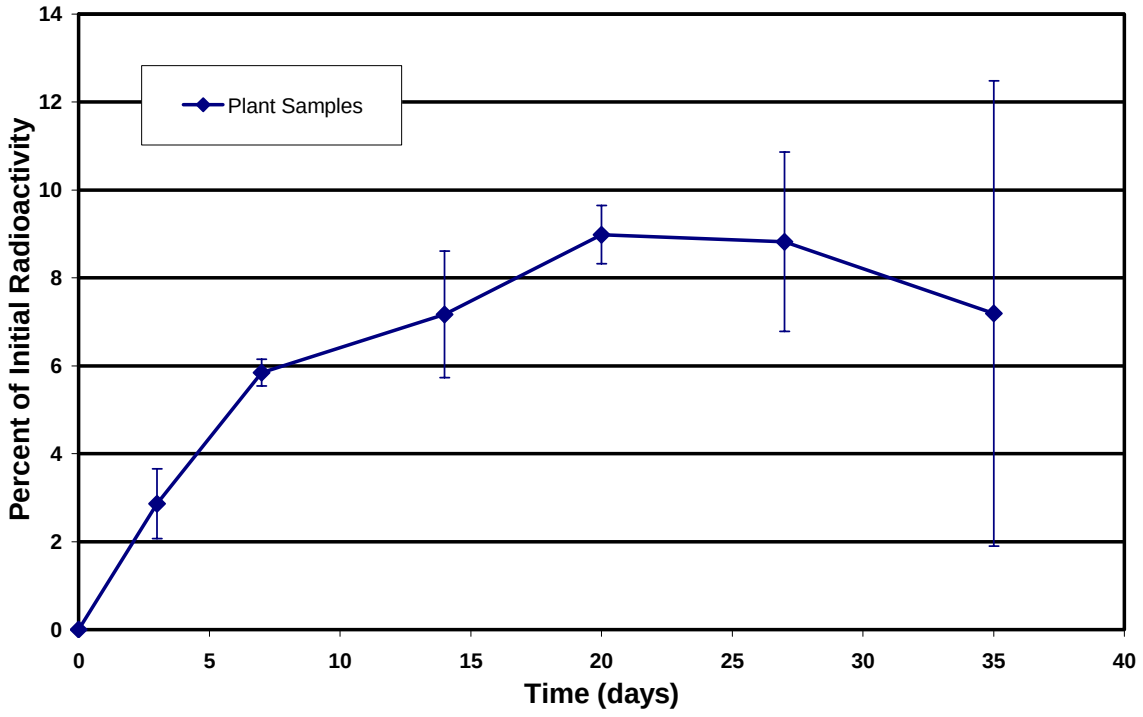


Figure 3.15. The above figure shows the percent of initial radioactivity bound to the plant matter increasing over time in flasks with living plants during the radio-labeled experiment. This shows that the plants are incorporating the RDX into the plant matter. Triplicate axenic *Arabidopsis thaliana* cultures were used for the living plant cultures.

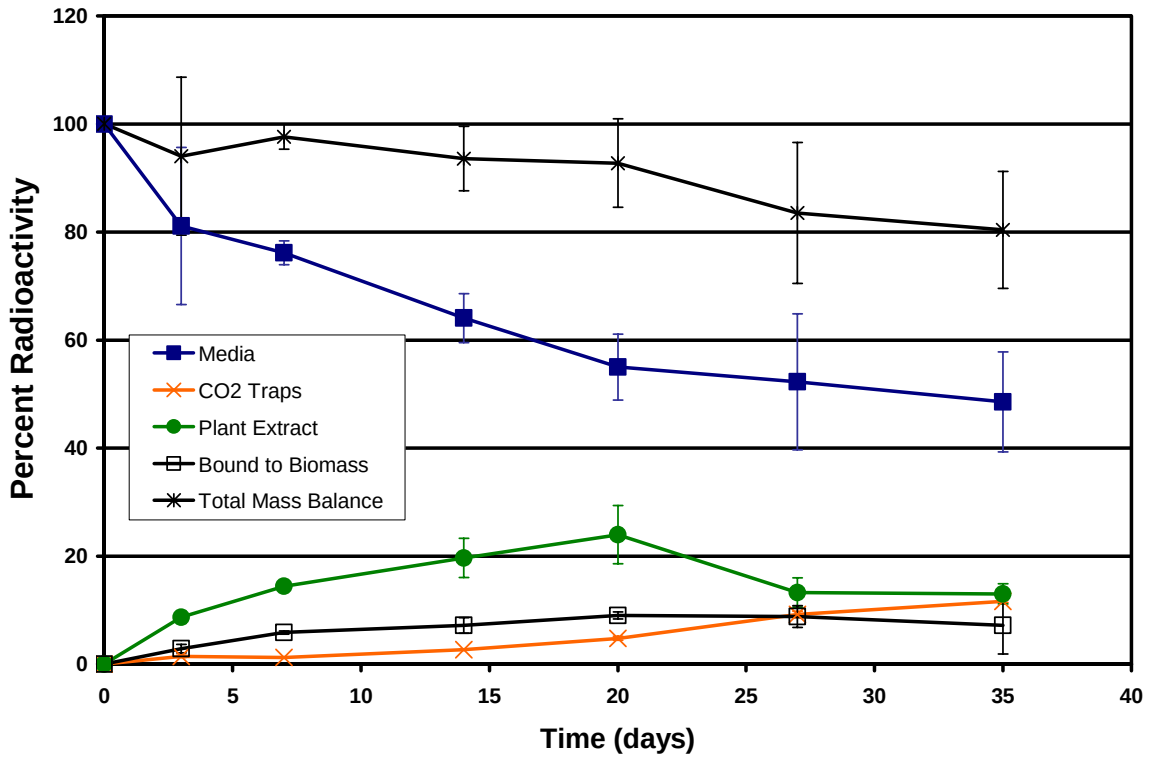


Figure 3.16. The figure above shows the total radioactivity from the mass balances for the living plant cultures in the radio-labeled experiment. The plants are taking the RDX from the media, and converting some of the RDX to CO_2. The remaining radioactivity is either in the extractable portions of the plant or incorporated into the plant biomass. This shows that the plants are actively metabolizing RDX. Triplicate axenic *Arabidopsis thaliana* cultures were used for the living plant cultures.

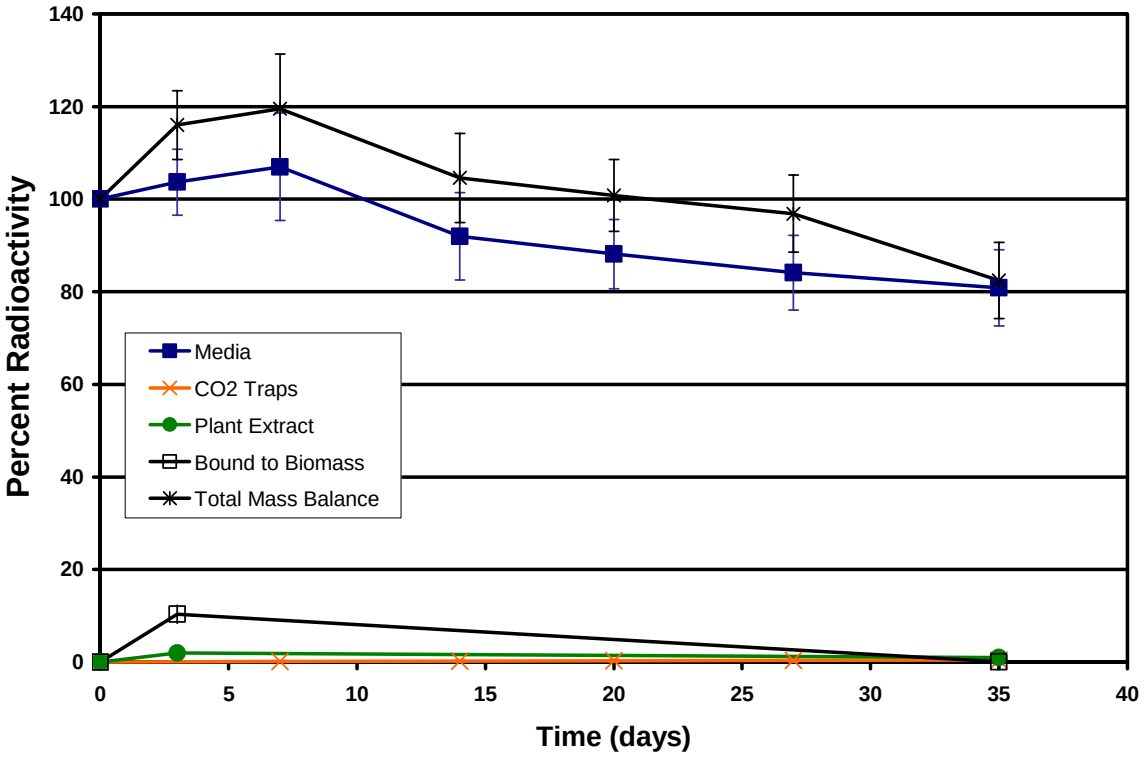


Figure 3.17. The figure above shows the total radioactivity from the mass balances for the heat-killed controls from the radio-labeled experiment. Very little radioactivity if removed from the media, and even less is converted to CO_2. Slight amounts of the remaining radioactivity are in the extractable portions of the heat-killed plants and incorporated into the heat-killed plant biomass. This shows that very small amounts of RDX are degraded without living plants. Triplicate axenic *Arabidopsis thaliana* cultures were used for the heat-killed plant cultures.

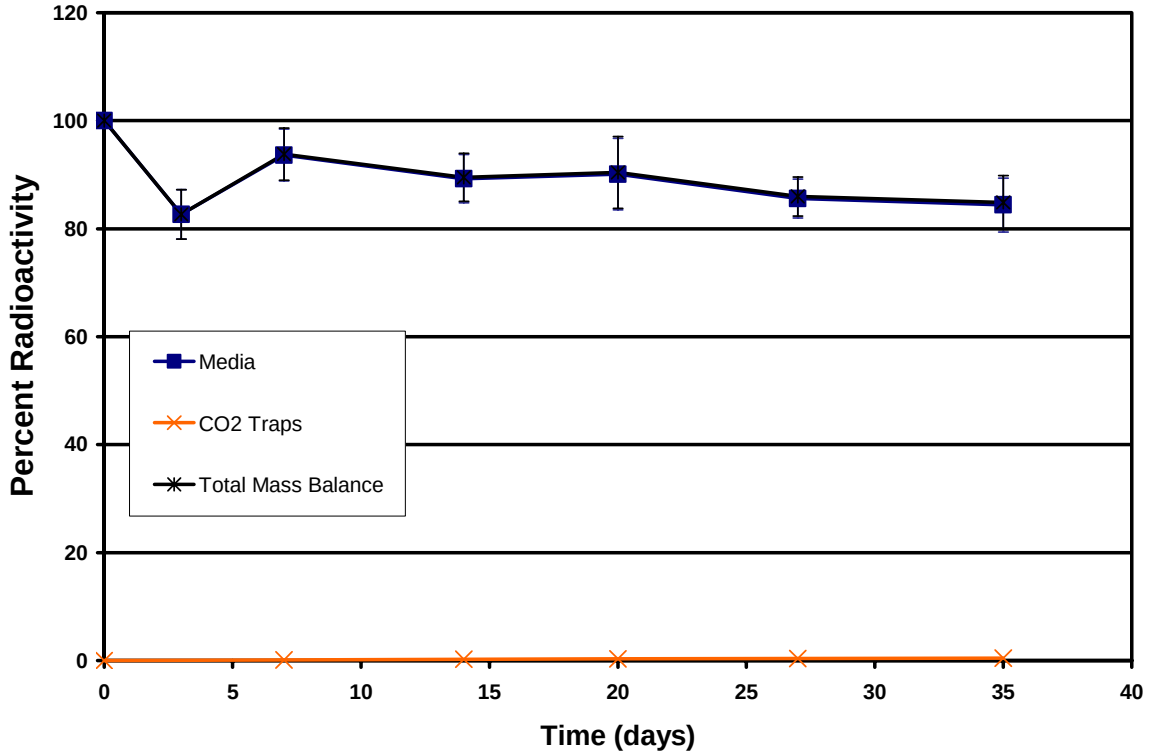


Figure 3.18. The figure above shows the total radioactivity from the mass balances for the light media controls from the radio-labeled experiment. The majority of the RDX remains in the media throughout the experiment. All of the radioactivity detected was from RDX. This shows that about 15% of the RDX was degraded without plants. Triplicate culture flasks were used for the light media controls.

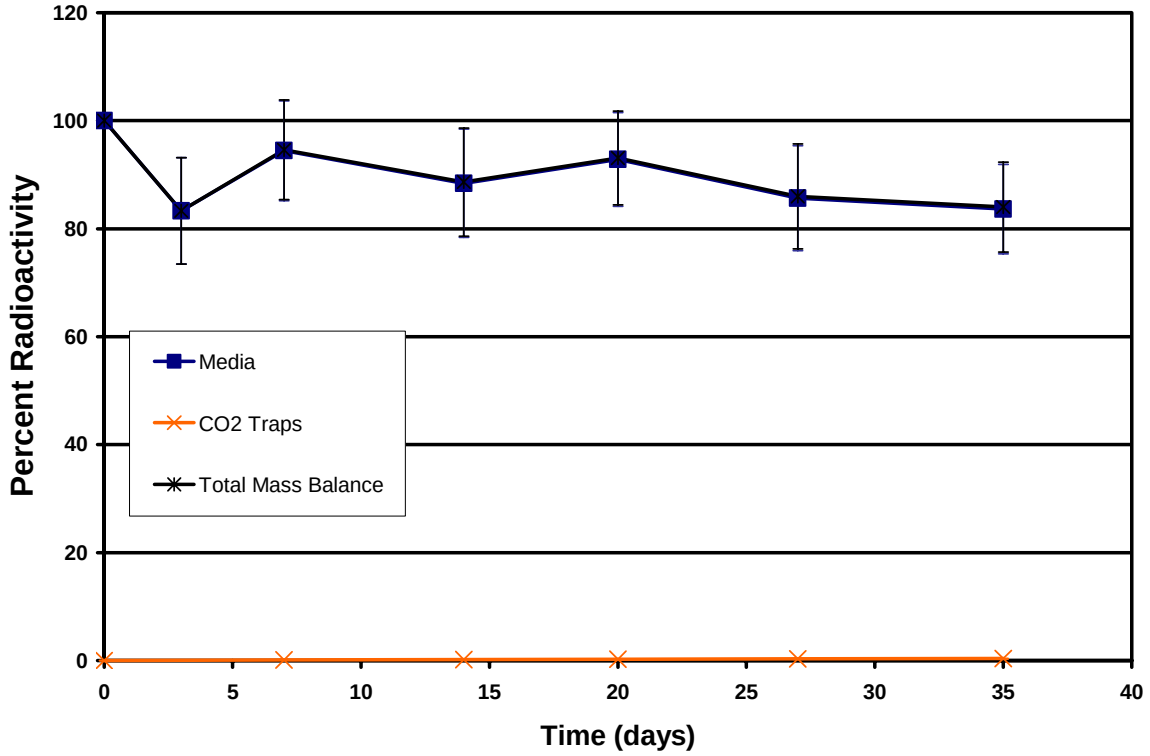


Figure 3.19. The figure above shows the total radioactivity from the mass balances for the dark media controls from the radio-labeled experiment. The majority of the RDX remains in the media throughout the experiment. All of the radioactivity detected was from RDX. Similar to the light controls (Figure 3.19), about 15% of the RDX is degraded over the experiment. Triplicate culture flasks were used for the dark media controls.

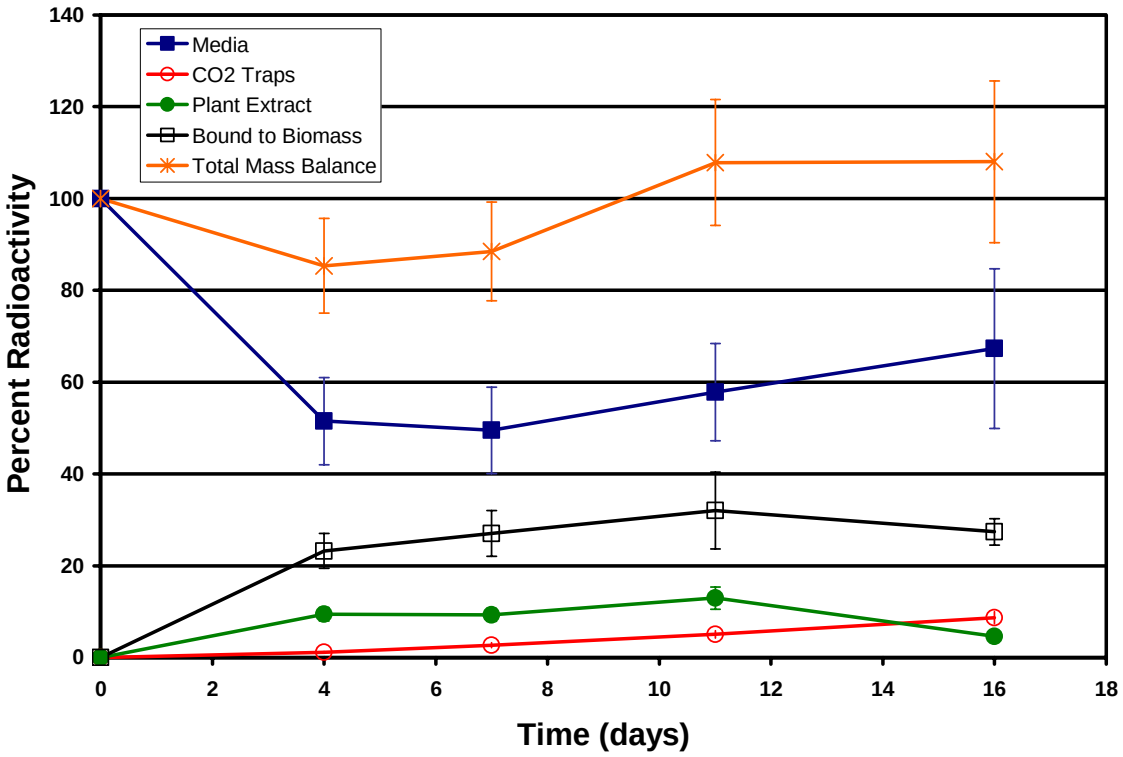


Figure 3.20. The figure above shows the total radioactivity from the mass balance for the living plant cultures from a prior radio-labeled experiment. The plants are taking the RDX from the media, and converting some of the RDX to CO_2. The remaining radioactivity is either in the extractable portions of the plant or incorporated into the plant biomass. This shows that the plants are actively metabolizing RDX. Triplicate axenic *Arabidopsis thaliana* cultures were used for the living plant cultures. This verifies that the results from the mass balance shown in Figure 3.16 are repeatable.

the conversion of RDX to MNX is the rate limiting step. The metabolites might be broken down as quickly as they are created, resulting in very low concentrations of the metabolites, which would explain the detection of only RDX and carbon dioxide. Media samples and plant intracellular extract samples could be concentrated down and reanalyzed with HPLC and the liquid scintillation counter to identify which RDX metabolites are present.

The results of the mass balances for the controls are shown in Figures 3.17, 3.18, and 3.19. These controls indicate that RDX is being degraded by the living plant cultures. The controls have less that 20% of the radioactivity removed from the media. This is much less than the 50% removed by the living plant cultures. The controls in this research also show that less than 1% of the RDX was mineralized to CO_2, which the living plant cultures mineralized over 10%. There is no difference between the amount of radioactivity left in the media for the light media controls and the dark media controls. This indicates that RDX was not degraded by light in this experiment. Just and Schnoor (2004) observed complete almost transformation of RDX in aqueous solution by light in 140 hours (41). However, their experiment used a large photon flux of 3900 μmol/(m^2*sec), which is about twice the amount generated by normal sunlight. The experiment was also conducted special far-UV cells to hold the aqueous solution, so that over 80 percent of the light was transmitted into the containers. The conditions used by Just and Schnoor were much different from the conditions used in this research, and could account for the large difference in the results. Additionally, the results of the experiment are repeatable. Figure 3.20 shows the results from a prior experiment with Arabidopsis. The same results for RDX uptake and mineralization were observed.

The results of the mass balance show how important the carbon dioxide trap was to the experiment. A significant amount of the initial radioactivity (11.64 percent ± 0.74) was converted to carbon dioxide after 35 days. This indicates that *Arabidopsis* is capable of completely mineralizing RDX to carbon dioxide in a liquid culture. The presence of complete mineralization is an important part of this study due to the fact that this is the first time that it has been shown with an axenic plant culture.

VanAken, *et al*., (2004), did a similar study on the phytotransformation of RDX in poplar tissue cultures. After 60 days, almost all of the RDX has disappeared from the media, and 17% was converted to CO_2. After 8 days, when approximately 50% of the RDX had

been removed from the media, about 12% of the RDX had been converted to CO_2 (42). This is very comparable to the rate of mineralization that was observed with *Arabidopsis*. It should be noted that bacteria was isolated from the poplar tissue culture and the bacteria may have been responsible for the mineralization of RDX. The *Arabidopsis* cultures showed that plants can metabolize RDX and it is possible that the poplar tissue cultures were metabolizing the RDX. In a bacterial study by Van Aken, *et al.* (2004), *Methylobacterium* was isolated from poplar tissue cultures and studied to determine how well it could metabolize RDX. After 40 days, the *Methylobacterium* had reduced the RDX from 20 mg/L to nondetectable levels, with 58% of the RDX being mineralized to carbon dioxide. When 50% of the RDX had disappeared from the media, about 30% had been completely mineralized to carbon dioxide. MNX was also observed early in the experiment and MEDINA was formed slowly over the course of the experiment (46). The bacterial study showed significantly higher levels of RDX transformation and mineralization than was observed with the poplar tissue cultures (42, 46). It is unclear if the poplar tissue cultures were responsible for the metabolism of RDX in the previous study by Van Aken, *et al.*, or if the phytosymbiotic bacterium was breaking down the RDX.

Fournier, *et al*., (2004), observed biodegradation of RDX by bacteria in aerobic cultures. *Rhodococcus* sp. strain DN22 mineralized up to 30% of the RDX after 26 days under aerobic conditions, with another 64% being converted to NDAB. The bacteria had difficulties converting the NDAB to carbon dioxide, so a large percentage of the RDX ended as NDAB (27). An anaerobic study by Zhao, *et al*., (2004), observed 50% of the RDX being mineralized to CO_2 in the presence of microcosms. The rate of mineralization in this study is also comparable to the mineralization observed with *Arabidopsis*. After 4 days, when 50% of the RDX was degraded, about 5% of the carbon had been converted to carbon dioxide (28). These studies show that the rate of conversion to CO_2 observed in *Arabidopsis* is comparable to other plants and bacteria.

No metabolites were observed in the media or in the plant extracts for *Arabidopsis*. VanAken, *et al*., (2004), did observe MNX and DNX in small percentages with poplar tissue cultures; the metabolites were fully degraded by the end of the experiment. After 8 days, 14% of the RDX had been converted to MNX. After 16 days, 4% of the RDX was converted to DNX. Polar metabolites, formaldehyde and methanol, were also observed increasing in

concentration during the experiment (42). These results are consistent with the phytoremediation pathway proposed by Schnoor and co-workers (Figure 2.4). Due to the low levels of metabolites that are present is plant cultures, it is likely that MNX and DNX were present in the *Arabidopsis* cultures, but the concentrations were too low for detection. Future work will include concentrating the media samples in order to reanalyze the samples for the nitramine metabolites, verifying that MNX and DNX are present in the *Arabidopsis* cultures. Formaldehyde and methanol may also have been in the *Arabidopsis* media samples. Standard analytical methods for detecting the nitramine metabolites are not capable of detecting the polar metabolites. Therefore, it was not possible to detect formaldehyde and methanol by the analytical methods used in this study. In the future, media samples could be concentrated and reanalyzed with methods developed for the detection of the polar metabolites to determine if they are present in the *Arabidopsis* cultures.

Conclusions

The growth curves showed that up to 35 mg/L RDX was not toxic to *Arabidopsis thaliana* by comparing the dried biomass of plants exposed to RDX with the dried biomass from the controls. The uptake curves indicated that RDX was taken up quickly and could be reduced to less than 30% after 21 days. ^{14}C-labeled studies showed that over 13% of the RDX was mineralized to carbon dioxide after 35 days. RDX was seen in the media and in plant extracts, but no metabolites were seen with HPLC. The mass balance indicated that the majority of the radioactivity was accounted for. These results indicate that RDX is quickly taken up by *the Arabidopsis* plants, and then slowly converted to the nitroamine metabolites. The nitroamines are then quickly broken down into smaller compounds, like carbon dioxide. This hypothesis is supported by the uptake of RDX, appearance of carbon dioxide, and the absence of the metabolites. Reanalysis of the samples needs to be performed to verify that MNX and DNX were present in the media samples. Metabolite feeding studies could also be conducted in the future to help confirm the RDX degradation pathway and determine transformation rates.

Chapter 4 - Screening Assay Determination and Screening Mutants for RDX

Introduction

The objective of this research was to use *Arabidopsis thaliana* mutants to determine if there are specific genes that promote RDX uptake and remediation and to establish its metabolic pathway in plants. Very little research has been focused on genetic mutations which enhance RDX uptake and metabolism. Important genetic sequences need to be identified to help understand more about the RDX pathway in plants. With understanding of these transformation pathways, native plants could be selected that would improve the transformation of RDX. Plants could be engineered to enhance transformation from knowledge of how genetic manipulation affects remediation. *Arabidopsis thaliana* was chosen as a model plant because of its small size, short life span, and relatively simple and fully-sequenced genome (47).

The approach to find important genes involved in the transformation pathways involved screening *Arabidopsis thaliana* mutants to determine if there were common genes that enhance remediation and/or limit the toxicity of the energetic materials to the plant, along with establishing a biochemical pathway for energetic materials. In other work in our research group, two libraries of *Arabidopsis* mutants (T-DNA and Enhancer-Trap) were screened to determine if any performed better than the wild-type plants in the presence of high TNT concentrations. Selected TNT mutants were genetically compared to determine if there are similarities between mutants that have an increased resistance to TNT toxicity as well as remediating TNT efficiently. Since the *Arabidopsis* genome has been sequenced, it has been possible to identify several genes involved in the metabolism of TNT.

Before *Arabidopsis thaliana* mutants could be screened and studied with RDX, the toxicity level for *Arabidopsis thaliana* wild-type plants had to be determined. By utilizing the fact that RDX is toxic to plants we used varying concentrations of RDX to screen *Arabidopsis thaliana* mutants for mutants that could survive high levels of RDX. Initial concentrations of RDX to study were estimated from previous phytoremediation work (41-45). Various concentrations of RDX were tested on *Arabidopsis* wild-type seeds to evaluate the toxicity effects that RDX has on the germination in solid media. The time of germination was recorded for each concentration of RDX. The size of the plants was monitored for

several weeks to determine how wild-type plants respond to growing in media containing RDX. The result of the wild-type screen was used to identify the conditions that should be used to screen mutant seeds. The concentration of seeds was also determined by the assay (1400 mg/L RDX and 2000 seeds per plate). These conditions were then used to screen *Arabidopsis* mutant seeds and select plants that perform better than the wild-type in the presence of high RDX concentrations. The results from the germination screen and the root length screen will be shown.

Materials and Methods

Chemicals

Bulk RDX in acetone (50 mg/ml) was purchased from AccuStandard (New Haven, CT). RDX standards (1mg/ml RDX in acetonitrile) were purchased from ChemService (West Chester, PA).

Half-strength Murashige and Skoog (MS) medium was used for all experiments. Medium consisted of 2.2 g of MS salt mixture, 3 mL of a 6% potassium phosphate monobasic (KH_2PO_4) solution, 1 mL of Gamborg's vitamin B_5 solution, 20 g of sucrose, and 0.5 g of 99% MES (2-morpholinoethanesulfonic acid) hydrate added to 1 L of water. The pH was adjusted to 5.8 with NaOH. Phytagel was then added to the media (2.0 g/L media) and the media was autoclaved (47).

The MS salt mixture was purchased from Gibco BRL Life Technologies (Carlsbad, CA). Potassium phosphate mono-basic was obtained in crystal form from Fisher Scientific (Hampton, NH). Gamborg's vitamin B_5 solution came from Sigma-Aldrich (St. Louis, MO). Sucrose was purchased from MP Biomedicals (Aurora, OH). 99% MES hydrate was obtained from Acros Organics (Morris Plains, NJ). Potassium hydroxide was also purchased from Fisher Scientific. Phytagel was obtained from Sigma-Aldrich.

All solvents, including acetonitrile, acetone, and ethanol, were purchased from Fisher Scientific.

Plants

Arabidopsis thaliana was grown axenically from seeds. Seeds were placed in a microtube and were sterilized using various methods. The seeds were then transferred to sterile Petri dishes with half-strength MS media. The plates were kept at 22 °C, under continuous light for all experiments (47).

Screening Assay Determination

Seeds were sterilized by one of two methods. The first method consists of applying 20% bleach to seeds for 15 min and then rinsing with sterile water three times. The second method sterilizes seeds by exposing seeds to chlorine gas for 3 hours. The chlorine gas was formed by combining 200 mL bleach with 20 mL concentrated HCl in a closed beaker. Seeds were in open microtubes in a tray suspended in the same container. The container with both the seeds and the gases was sealed and the bleach and HCl were gently mixed to form chlorine gas. Seeds were left in the chlorinated beaker for 3 hours.

Medium was autoclaved and then cooled in a 65°C water bath. RDX and acetone were added to the medium which was then poured into sterile petri dishes. After the petri dishes were then cooled to let the media solidify, the sterilized seeds were plated on the media. Lids were placed on the petri dishes, which were sealed with Parafilm to prevent evaporation and reduce the risk of contaminating the plates. Plates were then placed in a cold room at 4°C. After three days, the plates were then moved to the growth room to germinate, where they were kept at 22°C with continuous light. Seeds were monitored to record the time of germination and the size of the plants over several weeks. Some plants were then transferred to clean media, with no acetone or RDX, or to soil to observe if they would be able to fully mature after exposure to high RDX concentrations. Mutant seeds germinated in media in the growth room and then mutants were selected for transplantation to soil. Selected mutants were chosen on health and size in comparison to the majority of mutants and wild-type controls. Final selected conditions, concentration of RDX and concentration of seeds, allowed for the clear observation of variation in size and condition of plants in order to select mutants to transplant.

Testing for the germination of wild-type *Arabidopsis thaliana* was conducted at several different concentrations of RDX in *Arabidopsis* growth media. RDX levels from 0 to

2000 mg/L RDX were tested, while testing focused on concentrations from 1000 to 1500 mg/L RDX. Toxicity due to seed concentration was also tested. Toxicity of RDX to germination varied due to both RDX concentration and seed concentration, where 2000 mg/L RDX was the most toxic RDX concentration and lower seed concentrations also showed an increase in toxicity.

Wild-type seeds were grown in solid media containing various concentrations of RDX in order to determine the toxicity of RDX on germination. Concentrations of RDX in the media ranged from 0 mg/L to 2000 mg/L. RDX was first obtained in a solution of acetonitrile (1 mg/mL) from ChemService. RDX was also obtained in a more concentrated solution of acetone (50 mg/mL) from AccuStandard. Solvent concentrations were kept the same throughout a screening assay in order to compare the effect of the RDX only. Four replicates were used for each concentration of RDX studied. Two controls were used for the screening, unless noted. One control consisted of media with no solvent or RDX added, while the other control consisted of media with solvent only. The combined toxic effects of RDX and the solvent could be monitored by comparison with the first control. The second control permitted the toxic effects of RDX alone to be evaluated. This allowed us to observe the effect of several concentrations of RDX on germination, by comparing those seeds to the controls. Various concentrations of seeds were also used to observe the effect that seed concentration had on RDX toxicity. Seed density ranged from 50 to 2000 seeds per plate.

The first experiment was conducted with RDX in acetonitrile. Table 4.1 shows the conditions used for the different plates. Only one type of control was used to see the effect of acetonitrile alone on the seeds. The concentration of acetonitrile was kept the same by first adding RDX in solution to the media and then adding additional acetonitrile to the plates with lower RDX concentrations to equilibrate. Four replicates of each concentration were planted with seeds.

The next experiment used RDX dissolved in acetone. Four replicates of each concentration were planted with *Arabidopsis thaliana* WT seeds. Two types of controls were used. The first control verified that the seeds would germinate without the presence of RDX and acetone. The first control also showed that the seeds were not damaged or killed during sterilization and that any toxic effects were from RDX or acetone. The second control verified that the seeds would still germinate in the presence of acetone. Any toxic effects

that the samples showed when compared to the second control must have been from the presence of RDX alone. The concentration of acetone was kept the same by first adding RDX in solution to the media and then adding additional acetone, if needed, to equilibrate. The experimental conditions can be seen in Table 4.2.

Table 4.1. Preliminary experiment with RDX in acetonitrile.

2/2003	**[RDX]**	**[acetonitrile]**	**[seeds/plate]**
Control	0 mg/L	40 mL/L	50
Condition 1	10 mg/L	40 mL/L	50
Condition 2	20 mg/L	40 mL/L	50
Condition 3	30 mg/L	40 mL/L	50
Condition 4	40 mg/L	40 mL/L	50

Table 4.2. Experiment #1 with RDX in acetone.

6/23/2003	**[RDX]**	**[acetone]**	**[seeds]**
Control	0 mg/L	0 mL/L	50
Control	0 mg/L	5 mL/L	50
Condition 1	50 mg/L	5 mL/L	50
Condition 2	100 mg/L	5 mL/L	50
Condition 3	150 mg/L	5 mL/L	50
Condition 4	200 mg/L	5 mL/L	50
Condition 5	250 mg/L	5 mL/L	50

Four replicates of each concentration were planted with *Arabidopsis thaliana* WT seeds. Two controls were again used for the same reasons as previously stated. Again, any toxic effects that the samples showed when compared to the second control were from the presence of RDX alone. The concentration of acetone was kept the same by first adding

RDX in solution to the media and then adding additional acetone, if needed, to equilibrate. Seeds were transferred to the growth room after four days in the cold room.

Table 4.3. Experiment #2 with RDX in acetone.

7/3/2003	**[RDX]**	**[acetone]**	**[seeds]**
Control	0 mg/L	0 mL/L	50
Control	0 mg/L	40 mL/L	50
Condition 1	500 mg/L	40 mL/L	50
Condition 2	1000 mg/L	40 mL/L	50
Condition 3	1500 mg/L	40 mL/L	50
Condition 4	2000 mg/L	40 mL/L	50

Several additional experiments were conducted at concentrations between 1000 to 1500 mg/L RDX to determine which RDX concentration was the best for the *Arabidopsis* mutant screening. Tables 4.4 – 4.7 document details of each experiment.

Table 4.4. Experiment #3 with RDX in acetone.

7/14/2003	**[RDX]**	**[acetone]**	**[seeds]**
Control	0 mg/L	0 mL/L	50
Control	0 mg/L	30 mL/L	50
Condition 1	1000 mg/L	30 mL/L	50
Condition 2	1100 mg/L	30 mL/L	50
Condition 3	1200 mg/L	30 mL/L	50
Condition 4	1300 mg/L	30 mL/L	50
Condition 5	1400 mg/L	30 mL/L	50
Condition 6	1500 mg/L	30 mL/L	50

Table 4.5. Experiment #4 with RDX in acetone.

7/29/2003	**[RDX]**	**[acetone]**	**[seeds]**
Control	0 mg/L	0 mL/L	50
Control	0 mg/L	24 mL/L	50
Condition 1	1100 mg/L	24 mL/L	50
Condition 2	1125 mg/L	24 mL/L	50
Condition 3	1150 mg/L	24 mL/L	50
Condition 4	1175 mg/L	24 mL/L	50
Condition 5	1200 mg/L	24 mL/L	50

Table 4.6. Experiment #5 with RDX in acetone.

8/21/2003	**[RDX]**	**[acetone]**	**[seeds]**
Control	0 mg/L	0 mL/L	65
Control	0 mg/L	30 mL/L	65
Condition 1	1000 mg/L	30 mL/L	65
Condition 2	1100 mg/L	30 mL/L	65
Condition 3	1200 mg/L	30 mL/L	65
Condition 4	1300 mg/L	30 mL/L	65
Condition 5	1400 mg/L	30 mL/L	65
Condition 6	1500 mg/L	30 mL/L	65

The final experiment focused on concentrations of RDX from 1200 to 1500 mg/L RDX, with a concentration of 30 mL/L acetone. There were four plates at each RDX concentration. Three of the plates had 1000 seeds in each plate, while the fourth plate had 2000 seeds per plate. This final experiment was used to determine what concentration of seeds would work best to screen mutants. This experiment also showed what concentration of RDX would allow for the best screening of mutants. The goal of the mutant screen was to screen a large amount of mutants at once, at a concentration of RDX that would enable easy identification of which mutants were the most resistant to RDX. Ideally, a concentration of

RDX that kills or severely stunts most mutants was desired, allowing only the plants with the highest resistant to survive. Below, Table 4.8 summarizes the final experiment. Table 4.9 summarizes all seven of the experiments that were done to determine the conditions to use for the mutant seed germination screen.

Table 4.7. Experiment #6 with RDX in acetone.

8/21/2003	**[RDX]**	**[acetone]**	**[seeds]**
Control	0 mg/L	0 mL/L	245
Control	0 mg/L	30 mL/L	245
Condition 1	1000 mg/L	30 mL/L	245
Condition 2	1100 mg/L	30 mL/L	245
Condition 3	1200 mg/L	30 mL/L	245
Condition 4	1300 mg/L	30 mL/L	245
Condition 5	1400 mg/L	30 mL/L	245
Condition 6	1500 mg/L	30 mL/L	245

Table 4.8. Experiment #7 with RDX in acetone.

8/21/2003	**[RDX]**	**[acetone]**	**[seeds] - 3 plates**	**[seeds] - 1 plate**
Control	0 mg/L	0 mL/L	1000	2000
Control	0 mg/L	30 mL/L	1000	2000
Condition 1	1200 mg/L	30 mL/L	1000	2000
Condition 2	1300 mg/L	30 mL/L	1000	2000
Condition 3	1400 mg/L	30 mL/L	1000	2000
Condition 4	1500 mg/L	30 mL/L	1000	2000

Table 4.9. Summary of experiments with RDX in acetone.

	[RDX]	**[acetone]**	**[seeds]**	**Date plates were made**
Experiment # 1	50 to 250 mg/L	5 mL/L	50	6/23/2003
Experiment # 2	500 to 2000 mg/L	40 mL/L	50	7/3/2003
Experiment # 3	1000 to 1500 mg/L	30 mL/L	50	7/14/2003
Experiment # 4	1100 to 1200 mg/L	24 mL/L	50	7/29/2003
Experiment # 5	1000 to 1500 mg/L	30 mL/L	65	8/21/2003
Experiment # 6	1000 to 1500 mg/L	30 mL/L	245	9/2/2003
Experiment # 7	1200 to 1500 mg/L	30 mL/L	1000	10/7/2003

RDX Mutant Screen

T-DNA seed pools were ordered from the Arabidopsis Biological Resource Center (ABRC) Seed and DNA Stock Catalog. Numerous sets of T-DNA pools and lines were available. Columbia 2 and Columbia 7 WT strains both had pSKI15 vector - activation tag mutant pools and lines available. It should be noted that the screening assay was conducted with Columbia 0 WT. The seeds that were ordered were Columbia 2 WT (CS907) parent line with activation tag mutation (pSKI15 plasmid, 4x CaMV 35S promoter). The ordered seeds had more than 62,000 seed lines, distributed among the 208 pools. Seeds were donated to the ARBC catalog by Wolf Scheible and Chris Somerville. Each pool was shipped in a separate vial; each vial contained approximately 1000 to 2000 seeds. Pools were screened separately to allow for easier identification of mutants. Concentrations of 1400 mg/L RDX and 28 ml/L acetone with two-thousand seeds per plate were used to screen the seeds.

Root Length Screen

Root growth studies are very sensitive and can be used to evaluate toxicity at very low levels. Because roots are sensitive to toxic material, they are ideal for judging the healthiness of a plant and the growth rate. Based on this idea, *Arabidopsis thaliana* and 15 TNT mutants (generated by Dr. Hangsik Moon) were grown in the presence of RDX. This technique allowed seeds to germinate on half-strength MS media and then the seedlings were transferred to media containing RDX. The seedlings were measured after several days to

determine how the RDX had effected their growth. In order to evaluate TNT mutant seeds, both mutant and wild-type seeds were grown. Then, both mutant and wild-type seedlings were transferred to plates with media containing RDX. Throughout this experiment, final and initial lengths of the plant roots were recorded. This data was then used to identify any mutants that grew at a faster rate than the wild-type when RDX was present. Identifying the mutants that grow better in the presence of RDX may provide a better understanding of the RDX pathway. Because each mutant has been genetically transformed, it should be possible to determine which genes are involved in RDX uptake. Then, based on the results, the biochemical pathway can be adjusted to more accurately describe what happens to RDX.

The process of root length screening was very time consuming since it involves many precise steps. Plates containing MS media were made with and without RDX. Then the seeds were sterilized. Sterile seeds were placed on plates without RDX, and then were refrigerated at 4 °C in order to stop the dormancy and allow uniform germination. The plates were then transferred to a growth room, where they were kept at 25 °C. After four days, the seedlings were then transplanted to plates containing RDX. The seedlings were placed on the plate in a horizontal row. These plates were then placed vertically on a tray in the growth room to allow the roots to grow down towards the other end of the plate. The seedlings were then grown for several days and were measured to determine root growth after transplanting.

Due to the lengthy procedure, mutants that were previously selected for resistance to TNT were used in the root length studies. This allowed a small number of mutants to be studied more precisely. Fifteen mutants were selected to screen for RDX resistance from work on TNT resistance. The mutants included Enhancer Traps (ET) mutants and T-DNA mutants. Both types of mutants were genetically altered with a Tumor inducing (Ti) plasmid from Agrobacterium. For the ET mutants, a gene was introduced into the *Arabidopsis* genome. The insertion could enhance expression of nearby genes; if the mutation fell within a coding region, the gene would be destroyed. Seven ET mutants (ET # - 66, 121, 124, 197, 203, 223, and 224) were studied. On the other hand, modification to the T-DNA mutants always resulted in gene knockout. Eight T-DNA mutants (T-DNA # - 35, 43, 128, 148, 161, 185, 209, and 216) were studied.

Wild-type and mutant plant seeds were collected and one-hundred seeds of each type were sterilized in small vials. The vials were then placed in the sterile hood in order to

sterilize with ethanol and bleach solutions. To begin sterilization, a pipette was used to transfer 1 mL of 70% ethanol to each vial. The ethanol was left in the vials for approximately 7 minutes. During this time, each vial was shaken to make sure that each seed was exposed to ethanol. Next, the ethanol was removed with transfer pipettes. Vials were then filled with sterile water in order to rinse excess ethanol from the seeds. Each vial was shaken, and then the sterile water was removed with transfer pipettes. Next, 1 mL of a 50% bleach and Triton-X solution was added to the vials with a pipette. This solution was left in the vials for approximately 20 minutes while each vial was shaken. After this time, the bleach solution was removed with a transfer pipette and the seeds were rinsed three times with sterile water. Again, the sterile water rinse involved filling the vials with sterile water, shaking the seeds, and removing the water with transfer pipettes. Following sterilization, seeds were stored for a short amount of time in the 4 ^{o}C refrigerator until they could be plated.

To begin growing the *Arabidopsis* plants, both wild-type and mutant seeds were plated on half strength Murashige and Skoog (MS) media. This media was made with 2.2 g MS salt, 0.5 g MES (2-morpholinoethanesulfonic acid) hydrate, 20 g Sucrose, 3 mL 6% KH_2PO_4 (potassium phosphate monobasic) solution, and 1 mL Bamborg B_5 Vitamins in 1 L filtered water. The pH of the media was then adjusted to 5.8 with 0.5 N sodium hydroxide. Then, 0.40 g of Phytogel was added to five 250 mL beaker and 200 mL of media was added to each of these beakers. The beakers were covered with foil and autoclaved. While the media was still warm, it was poured into approximately 35 square plates and was cooled to 25 °C to solidify the gel.

Media containing RDX was created in the same manner. RDX was added to the media to achieve 200 mg RDX/L after removing from the autoclave. RDX could not be added to the media until after autoclaving due to its chemical instability. For this procedure it was important to add the RDX immediately after the media was removed from the autoclave to ensure that the RDX did not crystallize in the media.

After MS media plates had been made and seeds had been sterilized, the seeds were plated in a row on the MS media. For this process, 20 to 30 seeds were plated horizontally across the plate. Using a transfer pipette and sterile water, seeds were placed one by one across the width of the plate. Three plates were created for each mutant along with three

wild-type plates per set of mutant plates. After seeds were placed on each plate, the plates were sealed with Parafilm in order to maintain sterile conditions.

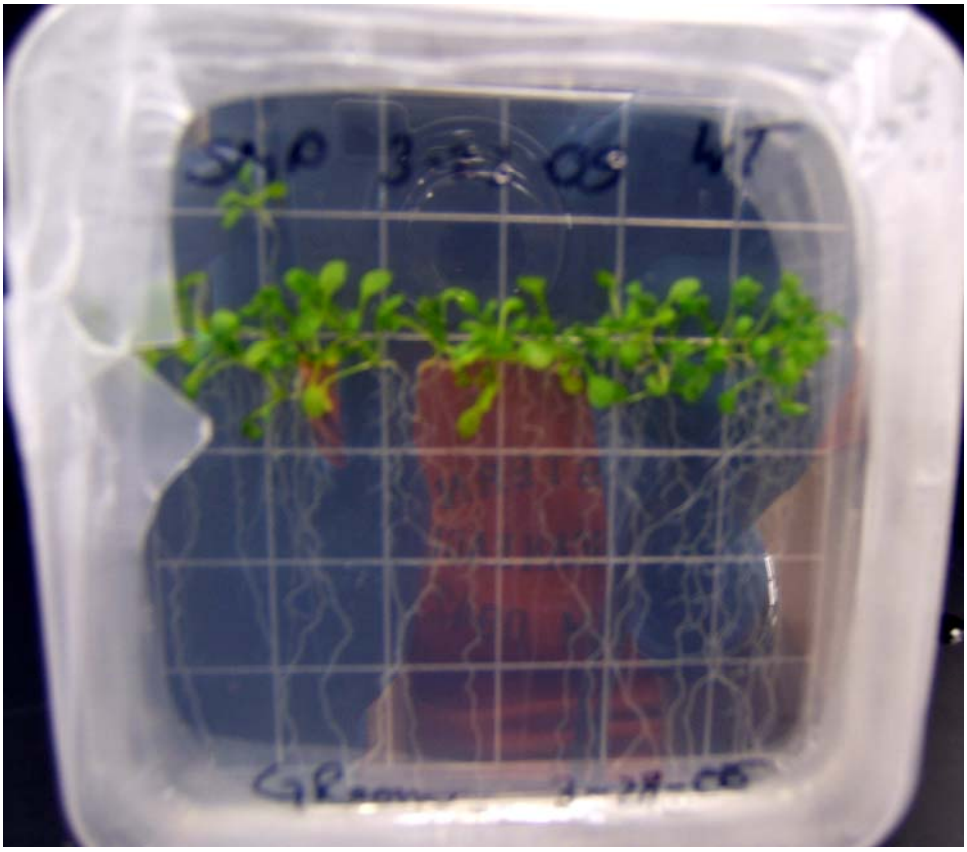


Figure 4.1. A plate from the root length screen is shown. These are eight day old Arabidopsis wild-type seeds. The seeds germinated in regular MS media, and then were transplanted to media containing RDX after four days. The root lengths were measured at four and eight days and were then compared to determine the toxicity on the root growth.

By starting the *Arabidopsis* seeds on MS media, normal germination was allowed to occur. If the seeds had been plated immediately on media containing RDX, seeds may not have germinated. Germination is a sensitive process therefore it was logical to allow the seeds to start growing under normal conditions before being exposed to RDX.

Plated mutants and their respective wild-type plates were then placed in the growth room for four days. In the growth room, a table was present that allowed the plates to be

stood up. This allowed the seeds to grow down the plates in a vertical fashion. Also, the room was kept at 26°C throughout the growth period.

After four days, the *Arabidopsis* plants were removed from the growth room and transplanted to media containing RDX. This was done in the sterile hood with tweezers that had been sterilized in the autoclave. When the plants were transplanted to the RDX media, 10 mutants were placed in a horizontal row across one half of the plate while 10 wild-type plants were placed in a row on the other half. Four plates were created in this manner for each mutant, and once again, each plate was sealed with Parafilm. Then the plates were turned over so dots could be made for the initial root lengths, and the plates were placed in the growth room for another four days.

After four days of growth in the RDX media, the plates were removed from the growth room. Each plant was then removed from the plate and measured on a ruler. These measurements along with the corresponding initial lengths were recorded for each plate. Two sample T-tests were conducted using JMP software to evaluate the performance of the mutants.

Results and Discussion

Screening Assay Determination

RDX was originally purchased in acetonitrile (1000 µg/mL). One experiment was done to test concentrations of RDX from 0 mg/L to 40 mg/L. All of these plates had 40 ml/L of acetonitrile. Four replicates were used for each concentration. Out of the 20 plates tested, only two plates germinated: one plate at 0 mg/L RDX and the other at 30 mg/L. This experiment was inconclusive about the toxicity of RDX, but showed that acetonitrile was too toxic to be used as a solvent for screening studies. This experiment also demonstrated the need for two controls, one with no RDX and no solvent, and a second control with only solvent. Two controls are necessary in order to be able to observe the toxicity of the solvent, as well as the toxicity of RDX.

Additional experiments had both types of controls and used RDX dissolved in acetone. All of the seeds in the first experiment with RDX in acetone germinated and appeared to grow equally well, regardless of RDX concentration. Concentrations of RDX

ranged from 0 to 250 mg/L. This data did not indicate any trends in growth versus RDX concentration which indicated that the concentrations of RDX used were too low to limit germination.

A second experiment was conducted with concentrations ranging from 0 to 2000 mg/L of RDX. After the seeds had been in the growth room for five days, the control without acetone appeared healthier than the rest of the samples. The control with acetone and the 500 mg/L RDX sample were doing equally well, and both were doing almost as well as the control without acetone or RDX. The 1000 mg/L RDX sample was showing slight retardation when compared to the 500 mg/L RDX sample. Neither the 1500 mg/L RDX sample nor the 2000 mg/L RDX sample had germinated yet. Results are shown below in Table 4.10.

Table 4.10. Germination rate experiment.

7/3/2003	**[RDX]**	**[acetone]**	**[seeds]**	**Results**
Control	0 mg/L	0 mL/L	50	germinated and growing well
Control	0 mg/L	40 mL/L	50	germinated and growing almost as well as first control
Condition 1	500 mg/L	40 mL/L	50	germinated, growing almost as well as first control
Condition 2	1000 mg/L	40 mL/L	50	germinated, growing almost as well as 500 mg/L
Condition 3	1500 mg/L	40 mL/L	50	no germination
Condition 4	2000 mg/L	40 mL/L	50	no germination

The acetone demonstrated some toxicity to the seeds at this concentration (40 mg/L). The concentration of acetone that was used in this experiment was the same concentration of acetonitrile that was used in the first experiment where only two plates germinated. This demonstrated that acetone is less toxic to *Arabidopsis* plants than acetonitrile.

Precipitation of RDX occurred during the experiments, but the reason for increased toxicity of the supersaturated solution is still unclear. Due to its poor solubility in aqueous solution, precipitation of RDX was first observed at 500 mg/L and significant precipitation occurred at levels above 1500 mg/L. Precipitation increased with increased concentration of RDX, with 1000 mg/L RDX having slight precipitation and 1500 mg/L RDX having significant precipitation.

Additional experiments were conducted focusing on 1000 mg/L to 1500 mg/L RDX. Both experiment 5 and 6 were placed in the growth room at the same time so that they could be compared. There were differences between experiments 5 and 6 based on concentration of seeds per plate, which appeared to affect the toxicity of RDX on germination rates and plant growth. At seven days old, the controls with no RDX for both the fifth and sixth experiments had the first set of leaves appearing, shown in Figure 4.2. All seeds had germinated at all concentrations in the sixth experiment, with 245 seeds per plate. In the fifth experiment, not all seeds had germinated yet at 1500 mg/L RDX. Plants were also larger in the sixth experiment than in the fifth experiment, due to the concentration of seeds.

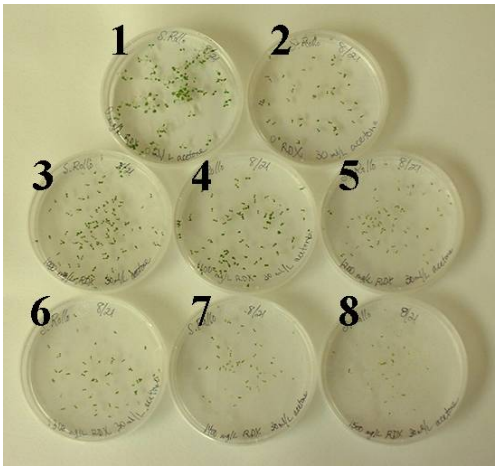
After ten days, the controls for both experiments had the second set of leaves appearing. Figure 4.3 shows that most concentrations had the first set of leaves in both experiments. In Experiment 6, the plants at both concentrations of 1100 mg/L RDX and 1200 mg/L RDX had the first set of leaves growing; the roots of these plants were very short, but the stems were long and the plants were a healthy color. This varies with Experiment 5, in that the plants at 1100 mg/L RDX and 1200 mg/L RDX had a first set of leaves which were barely visible; the root growth was again stunted, the stems were not as long, but the color was still normal. The majority of the plants were still larger in Experiment 6 than in Experiment 5.

At fourteen days old, the controls for both experiments had the third set of leaves growing. Figure 4.4 shows that the controls were also beginning to show signs of death: yellowing, turning dark, and wilting. Plants with RDX had either the first or the second set of leaves growing. At 1200 mg/L RDX, plants in Experiment 5 had a first set of leaves, while plants in Experiment 6 had a second set of leaves. The difference in the size of plants between the experiments appeared to reduce with time; very few of the plants were larger in Experiment 6.

Experiments 5 and 6 were conducted between 1000 and 1500 mg/L RDX at various seed concentrations. The toxic effects of these RDX concentrations on germination and plant growth appeared to be fairly similar to each other. Since the root length was severely stunted in these experiments, 24-day-old plants were transferred to media containing no RDX and to soil to determine if they would be able to develop to maturity. Developing to maturity is a requirement for selected mutants, since seeds need to be collected from the selected plants for future experiments. Plants that were transferred directly to soil had a difficult time adjusting with their minimal root length. Plants transferred to media without RDX has a faster growth rate, which allowed for normal root development. These plants could then be transferred to soil in order to fully mature. There was little difference between 1000 and 1500 mg/L RDX when observing germination and growth in RDX-containing media, but plants that were less stunted initially were able to develop much more rapidly when transferred to soil. Plants exposed to 1300 mg/L RDX did eventually develop in the soil, although they were still stunted. Plants exposed to 1400 mg/L RDX did not develop in the soil most likely due to severe root damage that had occurred.

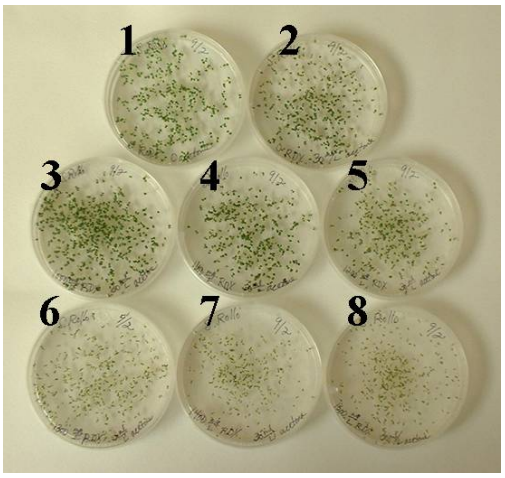
Transplanting the plants to soil helped determine the final concentrations of 1400 mg/L RDX and 28 ml/L acetone that would be used to screen the seeds. Two-thousand seeds will be screened on each plate. With higher concentrations of RDX, several toxic effects were observed, including stunted germination and plant growth. Although RDX stunted the germination of the seeds, all seeds eventually germinated at the RDX concentrations tested. Several experiments with wild-type seeds were conducted to determine the RDX concentration toxic to *Arabidopsis thaliana* seed germination and the concentration of seeds to use to screen mutant seeds. The germination of the seeds at higher RDX concentrations was severely delayed.

The experiments demonstrated the toxic effects of high RDX concentrations on the germination and growth of *Arabidopsis thaliana* wild-type seedlings. Higher concentrations of RDX demonstrated larger toxic effects on the germination rate and growth of the plants.



	[RDX]	[acetone]
Plate # 1	0 mg/L	0 mL/L
Plate # 2	0 mg/L	30 mL/L
Plate # 3	1000 mg/L	30 mL/L
Plate # 4	1100 mg/L	30 mL/L
Plate # 5	1200 mg/L	30 mL/L
Plate # 6	1300 mg/L	30 mL/L
Plate # 7	1400 mg/L	30 mL/L
Plate # 8	1500 mg/L	30 mL/L

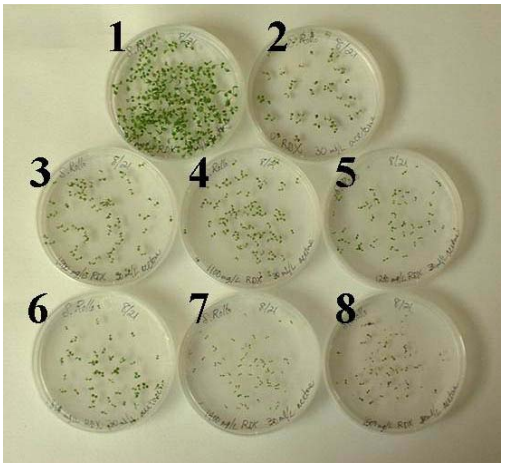
Figure 4.2a.



	[RDX]	[acetone]
Plate # 1	0 mg/L	0 mL/L
Plate # 2	0 mg/L	30 mL/L
Plate # 3	1000 mg/L	30 mL/L
Plate # 4	1100 mg/L	30 mL/L
Plate # 5	1200 mg/L	30 mL/L
Plate # 6	1300 mg/L	30 mL/L
Plate # 7	1400 mg/L	30 mL/L
Plate # 8	1500 mg/L	30 mL/L

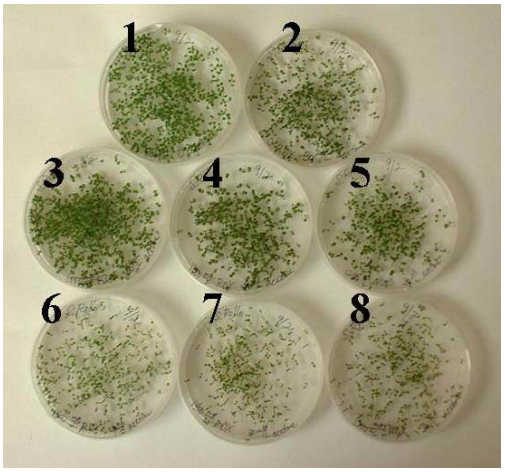
Figure 4.2b.

Figure 4.2. Plates from Experiment 5 and 6 shown seven days after the plates were placed in the growth room. Figure 4.2a shows Experiment 5 with 65 seeds per plate. Figure 4.2b shows Experiment 6 with 245 seeds per plate.



	[RDX]	[acetone]
Plate # 1	0 mg/L	0 mL/L
Plate # 2	0 mg/L	30 mL/L
Plate # 3	1000 mg/L	30 mL/L
Plate # 4	1100 mg/L	30 mL/L
Plate # 5	1200 mg/L	30 mL/L
Plate # 6	1300 mg/L	30 mL/L
Plate # 7	1400 mg/L	30 mL/L
Plate # 8	1500 mg/L	30 mL/L

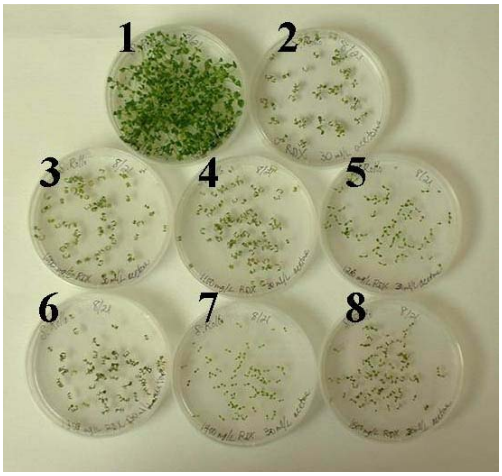
Figure 4.3a.



	[RDX]	[acetone]
Plate # 1	0 mg/L	0 mL/L
Plate # 2	0 mg/L	30 mL/L
Plate # 3	1000 mg/L	30 mL/L
Plate # 4	1100 mg/L	30 mL/L
Plate # 5	1200 mg/L	30 mL/L
Plate # 6	1300 mg/L	30 mL/L
Plate # 7	1400 mg/L	30 mL/L
Plate # 8	1500 mg/L	30 mL/L

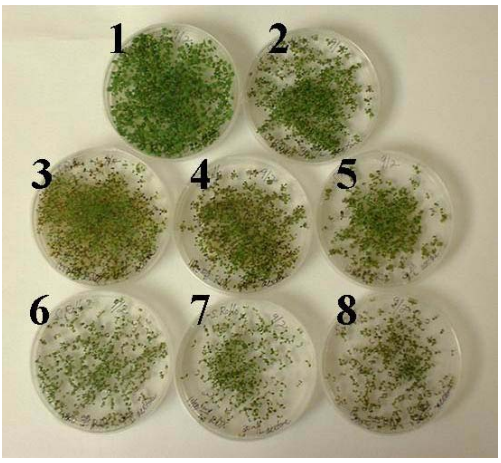
Figure 4.3b.

Figure 4.3. Plates from Experiment 5 and 6 shown ten days after the plates were placed in the growth room. Figure 4.3a shows Experiment 5 with 65 seeds per plate. Figure 4.3b shows Experiment 6 with 245 seeds per plate.



	[RDX]	[acetone]
Plate # 1	0 mg/L	0 mL/L
Plate # 2	0 mg/L	30 mL/L
Plate # 3	1000 mg/L	30 mL/L
Plate # 4	1100 mg/L	30 mL/L
Plate # 5	1200 mg/L	30 mL/L
Plate # 6	1300 mg/L	30 mL/L
Plate # 7	1400 mg/L	30 mL/L
Plate # 8	1500 mg/L	30 mL/L

Figure 4.4a.



	[RDX]	[acetone]
Plate # 1	0 mg/L	0 mL/L
Plate # 2	0 mg/L	30 mL/L
Plate # 3	1000 mg/L	30 mL/L
Plate # 4	1100 mg/L	30 mL/L
Plate # 5	1200 mg/L	30 mL/L
Plate # 6	1300 mg/L	30 mL/L
Plate # 7	1400 mg/L	30 mL/L
Plate # 8	1500 mg/L	30 mL/L

Figure 4.4b.

Figure 4.4. Plates from Experiment 5 and 6 shown fourteen days after the plates were placed in the growth room. Figure 4.4a shows Experiment 5 with 65 seeds per plate. Figure 4.4b shows Experiment 6 with 245 seeds per plate.

Germination was delayed longer for seeds exposed to higher RDX levels. It should also be noted that the seeds that germinated first, matured first. Age of the plants was determined from the time the seeds were placed in the growth room.

The results observed in this study can be summarized by three toxic effects. The first is that the concentration of RDX affected seed germination. With increasing concentrations of RDX, the toxic effects on germination also increased, which resulted in longer germination times. This study also showed that acetone was specifically toxic to root length, and these toxic effects also increased with increasing concentrations of acetone. The seed concentration is also an important factor to consider. Decreasing the concentration of seeds per plate increased the toxic effects of both RDX and acetone.

RDX Mutant Screen

The screening assay determination study determined that *Arabidopsis thaliana* mutant seeds should be screened with an RDX concentration of 1400 mg/L RDX with 2000 seeds per plate. Unfortunately, the seeds ordered from Arabidopsis Biological Resource Center had severe fungal contamination. Several sterilization protocols have been attempted to try to limit contamination. Increased sterilization reduced contamination, but also retarded the seed germination and growth. Customized sterilization methods reduced contamination from 100% of seeds screened down to 25%. Continued monitoring of the seeds was necessary to characterize seedling growth under the screening conditions.

Multiple sterilization methods were tested. A chlorine gas fumigation method was used for three hours and resulted in 100% contamination. The sterilization time was increased from 3 hours to 4 ½ hours, and was then further increased to six hours. After six hours of sterilization, 80% of the plates were still contaminated. Longer sterilization times were not possible with this method, since exposure to seven hours of chlorine gas caused a decrease in uniform germination.

A bleach solution was also used to sterilize seeds. Sterilization began with 20% bleach for 20 minutes, resulting in an 80% contamination rate. The sterilization was increased to a 50% bleach solution for 15 minutes, which resulted in a 60% contamination rate. Since the increased bleach sterilization method resulted in a 60% contamination rate, it

was concluded that this method was not sufficient to significantly lower contamination in order to screen seed effectively.

Since the contamination appeared to be from fungi, a method was developed to use fungicide to sterilize seeds. A 3% fungicide solution was used for 18 hours to sterilize seeds. However, this method was not sufficient enough to be used alone and resulted in 100% contamination. Seed germination was also noticeably slower due to the presence of fungicide. This prevented an increase in fungicide strength or exposure time.

A combination of sterilization methods was determined to be the best treatment for the severe fungal contamination. Ethanol, bleach and fungicide was used in combination to enhance the sterilization methods. Two variations of combinatorial treatments were tested to determine which method would best prevent the fungal contamination. The first method sterilized seeds with 70% ethanol for 5 minutes and then the seeds were sterilized again with 50% bleach for 15 minutes. Fungicide was also added to the media to help limit the growth of any fungi that was not killed by the ethanol and bleach sterilization. The seeds had 40% contamination. This contamination rate was still high, but was significantly lower than any of the sterilization methods had achieved alone.

A second combination of sterilization techniques was also evaluated. Ethanol and bleach sterilization times were increased to ensure a lower contamination rate. The second method sterilized seeds with 70% ethanol for 10 minutes and then the seeds were sterilized again with 50% bleach for 25 minutes. Fungicide was again added to the media to help limit fungal growth. Contamination dropped to only 25% of the seeds. This was the lowest contamination rate achieved during the germination rate mutant screen. The majority of screening occurred using the final contamination method described (70% ethanol for 10 minutes, 50% bleach for 25 minutes, with fungicide in the media).

Contamination proved to limit the progress of the mutant screen. The screen was also unsuccessful at identifying specific mutants. It was unclear if the method was capable of selection of mutants due to the qualitative characterization of a mutant plant's health. Root-growth studies seem to be more sensitive to subtle differences than germination screening that has been used so far.

Root Length Screen

From the final and initial root lengths, differences in root growth could be quantified based on percent change in root length (final length divided by initial length). T-tests were then used to statistically identify TNT mutants that had grown more than the wild-type. Table 4.11 displays the results that show significantly increased TNT mutant root growth when compared to the wild-type root growth.

From Table 4.11, it is seen that ET 121, T-DNA 128, and T-DNA 43 are the only mutants that show statistically larger root growth lengths when compared to the wild-type. The rest of the data did not show increased root growths length for the mutant or the increased root length was not statistically significant. The calculations display the average final and initial root growths for the mutants and the wild-type based on the summation of all recorded data. The statistical analysis is based on a 2 sample T-test that includes average growth based on an average of final lengths divided by initial lengths.

It was concluded that differences in root growth could be calculated a number of ways. Finding the average initial and final lengths was used to determine average percent growths for the wild-type and mutant. Differences between the average final and average initial root lengths were also found. The percent growth was a more precise way to compare wild-type and mutant root growth because the data is standardized. However, T-tests provided the most accurate analysis and should be used in future experiments.

Table 4.11. Comparison between average root growth for Wild-type *Arabidopsis* and genetically altered mutants. Larger root growth for the TNT mutant is justified by the confidence level.

	ET 121	**T-DNA 128**	**T-DNA 43**
Average Percent Growth-Mutant	397 %	498%	413 %
Average Percent Growth- Wild-type	337 %	380%	382 %
Confidence Level	93%	86 %	80 %

Statistical analysis can also be used to compare percent growths, but this method did not work with the data that was collected. Initially, final root lengths were going to be marked on the plate so that final and initial lengths could be measured directly from the plate. When roots grew together after 8 days of growth, it was necessary to remove the plants from the plates so that measurement could be with the ruler. Removing plants from the plates created problems because it was difficult to match up final lengths with initial lengths for each plant. Complications also arose when multiple plants grew together after being transplanted to the RDX media. In this case, only one initial length was marked for multiple plants, which created a potential source of error for the statistical analysis.

From both the statistical analysis and average percent analysis ET 121 and T-DNA 128 grew better than the wild-type. T-DNA 43, on the other hand, appeared to grow better than the wild-type based on the statistical analysis but not from the average percent growth. Experiments should be repeated for all three of these mutants in order to verify the results.

A control should also be grown during the retests. The control should include starting the mutant and wild-type on the MS Media as before, but they should be transplanted to regular media after four days rather than RDX media. This will identify mutants that grow better than the wild-type under normal conditions so that increased mutant growth is not mistakenly attributed to the presence of RDX.

Minor changes to the lab procedure could also reduce error. Increasing the concentration of RDX slightly could reduce growth length while also preventing root tangling. Otherwise, fewer plants should be grown on each plate so plants can be left in the plate for final measurements.

Overall, the results of this study appear promising. With a few additional experiments and simple changes to the experimental procedure, definite results can be obtained. Several other mutants are also available for testing, which could also lead to identification of other mutants that thrive better than the wild-type plants when exposed to RDX.

Conclusions

During these experiments, the toxicity of RDX on the germination of wild-type seeds was evaluated. A final concentration of 1400 mg/L RDX with 2000 seeds per plate was

identified as toxic enough to allow for identification of select mutants. Mutant seeds purchased from the ABRC were screened. Contamination problems, along with the qualitative type of screening, prevented any mutants from being selected from the germination screen. Root length screening was quantitative, but time consuming. Root length screening was conducted to test possible mutants selected from TNT screens. Three mutants (ET 121, T-DNA 128, and T-DNA 43) were selected from the root length screen and can be used for future uptake studies to determine metabolic rates. Low solubility and low toxicity of RDX proved to be difficult to work with. Sterile conditions were also difficult to achieve with the purchased seed stock. Future studies should focus on root length screening of a small sample of possible mutants. T-tests are a valid method to use to determine the success of a mutant's root growth.

Chapter 5 - Conclusions and Future Work

Phytoremediation of RDX by *Arabidopsis thaliana* was successful, with over 50% of the radioactivity being taken out of the media and over 11% of the RDX being completely mineralized to carbon dioxide after 35 days. This is a significant result; this is the first axenic plant culture study to show that plants can mineralize RDX. The RDX sorption rate was even faster in the initial uptake studies where the plants had constant air exchange, suggesting that respiration rates and the concentration of oxygen and carbon dioxide available to the plant play important roles in the conversion of RDX to carbon dioxide. The results of this study are consistent with the pathway (1) developed by Schnoor and his collaborators (Figure 2.3). Even though MNX and other metabolites were not detected with HPLC, it is likely that they were present in concentrations below detection limits. It should also be noted that RDX was not toxic to *Arabidopsis* at concentrations up to 35 mg/L RDX in liquid media. The toxicity of higher concentrations of RDX was not studied due to solubility limitations.

The toxicity level of RDX in the solid media used to grow *Arabidopsis thaliana* was evaluated to be near 1400 mg/L RDX. The high levels of RDX only stunted the wild-type plants and the seeds were always able to germinate in the presence of RDX. The germination screen was not an effective screening method for RDX due to the limited toxic effects observed. Over 100,000 mutant seeds (25% of the mutant library) were screened for germination effects and no mutant candidates were selected. Since the RDX only slightly affected the seedlings, a quantitative root length screening method was more likely to be successful at selecting mutants with important genetic mutations. Three preliminary mutants were selected from fifteen mutants that were evaluated with the root length screening method at 200 mg/L RDX. Additional mutants should be screened with the root length method to select plants with other important genetic mutations. Piecing together these key genetic sequences may help to shed light on the RDX phytotransformation pathway.

Future work should include the concentration and reanalysis of the media samples and plant extract samples from this study to determine if any metabolites are present. This would help quantify the amount of metabolites that are formed in *Arabidopsis*. Sample fractions could be collected from media and plant extracts for further analysis in order to

identify the unknown polar metabolites that can be seen in the radiographs (Figures 3.8 and 3.9). Methods for detecting formaldehyde and methanol from these samples need to be developed. The whole plant samples could also be bio-oxidized to verify the mass balance results for the plant extract and bound samples.

The three mutants that were selected from the root length screen need to be reevaluated to determine the growth rate without the presence of RDX. This data could be used to verify that the mutants are not affected by the toxicity of RDX in the media. Additional mutants selected for TNT could be evaluated for RDX using the root length screen method to discover other mutants with potential significance to the RDX pathway. Additional seeds could also be screened using the germination screen, but it unclear if this method would allow for the selection of mutant candidates.

APPENDIX A – Additional Figures from Mass Balance Experiments

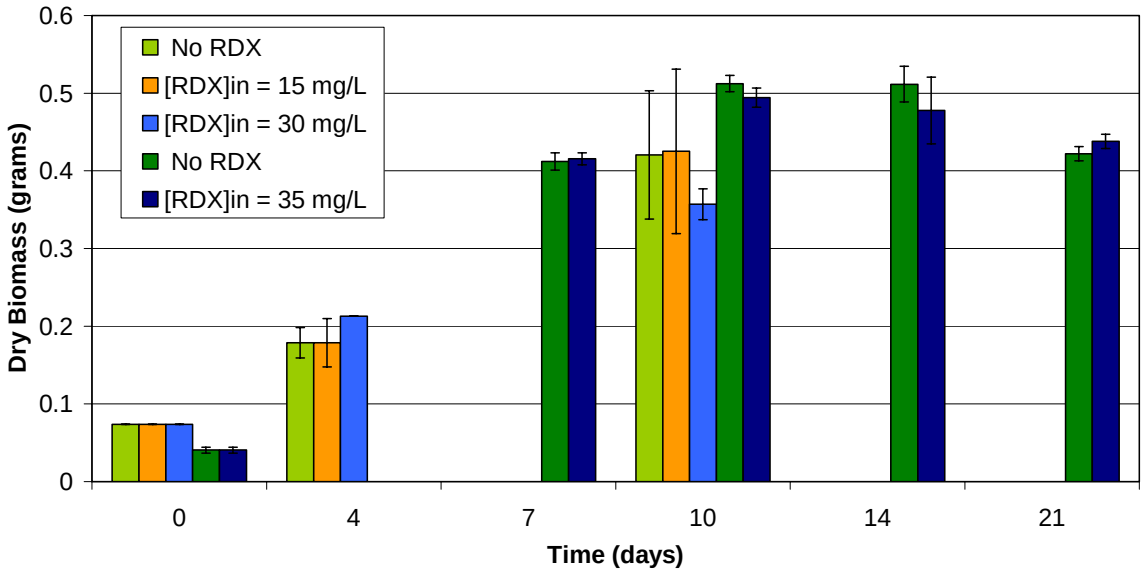


Figure A.1. Effect of several RDX concentrations on the growth of Arabidopsis seedlings. Different amounts of RDX were added to one week old seedlings. At the concentrations shown, RDX does not appear to have a toxic effect on the growth of the seedlings.

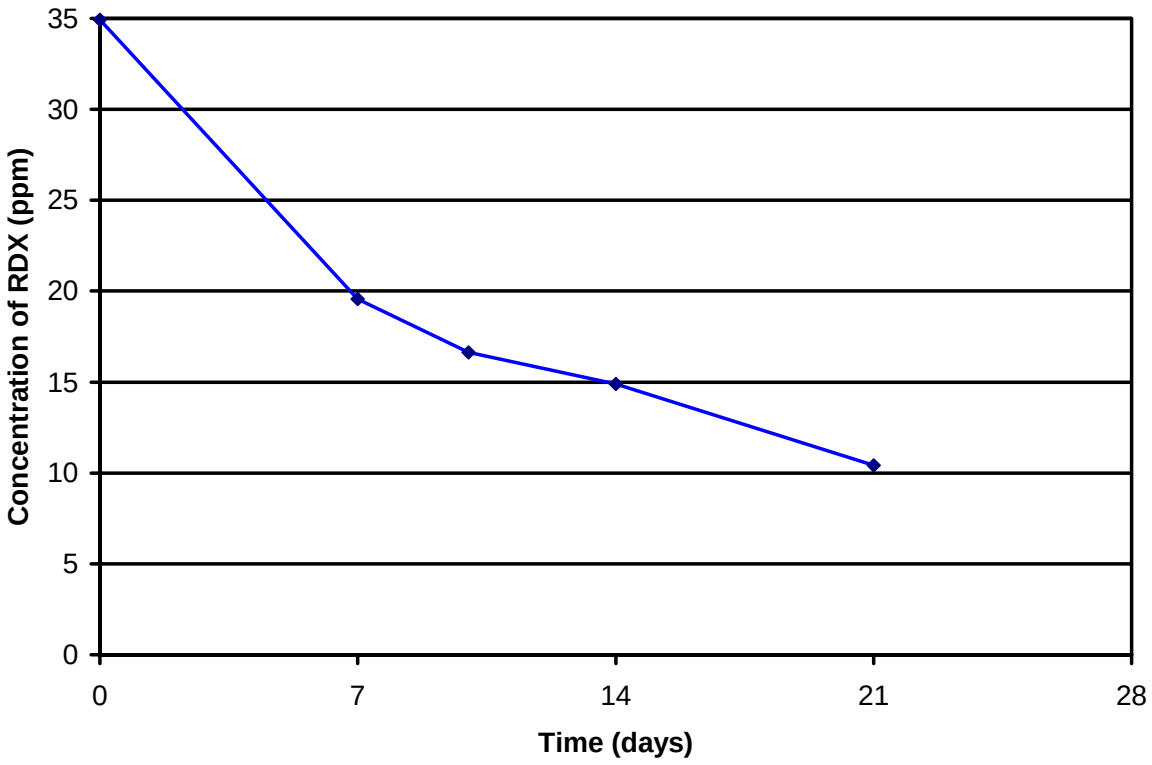


Figure A.2. RDX removal from Arabidopsis seedlings. Extracellular RDX is taken up by Arabidopsis seedlings. The plants were one week old when RDX was added to them.

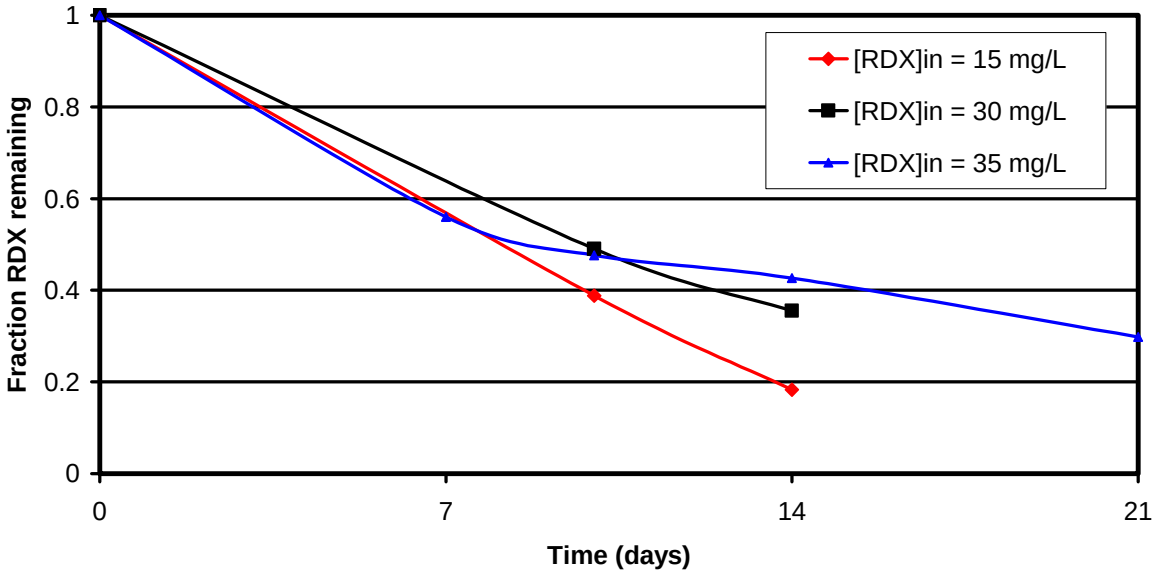


Figure A.3. Fractional RDX removal from Arabidopsis seedlings. Extracellular RDX is taken up by Arabidopsis seedlings. The plants were one week old when RDX was added to them.

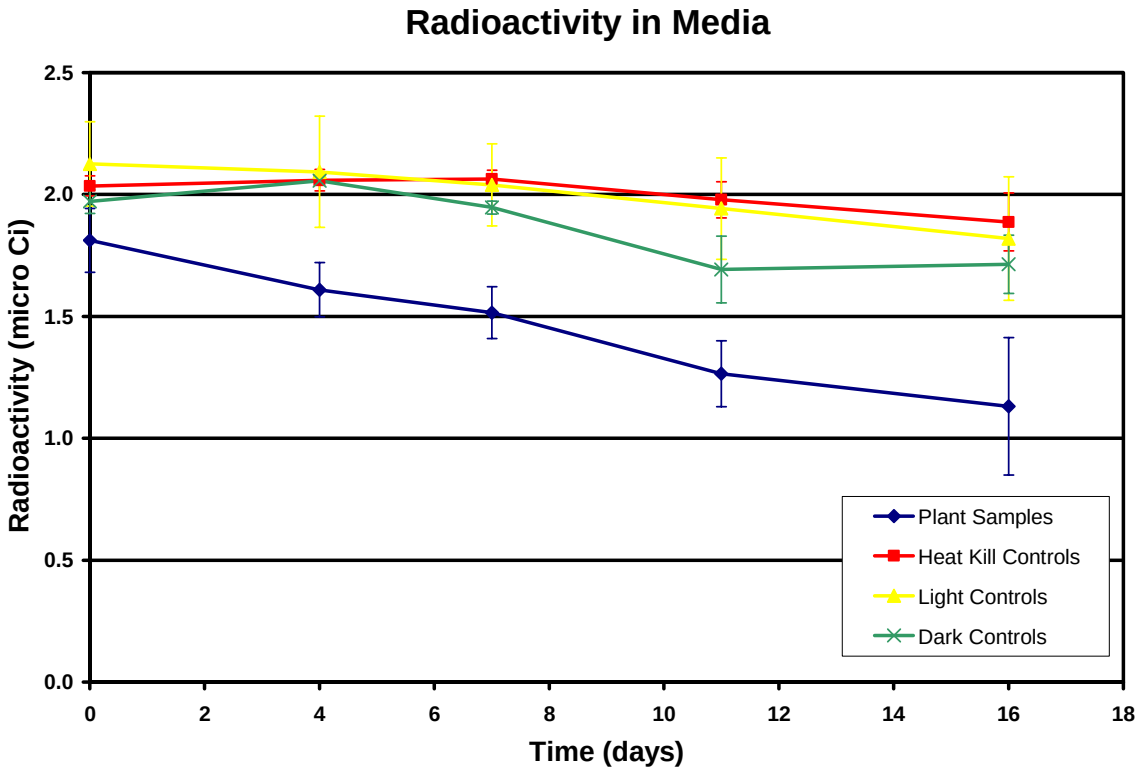


Figure A.4. The total radioactivity in the media decreases in all flasks during the experiment. The plant samples appear to remove the radioactivity from the media better than all of the controls.

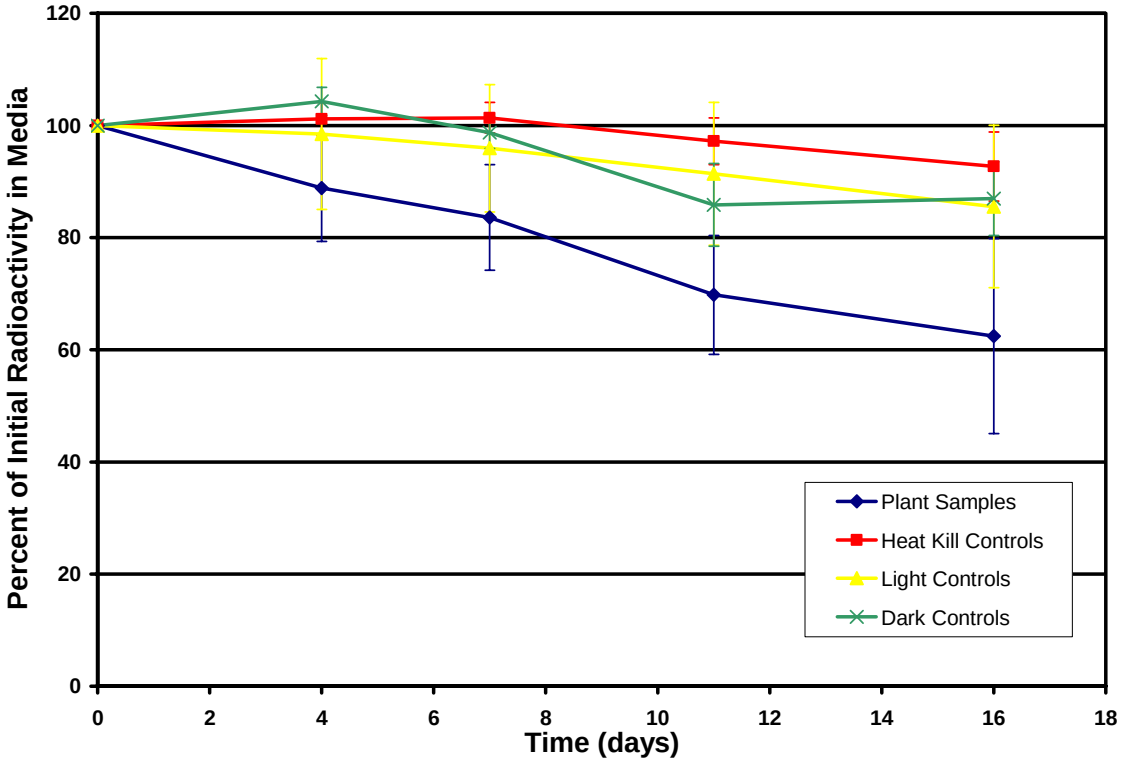


Figure A.5. The figure above shows that the fraction of total radioactivity in the media decreases in all flasks during the radio-labeled experiment. Radioactivity decreases much more rapidly in flasks with living plants. Triplicate axenic *Arabidopsis thaliana* cultures were used for the living plant cultures and the heat-killed controls. Triplicate culture flasks were sampled for the light media controls and the dark media controls.

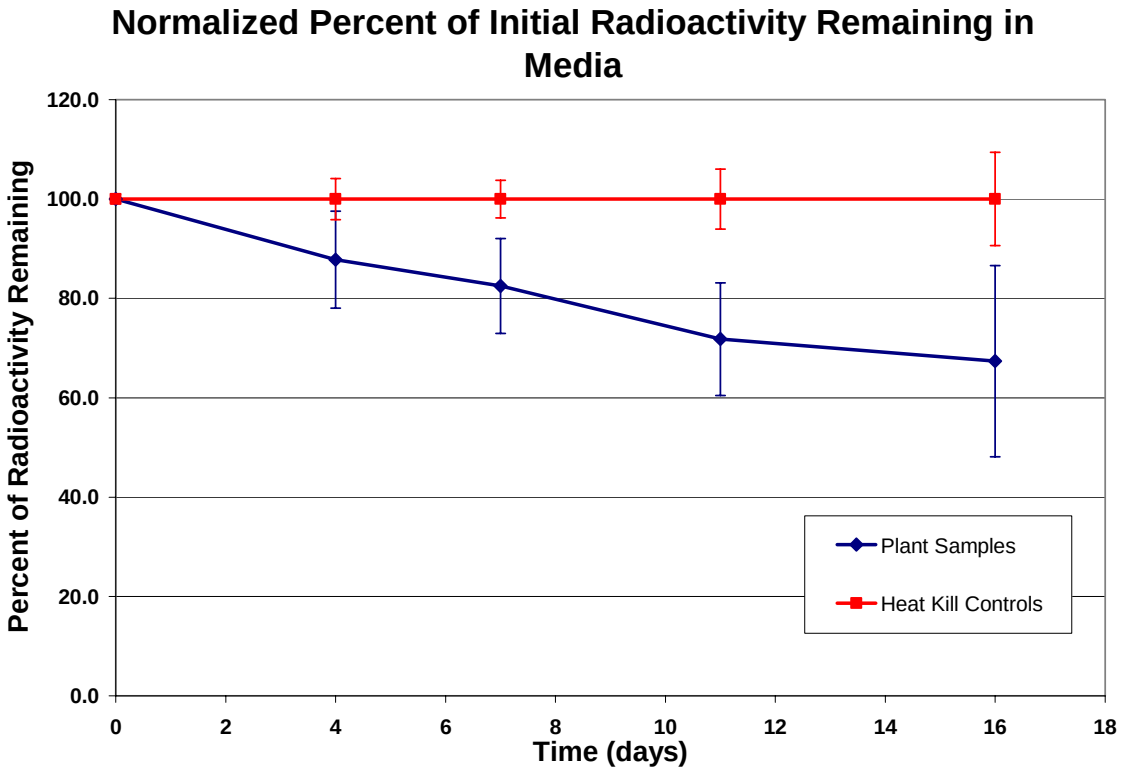


Figure A.6. The data is normalized so that the difference between the heat kill control and the plants can be seen. The plant samples show a decrease in radioactivity that shows how much RDX is being taken up by the plants.

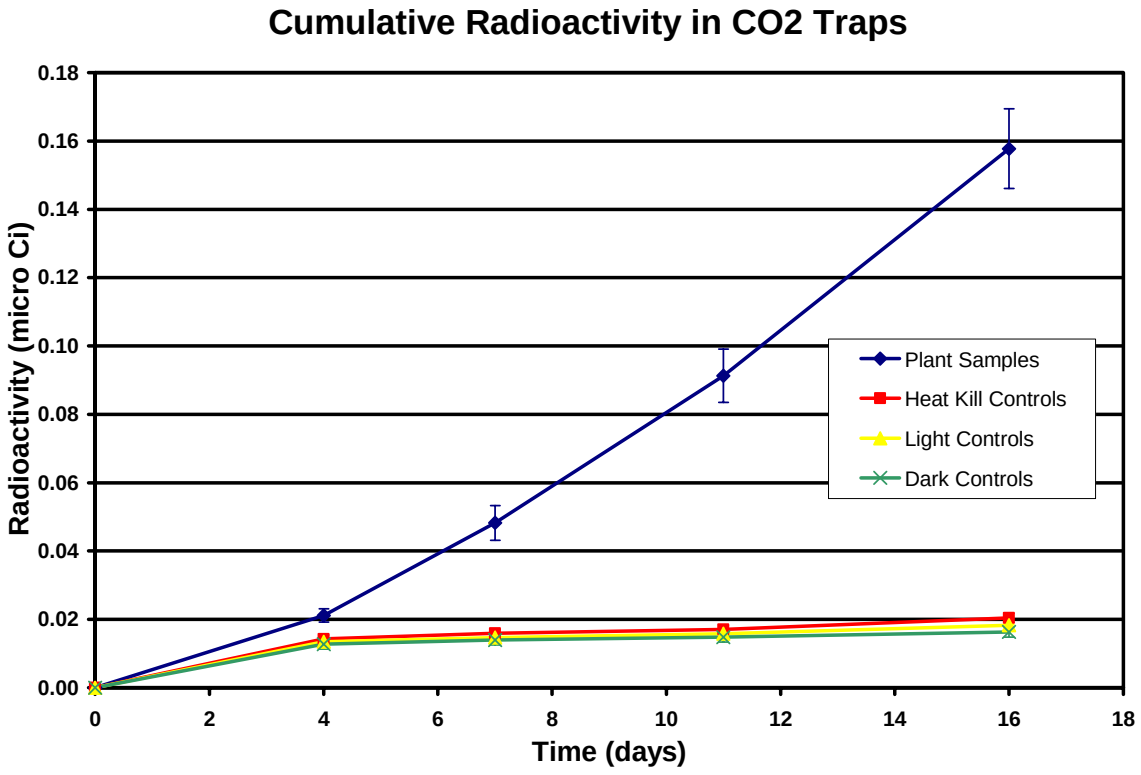


Figure A.7. The total radioactivity in the carbon dioxide traps increases in all flasks during the experiment. The plant samples appear to create significant amounts of radioactive carbon dioxide.

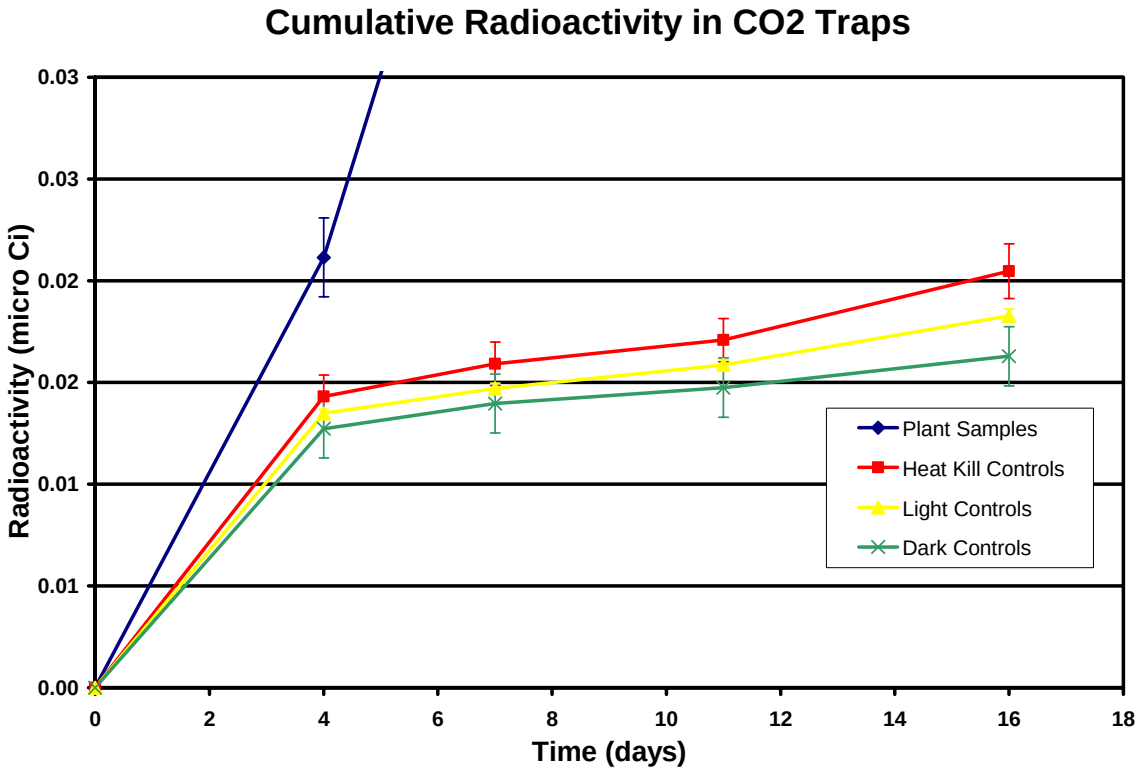


Figure A.8. The radioactivity in the carbon dioxide traps does not appear to differ much between the controls. This would suggest that the carbon dioxide is only being produced by metabolism of RDX.

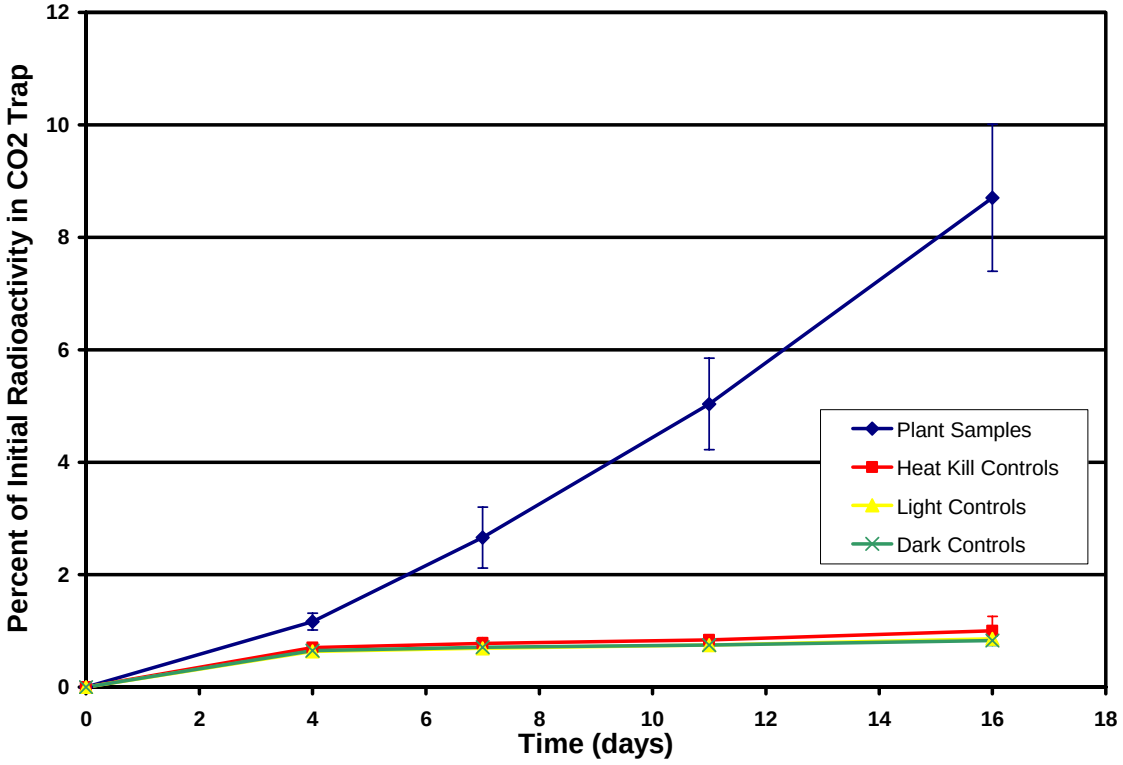


Figure A.9. The figure above shows the percent of radioactivity in the carbon dioxide traps increases in all flasks during the radio-labeled experiment. Flasks containing living plants convert RDX to carbon dioxide much quicker than controls, with over 8% of the initial radioactivity converted after 16 days. This is significantly more mineralization than the 1% that was observed in all of the control flasks. Triplicate axenic *Arabidopsis thaliana* cultures were used for the living plant cultures and the heat-killed controls. Triplicate culture flasks were sampled for the light media controls and the dark media controls.

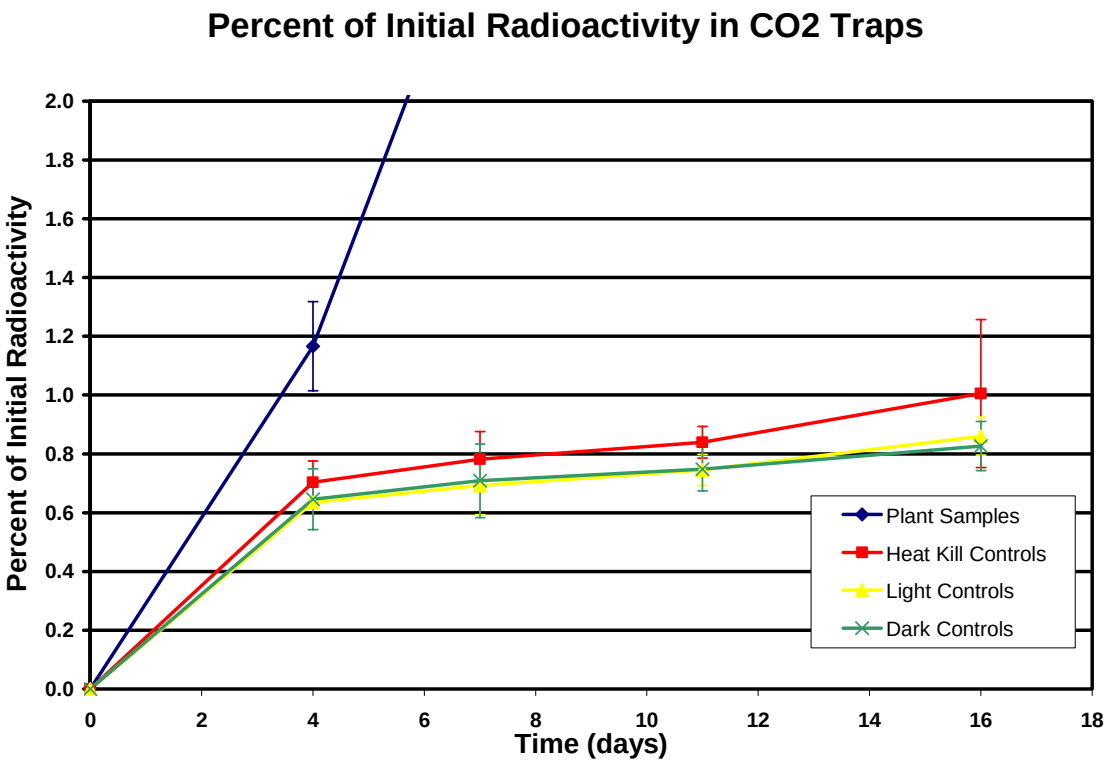


Figure A.10. The percent of radioactivity in the carbon dioxide traps increases in all flasks. Flasks containing living plants convert RDX to carbon dioxide much quicker than controls. There is very little difference between the controls.

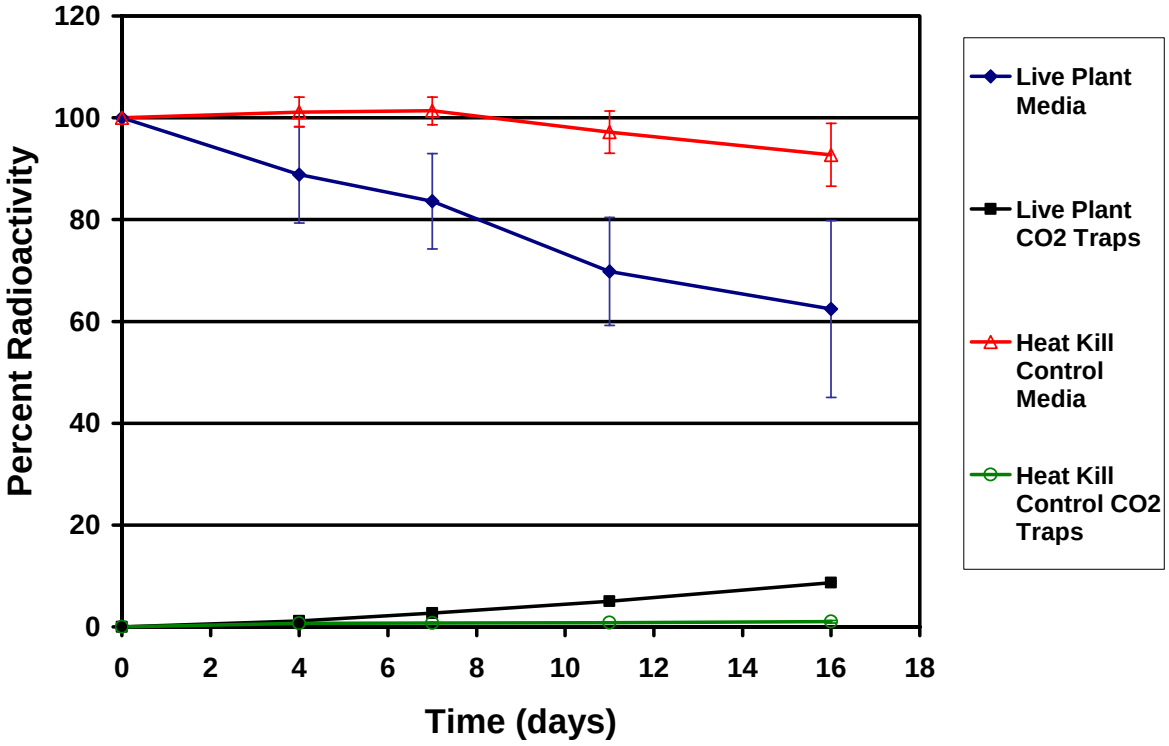


Figure A.11. The figure above shows that the total radioactivity in the media decreases faster and the ^{14}C-labeled carbon dioxide is being produced quicker in flasks with living plants. This shows that enzymatic activity is being used. Triplicate axenic *Arabidopsis thaliana* cultures were used for the living plant cultures and the heat-killed controls.

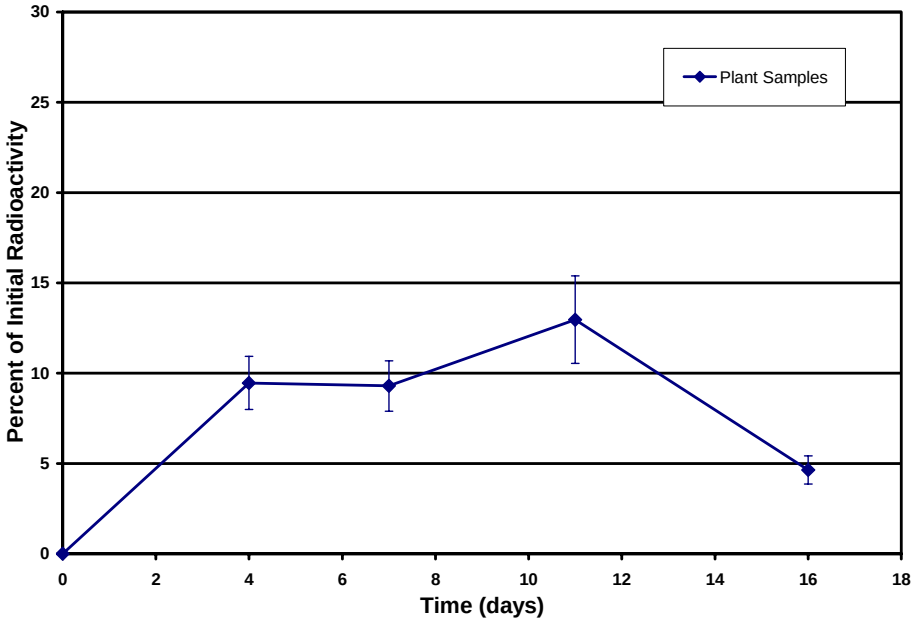


Figure A.12. The figure above shows that the total radioactivity in the plant extracts increases over time in flasks with living plants during both radio-labeled experiments. This shows that the plant is using enzymatic activity to take up the RDX. Triplicate axenic *Arabidopsis thaliana* cultures were used for the living plant cultures.

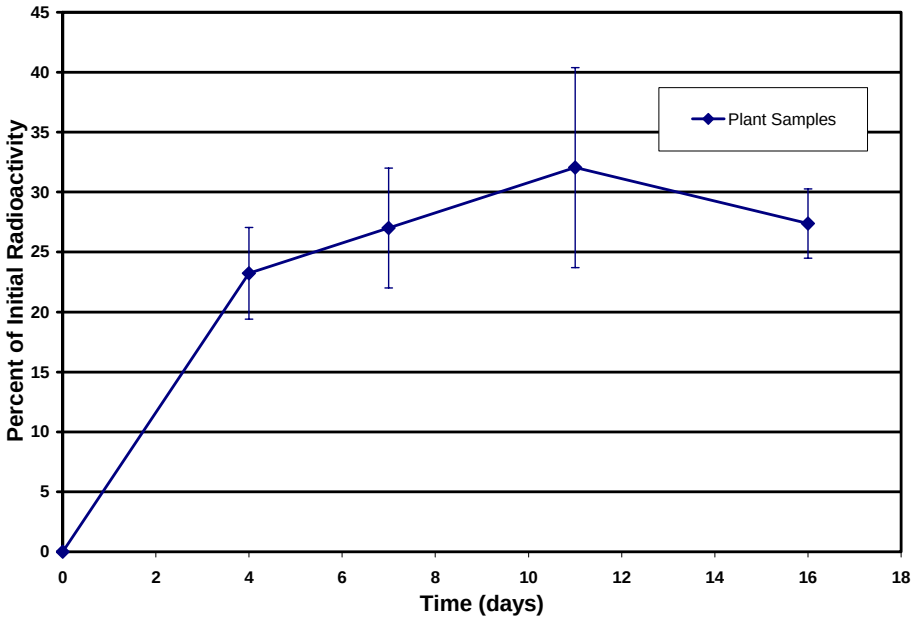


Figure A.13. The above figure shows the total radioactivity bound to the plant matter increasing over time in flasks with living plants. This shows that the plants are incorporating the RDX into the plant matter. Triplicate axenic *Arabidopsis thaliana* cultures were used for the living plant cultures.

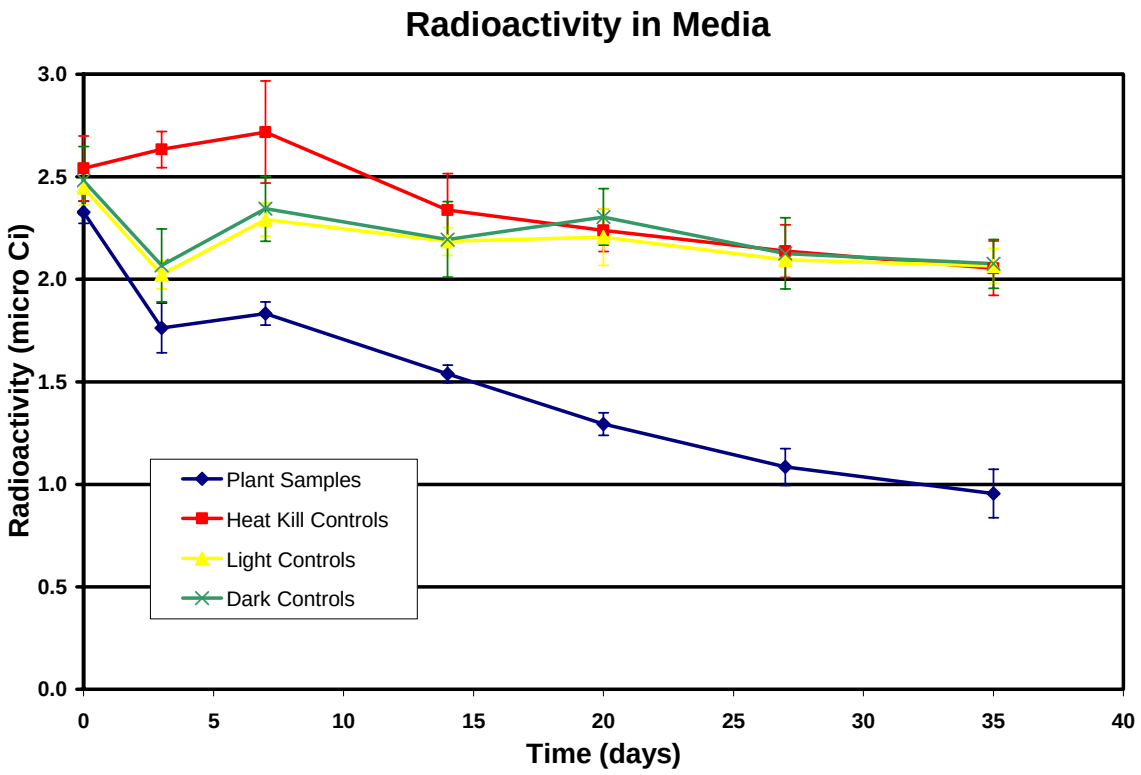


Figure A.14. The total radioactivity in the media decreases in all flasks after 35 days. The plant samples remove the radioactivity from the media better than all of the controls.

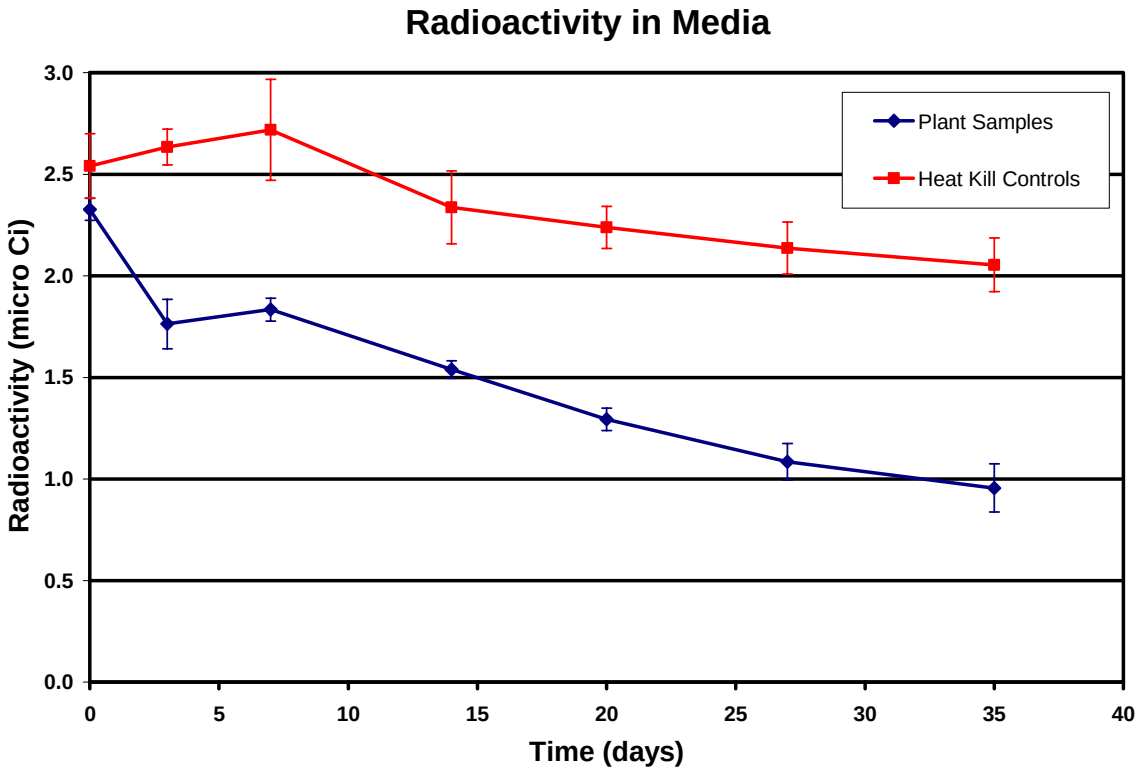


Figure A.15. More radioactivity is removed from the media by the live plants.

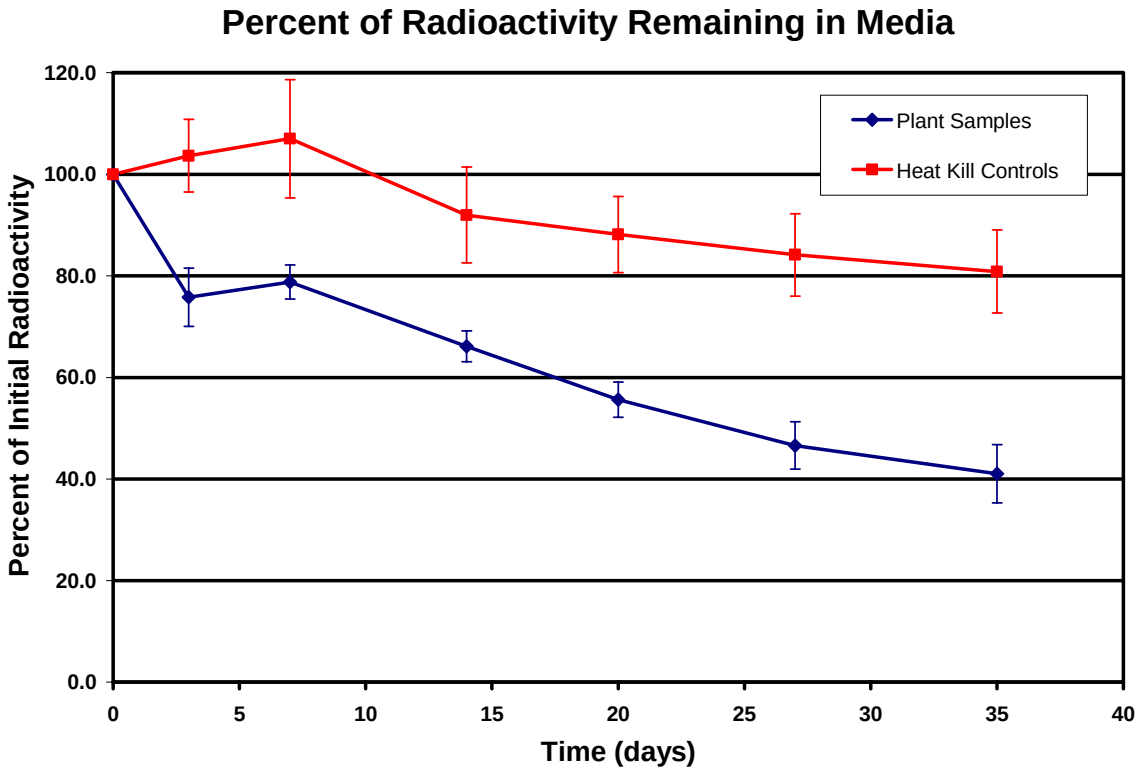


Figure A.16. More radioactivity is removed from the media by the live plants. 58% of the initial radioactivity is removed by the live plants, compared to the 19% removed by the heat kill controls.

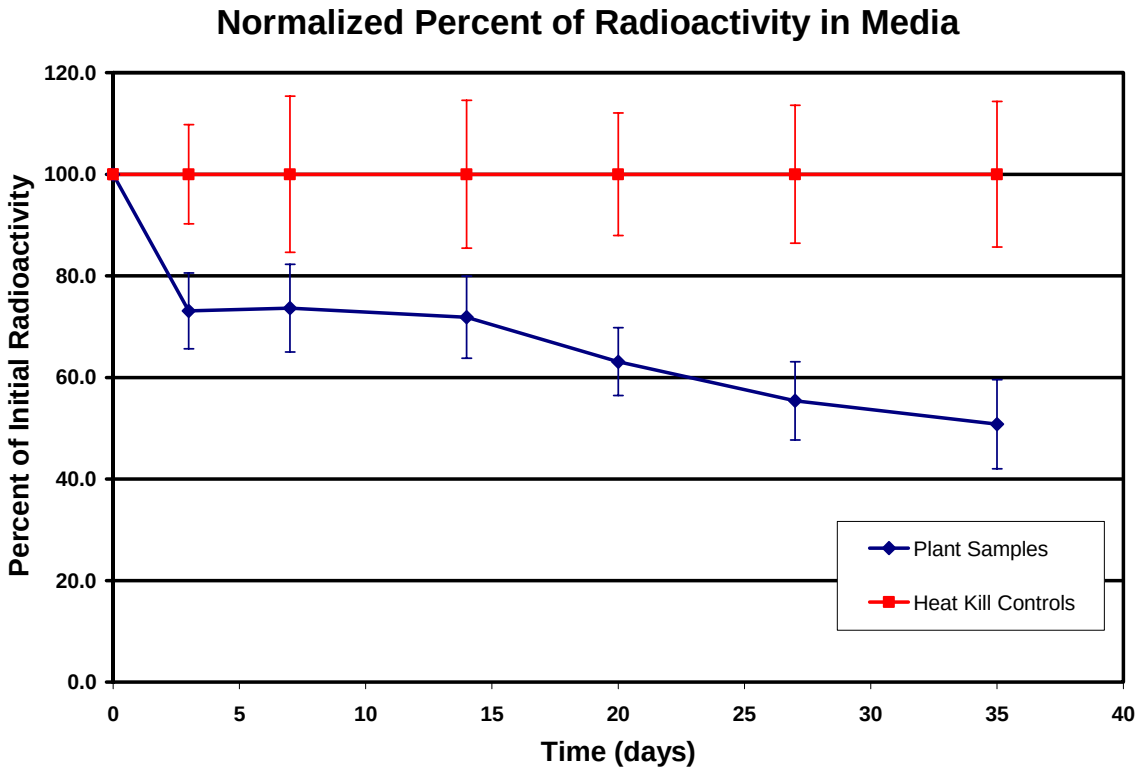


Figure A.17. The data is normalized so that the difference between the heat kill control and the plants can be seen. The plant samples show a decrease in radioactivity that illustrates over 50% of the RDX is being taken up by the plants after 35 days.

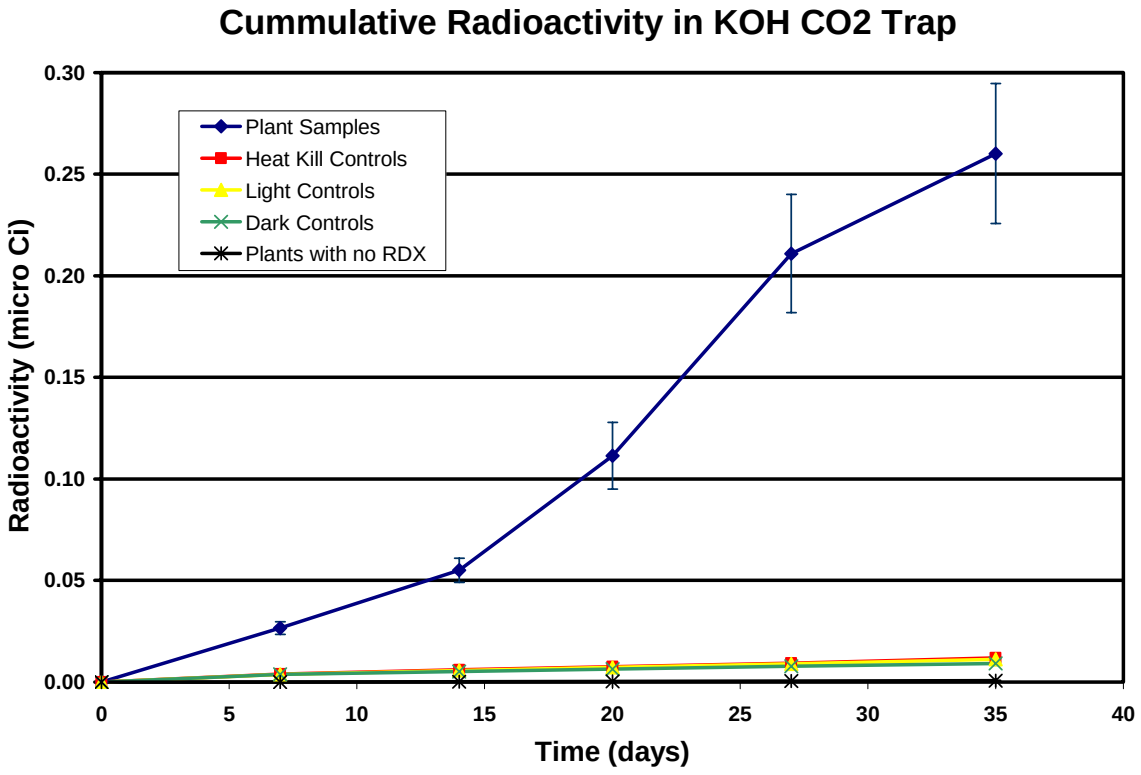


Figure A.18. The total radioactivity in the carbon dioxide traps increases in all flasks during the experiment. The living plants mineralize RDX to form significant amounts of radioactive carbon dioxide.

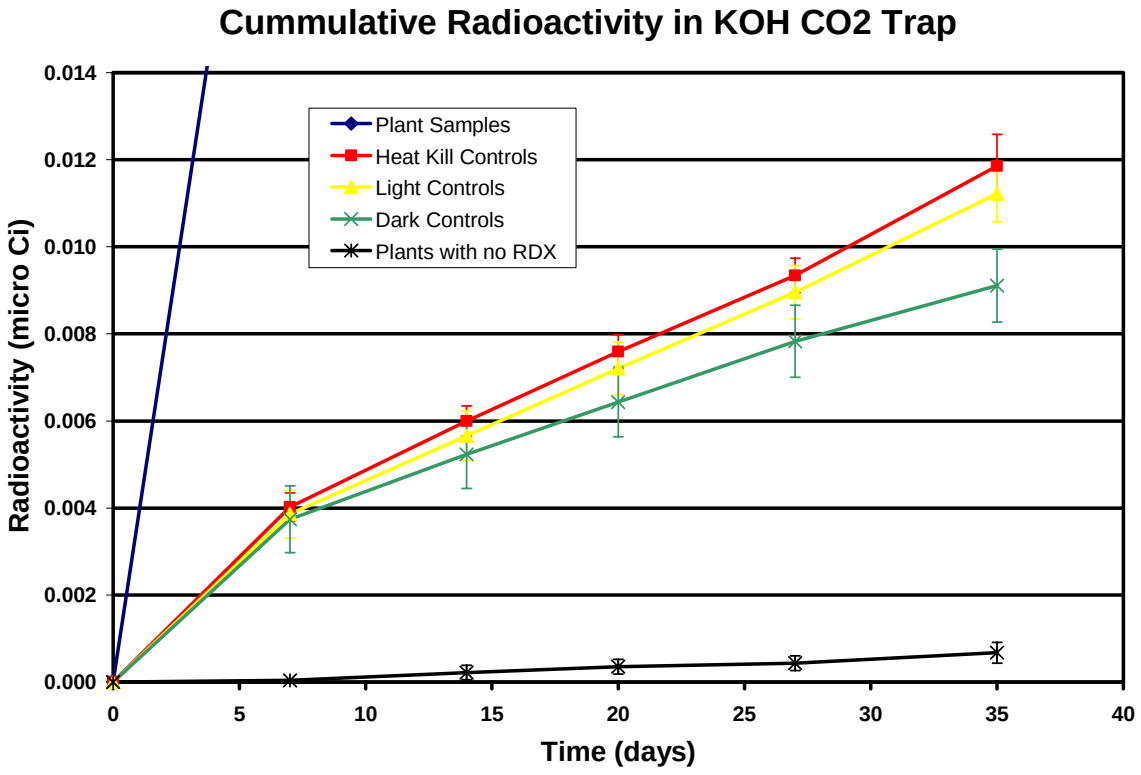


Figure A.19. The radioactivity in the carbon dioxide traps does not differ between the controls. This would suggest that the ^{14}C-labeled carbon dioxide is only being produced by metabolism of RDX by living plants.

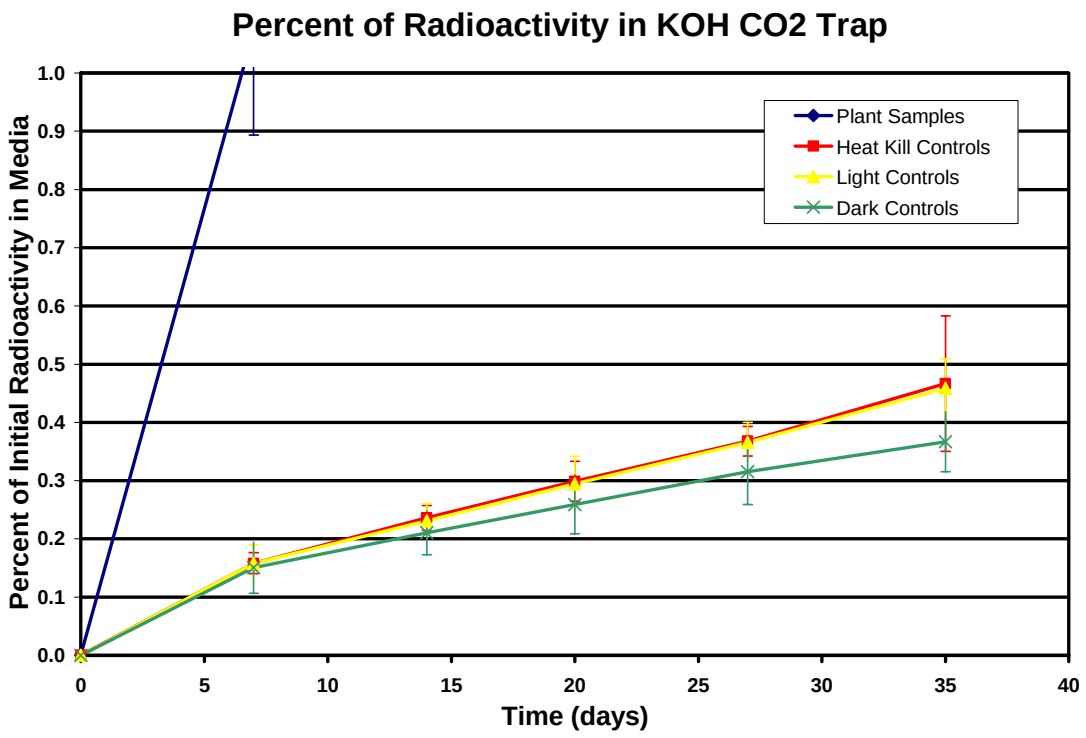


Figure A.20. Over 10% of the initial radioactivity was converted to carbon dioxide by living plants. This is significantly more mineralization than the 0.5% that was observed in all of the control flasks.

Table A.1. The unknown contaminant peak area from the flow scintillation radiogram. The contaminant is present in both the living plant cultures and the controls during the experiment. The peak does not increase or decrease in size over time. This indicates that the contaminant is stable over time and is not being formed from ^{14}C-RDX or broken down into ^{14}C-carbon dioxide.

	Time 0 – 0 days after adding RDX			Time 6 – 35 days after adding RDX		
	Living Plant Cultures	Light Media Controls	Dark Media Controls	Living Plant Cultures	Light Media Controls	Dark Media Controls
Unknown contaminant peak area	801	608	796	521	857	753
	796	784	690	932	771	829
	789	804	688	820	946	780
	759	737	804	937	978	953
	753			673		
Average (std dev)	779 (22.06)	733 (88.10)	745 (64.17)	777 (178.7)	888 (93.30)	829 (88.61)

Appendix B – Phytoremediation Glossary

Remediation - The process of trying to improve (pollution in the environment)

Degradation - The process of breaking down a compound

Biodegradation - Degradation using bacteria and other living organisms (plants)

Biotic - Produced or caused by living organisms

Abiotic - Not produced or caused by living organisms

Aerobic - Presence of oxygen

Anaerobic - Absence of oxygen

Clostriduium - A bacteria that is rod-shaped, gram-positive, spore-forming, chiefly anaerobic and very biochemically active

Amine - Organic compound containing NH_2, NH, or N

Amino - Organic compound containing NH_2

Nitroso - Containing NO

Nitrosamine - A nitroso group bonded directly to an amino nitrogen (containing an N-NO)

Nitro - Containing NO_2

Nitramine - A nitro group bonded directly to an amino nitrogen (containing an N-NO_2)

HPLC - High Performance Liquid Chromatography

LC - Liquid Chromatography

GC - Gas Chromatography

MS - Mass Spectroscopy

SPME - Solid Phase Microextraction

RAM - Radio Active Material Detector

Appendix C – Chemical Information

RDX standards (1mg/ml RDX in acetonitrile) were purchased from ChemService (West Chester, PA). Standards of the known RDX metabolites were purchased in powder form from Ron Spanngord at Stanford Research Institute (SRI) International (Menlo Park, CA) for HPLC.

Bulk RDX in acetone (50 mg/ml) was purchased from AccuStandard (New Haven, CT). Shipping requirements prohibited the purchase of bulk RDX in powder form. Additional safety requirements at Iowa State University were developed with Environmental Health and Safety. These included keeping the RDX refrigerated and fully dissolved in solution, as well as maintaining a log for RDX usage.

Bulk RDX was very hard to obtain due to recent increases in security of explosive materials. Quotes were obtained from AccuStandard, Cerilliant, and PerkinElmer for custom synthesis of concentrated RDX in solution. Other research groups acquired RDX from the government, while some researchers synthesized their own RDX. These government research facilities may also be a source for bulk RDX: US Army Toxic and Hazardous Materials Agency (Edgewood, MD), Battelle Columbus Division (Columbus, OH), Hallston Army Ammunition Plant (Kingston, TN).

Radio-labeled RDX was purchased from PerkinElmer (Boston, MA).

RDX

Registry Number: 121-82-4

Chemical Name: hexahydro-1,3,5-trinitro-1,3,5-triazine

MNX

Registry Number: 5755-27-1

Chemical Name: hexahydro-1,3-dinitro-5-nitroso-1,3,5-triazine

DNX

Registry Number: 80251-29-2

Chemical Name: hexahydro-1-nitro-3,5-dinitroso-1,3,5-triazine

TNX

Registry Number: 13980-04-6

Chemical Name: hexahydro-1,3,5-trinitroso-1,3,5-triazine

MEDINA

Registry Number: 14168-44-6

Chemical Name: Methylene-dinitramine or N,N’-dinitro-methanediamine

NDAB

Registry Number: 479422-92-9

Chemical Name: 4-nitro-2,4-diazabutanal or N-[(nitroamino)methyl]-formamide

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