FINAL REPORT

Engineering Transgenic Plants for the Sustained Containment and In Situ Treatment of Energetic Materials

SERDP Project ER-1318

JUNE 2009

Stuart E. Strand
Sharon L. Doty
University of Washington

Neil Bruce
University of York

This document has been approved for public release.

Strategic Environmental Research and Development Program

This report was prepared under contract to the Department of Defense Strategic Environmental Research and Development Program (SERDP). The publication of this report does not indicate endorsement by the Department of Defense, nor should the contents be construed as reflecting the official policy or position of the Department of Defense. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the Department of Defense.

Table of Contents

List of Acronyms iii
List of Figures and Tables iv
Acknowledgements vi
I. Executive Summary 1
Enterobacter cloacae nitroreductase reduction products 1
The phytotoxicity of TNT 1
Effect of transgenic tobacco on soil microbial community 2
Uptake of TNT and RDX by transgenic Arabidopsis 3
Production of transgenic Arabidopsis 3
Removal of RDX from soil leachate by transgenic Arabidopsis 3
Transgenic Aspen 4
Transformation of other plant species appropriate to training ranges 5
II. Objectives 5
III. Background 5
Products of TNT reduction 6
Phytotoxicity of TNT 7
Liquid culture studies of TNT uptake by plants 8
Interactions between plants and soil microbial communities 8
Arabidopsis expressing both XplA and NR 10
Production of transgenic Poplar 10
IV. Materials and Methods 11
Production of *35S-nfsI* tobacco 11
Tobacco germination studies 11
Liquid culture studies with NR tobacco 11
Amendment of soil with TNT 12
Ageing of TNT-Amended Soil 12
Growth of Plants in TNT-Amended Soil 12
Colony forming unit count 14
Biolog Eco plates 14
Genomic DNA extraction from soil 15
Amplification of 16S rDNA by PCR 15
Denaturing gradient gel electrophoresis 15
HPLC for detection of TNT and metabolites 16
Transforming Arabidopsis with *xplA*, *xplB*, *nfsI* and *3A4* 16
Liquid culture analysis of Arabidopsis expressing XplA and NR 16
Inhibition of XplA by TNT 16
Soil leachate studies on Arabidopsis expressing XplA and NR 17
Transformation of Aspen 17
Regeneration of transgenic Aspen 17
TNT and RDX uptake by transgenic aspen in nitrogen free media 18
TNT metabolism in *nfsI*-transformed aspen 18
Hardening off of Transgenic Aspen Line 10 18
V. Results and Accomplishments 21
Reduction products of *Enterobacter cloacae* NR 21
Quantitative RT- PCR analysis of *nfsI* expression in tobacco 25

Root morphology of seedlings exposed to TNT 25
Root Tolerance Index to TNT 30
Phytotoxicity of TNT during vegetative growth 30
Soil studies on NR-expressing tobacco 33
Fate of TNT and transformation products following plant uptake 34
Soil characterization 35
Colony forming unit count 36
Substrate utilization profiles 38
Genetic diversity 48
Characterization of XplA expressing Arabidopsis 55
Arabidopsis expressing NR 57
Arabidopsis lines expressing XplA and NR 59
Inhibition of XplA by TNT 61
Soil studies on NR expressing Arabidopsis 68
Soil leachate studies on XplA and NR Arabidopsis 68
Characterization of XplB expressing Arabidopsis. 69
Liquid culture analysis of Arabidopsis expressing XplA and XplB 69
Production of 3A4, CPR, XplA and NR expressing Aspen 71
RDX removal by best aspen root lines in nitrogen free media 72
TNT and RDX uptake by transgenic aspen in nitrogen free media 72
New NR Transgenic Aspen 75
Multigene Transgenics 75
TNT metabolism in nfsI-transformed aspen 76
RDX Metabolism by Transgenic Aspen Line 10 77
Hardening off of Transgenic Aspen Line 10 78
VI. Concluding Summary 79
TNT toxicity studies on NR expressing tobacco 79
Growth of NR-expressing tobacco plants on TNT-contaminated soil 79
Comparison of the microbial communities of bulk soil 80
Comparison of the microbial communities of rhizosphere soil 81
Characterization of XplA expressing Arabidopsis 84
Transformation of INRA 717 aspen with xplA and nfsI using A. rhizogenes 86
Appendices 87
List of Technical Publications 87
Other Technical Material 87
References 89

List of Acronyms

ADNT	Aminodinitrotoluene
cDNA	Complimentary DNA
DGGE	Denaturing gradient gel electrophoresis
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
GTase	UDP-glycosyltransferase
GTN	Glycerol trinitrate
HADNT	Hydroxlyaminodinitrotoluene
HMX	High metling explosive (octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine)
HPLC	High performance liquid chromatography
INRA	French National Institute for Agricultural Research
MEDINA	Methylendinitramine
MG	Mono Glucoside
MS	Mass Spectrometry
NDAB	4-nitro-2,4-diazabutanal
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
nfsI	Gene encoding TNT-detoxifying nitroreductase from *Enterobacter cloacae*
NR	TNT-detoxifying nitroreductase enzyme from *Enterobacter cloacae*
PCR	Polymerase chain reaction
PETN	Pentaerythritol tretranitrate
RDX	Royal demolition explosive (hexahydro-1, 3, 5-trinitro-1,3,5-triazine hexahydro-1,3,5-trinitro-1,3,5-triazine
SERDP	Strategic Environmental Research and Development Program
TNT	2,4,6-Trinitrotoluene
UDP	Uridine Diphosphate
UY	University of York
UW	University of Washington
XplA	RDX-degrading enzyme from *Rhodococcus rhodochrous* 11Y
xplA	Gene encoding RDX-degrading enzyme from *Rhodococcus rhodochrous* 11Y
XplB	Reductase encoded adjacently to *xplA* in *Rhodococcus rhodochrous* 11Y genome
xplB	Gene for reductase encoded adjacently to xplA in *Rhodococcus rhodochrous* 11Y genome

List of Figures and Tables

Table 1 Development of lateral roots of untransformed wild type and transgenic seedlings germinated on TNT. ... 26
Table 2 Root tolerance index of tobacco seedlings after exposure to TNT ... 30
Table 3 Effects of TNT toxicity on the vegetative growth of tobacco ... 31
Table 4 ANOVA testing for association of CFU per gram soil, with TNT concentration and soil type ... 38
Table 5 ANOVA testing for association of average AAT with TNT concentration and soil type 42
Table 6 Eigenvalues, percentage variance and number of loadings ... 42
Table 7 The factor solution is presented with significant loadings for each factor emboldened.. 43
Table 8 Substrates with significant loadings for PC1 and PC2 ... 47
Table 9 Bacterial species with related sequences identified from DGGE bands ... 53
Table 10 Characterization of transgenic hybrid aspen lines ... **Error! Bookmark not defined.**
Table 11 Uptake of RDX by transgenic hybrid aspen lines, *P. tremula* x *P. alba* (INRA 717-1B4) transformed with *3A4*, *cpr, xplA* and *nfsI*. ... 72
Table 12 Uptake of TNT and RDX from hydroponic nitrogen free media by transgenic aspen. Tall cuttings were grown in vials containing TNT(0.05 mM) and RDX(40µg/ml; 0.181 mM) in nitrogen free media and allowed to grow. Experiment was performed in triplicate ... 72

Figure 1 Proposed pathway of aromatic compound reduction by nitroreductase (NR) ... 6
Figure 2 Proposed pathway of RDX degradation by *Rhodococcus rhodochrous* 11Y ... 9
Figure 3 Schematic of the soil columns for growth of tobacco in TNT-amended soil ... 13
Figure 4 Outline of TNT-amended soil experiment design ... 13
Figure 5 Transformation of TNT by *Enterobacter cloacae* nitroreductase ... 22
Figure 6 Analysis of TNT transformation products from *Enterobacter cloacae* nitroreductase.. 23
Figure 7 Characteristic UV/vis spectra of 4-HADNT produced by *Enterobacter cloacae* nitroreductase ... 24
Figure 8 Reduction of TNT to 4-HADNT by *E. cloacae* nitroreductase ... 24
Figure 9 Relative quantitative levels of *nfsI* expression in transgenic plant lines ... 25
Figure 10 Root morphology of seedlings germinated on TNT ... 26
Figure 11 Morphology of transgenic NR 3-2 tobacco roots grown on TNT. ... 28
Figure 12 Morphology of transgenic NR 3-2 tobacco seedlings germinated on TNT. ... 29
Figure 13 Toxicity of TNT on liquid culture grown tobacco ... 32
Figure 14 Tobacco plants grown in TNT-amended soils for 12 weeks ... 34
Figure 15 Root and shoot lengths of NR expressing tobacco grown in TNT contaminated soil . 34
Figure 16 Concentrations of TNT and transformation products within soil ... 35
Figure 17 Soil pH across a range of TNT concentrations for different soil samples ... 36
Figure 18 Colony forming unit count per gram of soil after six days incubation ... 37
Figure 19 Time courses of substrate utilization in TNT contaminated soil ... 40
Figure 20 Average AAT for EcoPlate incubations from soil samples ... 41
Figure 21 Principle component analysis of the Biolog EcoPlate data ... 45
Figure 22 Principal component 1 scores for soil amended with TNT ... 46
Figure 23 Agarose gel of community genomic DNA extracted from soil samples ... 49
Figure 24 16S rDNA PCR amplification products ... 49
Figure 25 DGGE of 16S rDNA products TNT amended soil ... 50

Figure 26 Pearson correlation of lanes from DGGE analysis 51
Figure 27 Sequence alignment of excised and sequenced bands from DGGE gels 52
Figure 28 Phylogenetic tree of DGGE-related sequences 54
Figure 29 Transcript expression of *xplA* and XplA protein levels..... 55
Figure 30 Uptake of RDX from liquid medium by Arabidopsis seedlings. 56
Figure 31 Root lengths of NR expressing Arabidopsis grown on TNT 57
Figure 32 TNT Uptake from liquid culture by NR expressing Arabidopsis..... 58
Figure 33 Uptake of RDX and TNT by Arabidopsis expressing both XplA and NR from liquid culture 60
Figure 34 Uptake of RDX by Arabidopsis expressing both XplA and NR from liquid culture Ten-day-old seedlings were dosed with 180 µM RDX. WT, untransformed wild type. Results are means ± s.e.m. of five replicate measurements. 61
Figure 35 Uptake of RDX and increasing concentrations of TNT from liquid culture by Arabidopsis expressing both XplA and NR 62
Figure 36 Inhibition of purified XplA activity by TNT 63
Figure 37 Arabidopsis expressing XplA grown on RDX-contaminated soil 64
Figure 38 Removal of RDX from contaminated soil by XplA expressing Arabidopsis 65
Figure 39 Levels of RDX in soil leachate from Arabidopsis expressing XplA..... 66
Figure 40 Growth of XplA expressing Arabidopsis lines on RDX contaminated soil 67
Figure 41 Growth of NR expressing Arabidopsis on TNT contaminated soil 68
Figure 42 Levels of RDX in soil leachate from *35S-xplA-nfsI* Arabidopsis lines 69
Figure 43 RDX uptake from liquid culture by Arabidopsis expressing XplA and NR 70
Figure 44 Transgenic aspen expressing XplA (Line 10) plants dosed with RDX..... 73
Figure 45 Transgenic aspen expressing *xplA* (Line 10) plants 74
Figure 46 RDX uptake by hydroponic cultures of Line 10 aspens transformed with *xplA* compared to vector control plants and media controls with no plants..... 74
Figure 47 NR transgenic aspen root line WK..... 75
Figure 48. Line H triple transgenic aspen with NR, CPR, and xplA. 76
Figure 49 TNT products detected in transgenic aspen expressing NR..... 77
Figure 50 Schematic of proposed method for phytoremediating TNT and RDX from military training ranges using genetically modified plants 85

Acknowledgements

Professor Neil Bruce
Dr. Liz Rylott
Dr. Nerissa Hannink
Dr. Emma Travis
Dr. Susan Rosser
Dr. Debs Rathbone
Others...

University of York, York, UK

Dr. Sharon Doty
Dr. Zareen Khan
Allison Moore
Azra Vajzovic
Undergraduates Anwar Zaman, Dilom Fesaha, Sandy Li, and Leonid Kisselev
Mychajlo Eliaszewsky
Caitlin Pensak

University of Washington

I. Executive Summary

Explosives from manufacture and military use now contaminate large areas of land and ground water across the United States and Europe. These energetic compounds are both toxic and recalcitrant to degradation and removing them is a difficult and massive logistical task. While phytoremediation is a technique that potentially offers an environmentally friendly, low-cost alternative to current remediation techniques, the use of this method is hindered by the phytotoxicity of TNT and the low inherent metabolic abilities of plants towards these xenobiotic compounds. Bacterial enzymes capable of detoxifying these energetic compounds have been identified, and further characterization is presented here. These genes have been introduced into the model plant species, tobacco and Arabidopsis where, in combination, they confer tolerance to TNT and the ability to detoxify TNT and degrade RDX. These studies, shown below, provide valuable information on the ability of transgenic plants to remove explosives from soil and ground water. The knowledge gained using these model species has been essential in the production and characterization of more robust tree species, Aspen and poplar hybrids, for both laboratory and field testing.

***Enterobacter cloacae* nitroreductase reduction products**

We have previously found that expression of *Enterobacter cloacae* nitroreductase (NR) confers tolerance to TNT by rapidly transforming TNT to hydroxylamino derivatives [1]. These can subsequently be conjugated by the plant to sugars. In work presented here a time-course HPLC-based assay was conducted which revealed that the major product of TNT reduction was the 4-isomer of hydroxylamino-dinitrotoluene (4-HADNT). This was confirmed by comparison of retention times and UV/vis spectra to authentic standards of 4-HADNT.

The phytotoxicity of TNT

The ability of untransformed wild type tobacco to germinate on TNT was not substantially affected at concentrations up to 0.05 mM TNT (11mg/L). This inherent level of tolerance could be conferred by the expression of an endogenous NR. However, the expression of bacterial NR in transgenic seedlings greatly enhanced germination. The tolerance of germinating seedlings to TNT was found to be positively correlated with *nfsI* expression. The most tolerant transgenic line, NR 3-2 was able to germinate at 0.25 mM (56 mg/L) TNT, a level which inhibited germination of untransformed wild type tobacco seeds. This is encouraging as a phytoremediation application may begin with seeds that would need to be tolerant to TNT to germinate. Germination studies with TNT are few; however, the tolerance conferred by the presence of *nfsI* in line NR 3-2 compares favorably to that of a range of wild grass species tested including tall fescue (*Festuca arundinacea Schreb.*)[2], switchgrass and smooth bromegrass [3]. When grown in liquid culture transgenic NR 3-2 plants increased in biomass when in the presence of 0.5 mM (113 mg/L) TNT. This level of TNT tolerance has not been previously reported for any plant species and is above the aqueous solubility limit of TNT (approximately 100mg/L). This indicates that transgenic plants expressing NR activity would be able to withstand the highest concentrations of TNT encountered in ground water from training ranges.

Soil studies of TNT tolerant transgenic tobacco

The NR-expressing tobacco plants exhibited increased tolerance to TNT in soil compared to the untransformed, wild type plants. In uncontaminated soil, both transgenic and untransformed

wild type plants grew well, with no apparent differences in growth or morphology. In soil contaminated with 150 mg/kg TNT, untransformed wild type plants exhibited stunting of roots, and chlorosis of leaves, whilst NR plants showed dramatically increased tolerance to the toxic effects of TNT. With increasing TNT concentration, the phytotoxic effects of TNT on both untransformed wild type and transgenic plants increased. At the highest level of TNT (4000 mg/kg) wilting and necrosis was observed in the untransformed wild type plants, while the NR expressing line was still exhibiting signs of tolerance.

Effect of transgenic tobacco on soil microbial community

The effect of growing tobacco in TNT-contaminated soil on the microbial community structure and function of soil was investigated using colony forming unit (CFU) enumeration, carbon substrate utilization patterns (Biolog Ecoplates) and denaturing gradient gel electrophoresis (DGGE) analysis. Monitoring CFUs from soil in the bulk and unplanted samples revealed there was a shift in the microbial community from slower growing k-strategists towards faster growing r-strategists as the concentration of TNT increased. Pseudomonads, of which many species are known to transform TNT, were heavily represented in soils with high levels of TNT contamination. At the highest concentrations of TNT, the overall culturable bacterial count dropped significantly in unplanted and bulk soil samples from untransformed wild type and NR expressing plants.

The main effects of the plants on the microbial community occurred in the rhizosphere. In uncontaminated soil, there was no difference in the levels of culturable bacteria in the rhizosphere sampled from NR expressing plants compared to that frpm untransformed wild type plants. Extremely little is known about the impact of transgenic crops on soil ecosystems and this result has important implications for the use of the transgenic tobacco in the environment. At 150 and 500 mg TNT/kg soil there was a reduction in the CFUs from untransformed wild type rhizosphere soil compared to NR-expressing tobacco rhizosphere soil, there was also a dramatic decrease in the amount of rhizosphere soil from untransformed wild type plants. At the highest levels of TNT contamination, (1500 and 4000 mg TNT/kg soil) only NR-expressing plants had sufficient roots to enable study of the rhizosphere, due to the phytotoxicity of the TNT to the untransformed wild type tobacco. Even at these high concentrations of TNT amendment, the culturable bacterial count remained relatively high in the samples from the NR plants. This is likely to be due to a combination of the relatively healthy NR plants being able to produce microbial-enhancing root exudates, and localized removal and detoxification of TNT by the plant roots. There was not enough rhizosphere soil available to determine TNT concentration in these samples. Analysis of TNT concentrations in a combination of rhizosphere and bulk soil revealed that concentrations of TNT within the soil of NR-expressing tobacco were, in general, lower than that from untransformed wild type tobacco soil.

Carbon substrate utilization patterns indicated that the overall metabolic activity was, in general, higher for the rhizosphere soil compared with the bulk soil of both NR expressing and untransformed wild type plants. DGGE analysis showed that the genetic diversity of the rhizosphere soil from untransformed wild type and NR expressing tobacco plants did not vary significantly. The concentration of TNT with which soil was contaminated was observed to impact on the genetic diversity of the soil microbial community. Sequencing of DGGE gel bands from TNT-contaminated samples showed that they were all closely related to the Bacteriodetes division. Bacteriodetes increased as the concentration of TNT in the soil rose, they are known to be less susceptible to TNT toxicity than the general bacterial population and might be playing an

active role in taking up and transforming TNT. It is possible that rhizodegradation of TNT occurs via an increase in carbon via root exudates leading to more efficient metabolism of TNT by the bacteria present in the rhizosphere. This study shows that plants engineered for the phytoremediation of TNT can increase the functional and genetic diversity of the rhizosphere bacterial community in acutely polluted soil compared to untransformed wild type plants.

Uptake of TNT and RDX by transgenic Arabidopsis

Transgenic Arabidopsis lines expressing the cytochrome P450, XplA, from the RDX-degrading *Rhodococcus rhodochrous* strain 11Y, and nitroreductase, NR, from *Enterbacter cloacae* were produced. The XplA-expressing lines removed 180 μM RDX from liquid culture in seven days, whilst there was no significant removal of RDX by the untransformed, wild type Arabidopsis. 180 μM is over three times the concentration measured in waste water from manufacturing sites[4] and is close to the aqueous solubility limit for RDX (at 25 ºC) [5]. Additionally Arabidopsis expressing NR exhibited similar tolerance to that observed for the tobacco expressing NR. The transgenic lines having the highest rates of uptake (lines *35S-xplA*-6 and -10 and *35S-nfsI-1*) were crossed to produce plants that take up both RDX and TNT from liquid culture. However, our liquid culture studies showed that there was a two-day lag prior to any RDX removal from the media by the *35S-xplA-nfsI* plants. This was not observed in the *35S-xplA*-only line. This delay corresponded with the time taken by the *35S-xplA-nfsI* line to remove all the TNT from the media. To investigate this further, we conducted liquid culture experiments with a range of TNT concentrations in the presence of 180 μM RDX. The results showed that the delay in RDX uptake increased with increasing TNT concentration, with no delay in RDX uptake in the absence of TNT. Although the *35S-xplA-nfsI* lines removed all the TNT from the flasks and remained green at lower concentrations of TNT, at the two highest concentrations of TNT tested (175 μM and 250 μM), the *35S-xplA-nfsI* lines became bleached and necrotic. At these higher concentrations the *35S-nfsI* lines were able to remove TNT from the media and remain green. These results indicate that the presence of TNT inhibits RDX uptake and that the two different explosives could have overlapping detoxification pathways. We have demonstrated that the activity of purified NR is not altered by the presence of RDX; however, our studies show that XplA activity is inhibited by the presence of TNT.

Production of transgenic Arabidopsis

Our initial target was to engineer tobacco plants capable of detoxifying RDX. However, we decided to use Arabidopsis as our model system as it offers several favorable benefits over tobacco. Arabidopsis has a shorter life cycle and a more efficient transformation system, enabling homozygous transgenic lines to be produced significantly more quickly than for tobacco. The transformation process (floral dipping) does not require aseptic, tissue culture techniques, and is less labor intensive. Furthermore, the genome of Arabidopsis has been fully sequenced and annotated and there are many genomics tools available including microarrays, knock-out lines and mutant populations that will be valuable in studying the effects on plant gene expression, and elucidating the pathway of RDX detoxification in plants.

Removal of RDX from soil leachate by transgenic Arabidopsis

We have developed a method to determine RDX uptake from soil leachate by Arabidopsis, and used this to measure RDX uptake in untransformed wild type, XplA and NR-expressing transgenic Arabidopsis. The five lines expressing XplA activity removed significantly more (53-

80 %) RDX from the soil leachate than either untransformed wild type plants (30 %) or plants expressing only NR (28 %). The *35S-xplA-nfsI* double transgenic lines did not perform as well as expected, removing less (20-47 %) than the *35S-xplA* lines. This was not predicted as these lines removed RDX from liquid culture at rates similar to the *35S-xplA* parental lines.

In the *R. rhodochrous* 11Y genome, there is a reductase, *xplB*, encoded adjacently to *xplA*. To test if expression of XplB activity could increase the rate of RDX degradation in Arabidopsis expressing XplA, we transformed *35S-xplA*-line10, our fastest RDX-degrading line, with *xplB* and characterized five lines independent for *35S-xplB.* In addition, five, independent lines containing *35S-xplB* -only as control lines were analyzed. Transgene expression of both transgenes was confirmed using RT-PCR. Liquid culture studies were performed using 180 µM RDX. As predicted, RDX levels did not decrease significantly in the flasks containing the untransformed plants, or the *35S-xplB*-only lines, while the *35S-xplA*-line 10 plants removed all the RDX from the media within four days. The five *35S-xplA-xplB* lines exhibited uptake rates significantly higher than the *35S-xplA*-line with all five lines removing the RDX within one day and lines *35S-xplA-xplB*-2 and 27 removing 50 % of the RDX within four hours. The ability of XplA and XplB expressing lines to remediate saturating levels of RDX from soil leachate is particularly significant; this directly addresses the key problem of RDX contamination in ground water on military training ranges.

Transgenic Aspen

Green callus and small plantlets were regenerated from our two best XplA transgenic lines, Line 10 and Line L. These root lines had the highest removal rate of RDX (69% and 74%, respectively, compared to nearly no removal by the vector control lines. The plants grew well and were used for RDX uptake experiments.

Our two best xplA transgenic root lines, Line 10 and Line L, which had the highest removal rate of RDX (69% and 74%, respectively, compared to nearly no removal by the vector control lines) were tested as rooted cuttings in hydroponics. Line 10 plants grew more vigorously than Line L, so we did our RDX uptake experiments with only Line 10 compared with vector control plants. The tested plants were healthy and each had some roots. The plants were exposed to approximately 25 ppm RDX, and the concentration of RDX was monitored over ten days. The rooted Line 10 aspen removed an average of 43% of the RDX compared with only 10% by the vector control plants. There was no removal of RDX in the unplanted vials. The difference in RDX removal between the xplA line and the vector controls was seen as early as two days. This was our first experiment in which there was strong removal of RDX by whole aspen plants.

We have continued to transform more aspen with *nfsI* and *xplA*. One of the new lines, WK, displayed good growth on plates containing TNT. Genomic preps and PCR revealed that this line was transgenic only for *nfsI* and not for *xplA*. We incubated WK and vector control roots in medium containing TNT, and monitored TNT removal by HPLC. After only 24 hours, line WK removed 98% of the TNT compared with 59% for the non-transgenic control even though there was one third less root mass in the WK vials.

Our previously described two triple lines, Line 6 and Line H, have improved TNT and RDX uptake compared to the vector control plants. Line 6 and Line H were used for metabolite studies. Unfortunately, the TNT and RDX uptake rates are not as high with these lines as with our best lines transformed with either *xplA* (Lines 10 and L) or *nfsI* (Line WK).

The metabolism of TNT by *nfsI*-transformed aspen (Line 6 clones) was studied with the analysis of ADNT and hydroxyamino dinitrotoluene (HADNT) metabolites. TNT was degraded

quickly. A small peak at the retention time of the peak for 4-Amino-2,6-dinitrotoluene, 4 ADNT. We spiked our samples with 10 μg/ml of 4 ADNT and saw an increase in the area of the peak at 6.8 minutes retention time, the 4 ADNT peak retention time.

Unfortunately, the growth characteristics of aspen transformed with *A. rhizogenes* were poor, and the plants were unsuited to mass propagation. We are continuing to transform INRA 717 aspens using disarmed *A. tumefaciens.* Several transformed plantlets are developing and will be evaluated for their ability to degrade RDX.

Transformation of other plant species appropriate to training ranges

In the CU-1318 proposal we planned to transform other tree species (black locust, aspen, and conifer). After visiting Eglin, we realized that fire resistant grasses are better adapted to training range conditions. Accordingly, are collaborating with a scientist with experience in grass transformation (Mr Antonio Palazzo, ERDC-CRREL US Army). Species will be selected on the basis of their wide geographical distribution and resilience to fire. Agrobacterium and ballistic methods for transformation will be evaluated. One species with desirable transformation characteristics will be selected for further work. Genes for TNT and RDX transformation will be introduced and the transgenic grass tested for toxicity and metabolite fate as described above. Three grass species have been selected for transformation under the new SERDP project, ER-1498, starting in April 2006.

The project accomplished both objectives; developing and characterizing the ability of transgenic Arabidopsis and tobacco to degrade or detoxify the explosives RDX and TNT. The insight gained from studying the responses in these model plant systems has been key to the production of transgenic poplar lines that are now available for glasshouse and field trials.

II. Objectives

The first objective of this project was to engineer model plant species to express bacterial genes to detoxify or degrade explosive compounds TNT and RDX. The mode of action of these bacterial enzymes and the ability of the transgenic plants to detoxify or degrade the explosives was to be analyzed.

The second objective was the production of robust transgenic plants with desirable structural and biological characteristics that can be used to contain and degrade energetic materials. Poplar trees which are already used in phytoremediation and easily transformable were the target species. These plants were to be developed for future glasshouse and field trials.

III. Background

During the last two decades, significant progress has been made in the treatment and containment of soils contaminated with explosives, but existing methods are intrusive and expensive. Containment of explosives in sites such as training ranges that receive periodic inputs of energetic materials, using existing technologies, continues to be demanding and very expensive. While it may be feasible to prevent their horizontal movement using physical barriers,

vertical migration of the more mobile compounds such as RDX and HMX is a matter of major concern.

Phytoremediation is generating significant interest as an economical and self-sustaining alternative for containment and clean up of toxic compounds. Plants have been shown to promote uptake, degradation and sequestration of TNT, but the activities are low and RDX is generally only partially transformed and RDX residuals bind to plant tissues. In addition, phytoremediation is limited by the phytotoxicity of TNT and other munitions pollutants. These limitations can be addressed by the use of genetic engineering whereby genes that encode enzymes active against xenobiotic compounds are expressed in plants.

Products of TNT reduction

The *Enterobacter cloacae* nitroreductase (NR) belongs to a family of oxygen-insensitive nitroreductases including members from *Escherichia coli* strain B [6] and *Salmonella typhimurium* [7]. It shares over 80 % amino acid sequence identity with both of these enzymes [8, 9]. Interestingly the TNT reduction products detected from these bacteria are different: the hydroxylamino derivative was detected from *E. coli* strain B whilst the amino derivative was detected from *S. typhimurium* cultures. As yet, it is not known if the NR protein structure is involved in determining whether the final reduction product will be a hydroxyl or an amino derivative. Hydroxylamino derivatives are rarely reported in plant studies of TNT transformation and as hydroxylamino derivatives are significantly less stable than the amino derivatives, this may be a factor.

The original pathway of TNT reduction [10] proposed that prior to the production of the hydroxyl derivative, a nitroso derivative would be formed (Figure 1). This was proposed from indirect evidence of nitroso dinitrotoluene production from the isolation of azoxy dimers (which are formed from condensation between nitroso and hydroxylamino groups). The nitroso derivative itself is rarely reported possibly due to the observation that reduced nicotinamide and flavin cofactors can reduce nitroso groups in the absence of enzyme catalysis [11]. Before the start of this project, the biological formation of nitroso derivatives of TNT had not been conclusively demonstrated although the production of both 2 and 4 isomers of the nitroso compound from 2,4 dinitrotoluene had been reported [12]. The isomers were found to be very unstable and were produced from microbial transformation using a mixed culture from activated sludge.

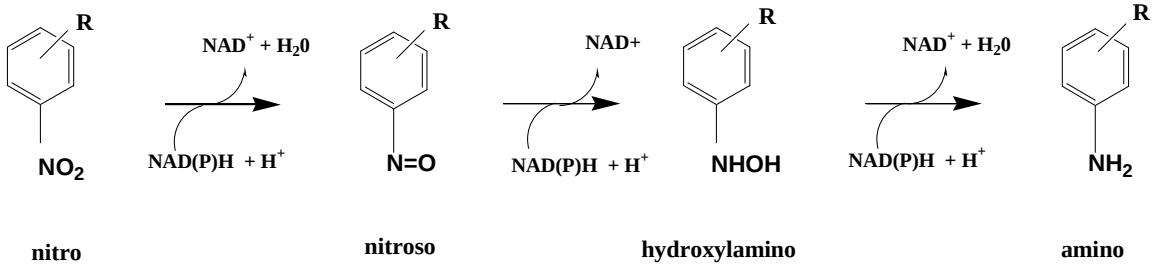


Figure 1. Proposed pathway of aromatic compound reduction by nitroreductase (NR) (Bryant and DeLuca, 1991).

We have studied the products of TNT reduction by the *E. cloacae* strain NCIMB10101 NR to establish the identity of TNT transformation product isomers and to make future comparison between any bacterial nitroreductase products from transgenic plants and plant endogenous nitroreductase activities. This was performed using HPLC-based time course assays and by comparisons with authentic standards.

Phytotoxicity of TNT

Plant NR activity against TNT has been observed in various studies, with a range of plant species including terrestrial [13, 14] and aquatic plants [15, 16]. In a study of plants with enzyme activity against TNT, NRs were found in 20 % of plants tested [17]. Germination studies on TNT toxicity are few. When the grass tall fescue (*Festuca arundinacea.*) was exposed to 7.5 mg/L (0.033 mM) TNT the growth of the primary root was affected and the radicle did not have as many root hairs present. On higher TNT concentrations such as 60 mg/L (0.26 mM) TNT, the radicle, if present, was very small and lacked secondary roots [2]. Later work by other researchers, also on grasses, suggests that the root growth rate of switchgrass (*Panicum virgatum*) was reduced at concentrations above 15 mg/L (0.066 mM). At 60 mg/L (0.26 mM) only a primary root was present which lacked root hairs. After 14 days at concentrations above 30 mg/L (0.13 mM) necrosis was observed on roots and shoots. Smooth bromegrass (*Bromus inermis*) root development was more affected by TNT with concentrations above 7.5 mg/L (0.33 mM) affecting root growth rate. The differences in TNT tolerance highlighted by these studies indicate that TNT tolerance at germination is species-dependent, as has been suggested for TNT-tolerance during vegetative growth [3].The ability of grasses to germinate was not substantially affected up to 0.05 mM TNT [18]. Interestingly, at levels of TNT below the toxicity threshold (<0.025 mM), enhanced growth has been reported in a number of species [19]. This phenomenon, termed “hormesis”, has been observed in many areas of toxicological study and occurs for example, when the compound mimics the effects of a growth hormone at low doses. This results in a non-linear dose response whereby low-level exposure to a toxicant causes beneficial effects, and detrimental effects are observed at higher concentrations [20]. Hydroponically grown cotton and corn were observed to have stimulated growth rates when exposed to sublethal doses of the herbicide, and auxin analogue 2,4-D [21]. The presence of TNT also reduced the formation of root hairs and lateral roots in grasses, and this may be due to variations in seed structure [2, 22]. Switchgrass was found to germinate better than smooth bromegrass possibly because the switchgrass seed coat is more impervious to TNT, thus physically restricting TNT movement into the embryo [22].

As the precise mode of TNT phytotoxicity is unknown, the exact benefit of the NR gene *nfsI* expression is unclear. In the proposed pathway of nitroaromatic reduction by NR (Figure 1) the nitro group is reduced, via a nitroso intermediate to hydroxylamino and amino forms. This functionalizes the compound, making further detoxification processes such as conjugation and compartmentation possible. Prior to the start of this project, we have shown that expression of *nfsI* in tobacco confers increased tolerance to TNT [1]. As part of this project, a thorough investigation on the ability of these plants to detoxify TNT has now been performed and is presented below.

Liquid culture studies of TNT uptake by plants

The reduction products of TNT are frequently observed when plants are grown in any media containing this explosive. The most common transformation products of TNT produced by plants, are the monoamino-dinitrotoluenes (ADNTs) which have been observed in the tissue of aquatic and terrestrial plants, monocotyledons and dicotyledonous plants [3, 16, 23]. TNT transformation products are also observed in soil and axenic growth media associated with plants [3, 24].

Whilst studying uptake and transformation of TNT from soil is essential to determine the viability of any phytoremediation application, axenic studies must also be performed to exclusively identify the contribution of the plant to the process. It is important to identify the pathway(s) of plant transformation of the contaminating compound as products of this transformation may also be toxic. The eventual fate of these products is also of interest in order to determine if the products are secreted into the environment or sequestered within the plant. A range of microbes are known to transform TNT and when present in a plant transformation study will also contribute to the removal and transformation of TNT from the environment, thus complicating analysis. This factor has been encountered in many experiments [25, 26]. The aim of this work was to examine the uptake and fate of TNT in untransformed wild type and transgenic plants. It was envisaged that the use of axenic liquid culture conditions would better explain any differences in metabolite production. Liquid culture experiments were also used for the initial mass balance/metabolism studies in order to increase the accuracy of the determination of the fate of TNT and RDX metabolites by avoiding sorption onto soil components and minimizing microbial activities.

Interactions between plants and soil microbial communities

Previous studies suggest that the main effects of the plants on the microbial community, occurred at the root-soil interface, i.e. the rhizosphere [27, 28]. This is termed the rhizosphere effect. Scale is an important concept when studying soil microbial communities. It is therefore necessary to consider localized effects in soil; for instance, it has been shown that rhizosphere communities vary spatially in a radial direction from the root surface as well as varying along the root axis [29].

There are a variety of ways through which plants are considered to alter the microbial community of the soil [30]. The main impact is through release of root exudates by the plants, accounting for between 15 and 40 % of the total carbon derived from photosynthesis, was shown to be responsible for an increase in the bacterial community [31]. Plants release a variety of compounds, including oxygen, water and ions, into the soil. The majority of root exudates consist of carbon-containing compounds. All roots have the ability to secrete both high and low molecular weight molecules into the rhizosphere [32]. The low molecular weight compounds include sugars and simple polysaccharides, amino acids, organic acids and phenolic compounds. High molecular weight compounds, including compounds such as flavenoids, enzymes, fatty acids, growth regulators, nucleotides, tannins, carbohydrates, steroids, terpenoids, alkaloids, polyacetylenes and vitamins, are released in large quantities. Since plant roots are the main source of carbon within the soil, and therefore the main source of energy or food for living organisms, the region of soil surrounding roots tends to resemble an ‘oasis in a desert’. The root exudates of tobacco plants have not been extensively characterised, however tobacco plants have been observed to exude a range of organic acids [33]. A second way in which plants effect the rhizosphere is through root surfaces providing an anchor for assemblage of microbial

communities, analogous to the formation of biofilms [34]. A third way in which plants can influence the microbial community of the rhizosphere is through alteration of the soil chemistry and physical conditions [35]. For instance, the redox potential or pH can be changed through respiration; this can result in an increase in the reductive reactions in the soil as electrons are generated through the microbial metabolism of root exudates. In addition, the inorganic nutrient availability is affected by the uptake of minerals by the plant.

Degradation of RDX by cytochrome P450s

RDX is classified as a priority pollutant by the Environmental Protection Agency (EPA) and contamination on military training ranges is of concern; use of RDX has been restricted by the EPA at the Massachusetts Military Reservation of Cape Cod where RDX contamination is threatening drinking water sources [36]. Microorganisms present in soil with high levels of RDX contamination have been found to degrade RDX, but lack sufficient biomass or metabolic activity to degrade this compound before it leaches through soils polluting groundwater.

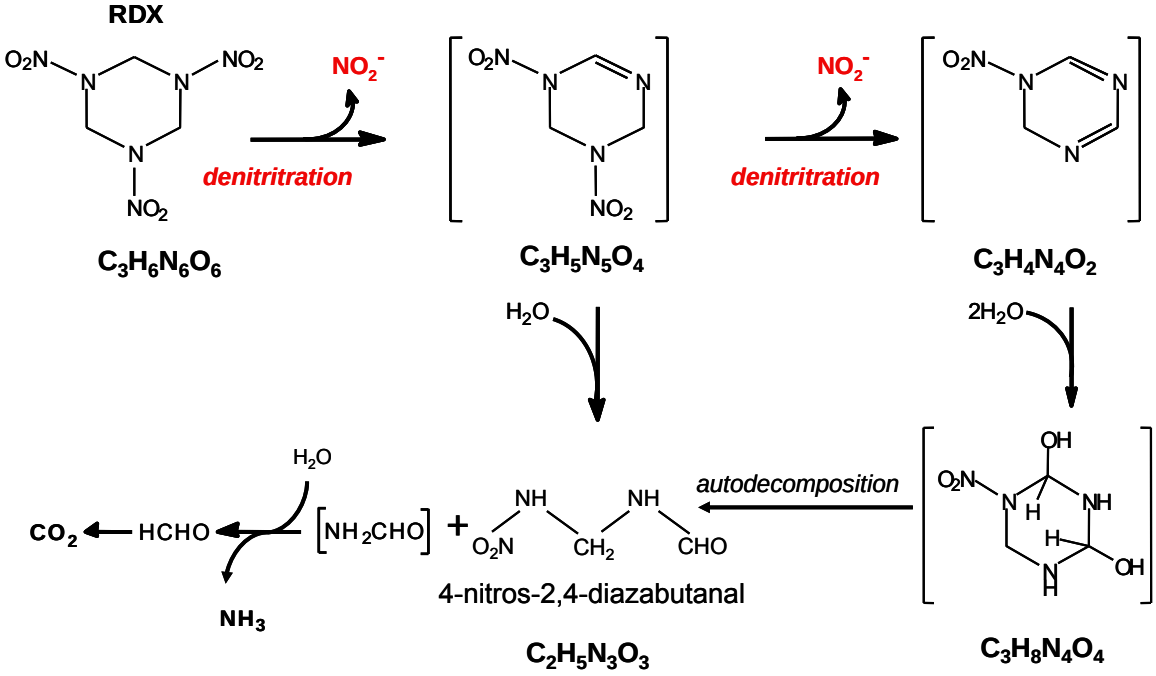


Figure 2. Proposed pathway of RDX degradation by *Rhodococcus rhodochrous* 11Y
Proposed by Fournier et al. 2002, 2004 [37, 38]

The aerobic degradation of RDX by *Rhodococcus rhodochrous* strain 11Y is catalyzed by a cytochrome P450 and we have now cloned the gene, x*plA*, encoding this activity [39]. The degradation is thought to proceed by initial denitration, followed by ring cleavage, yielding nitrite, formaldehyde and 4-nitro- 2,4-diazabutanal (Figure 2) [37, 38]. So far, *xplA* and *xplB*, a reductase encoded adjacently to *xplA* have been found only in *Rhodococcus* and related bacteria isolated from RDX-contaminated soil; this indicates that the RDX-degrading ability of XplA could have evolved under this selective pressure. The x*plA* gene was introduced into Arabidopsis and the uptake of RDX from liquid culture tested. Additionally, transfomants expressing a full-

length cDNA clone encoding the cytochrome P450 3A4 from the lab of Frank Gonzalez, the gene encoding the poplar cytochrome P450 oxidoreductase (CPR) from Carl Douglas and *xplB* were made. It has been shown that overexpression of the *3A4* and an oxidoreductase together, results in superior RDX degrading activity when compared to the P450 alone.

Arabidopsis expressing both XplA and NR

Since military ranges are often contaminated with more than one explosive it is desirable to engineer plants that can be used to treat both TNT and RDX contaminated soil. Towards this end both *nfsI* and *xplA* were expressed in plants and the ability of these plants to phytodetoxify mixtures of explosives evaluated.

Production of transgenic Poplar

The "supervirulence" plasmid, pTVK291, has been used in many studies to increase transformation frequencies of recalcitrant plants, including rice and maize. This plasmid carries kanamycin resistance for selection in bacteria, so it will be compatible with the binary vector pART27. Since it is not compatible with our other binary vector, pKH200, we have already subcloned the "supervirulent" genes into a vector (pUFRO47) that has gentamicin resistance for bacterial selection.

The newly developed plasmid, pJS96, which imparts hypervirulence on Agrobacterium will also be used. This plasmid expresses the pilus subunit gene of *Caulobacter crescentus*, resulting in a striking enhancement in transformation frequencies. It has a spectinomycin/streptomycin resistance gene for selection in Agrobacterium, thus making it compatible with pKH200-based binary vectors but not with pART27-based ones. We will subclone the Ptac-pilA construct into pUFRO47 as we did for the supervir genes.

The majority of reports for poplar transformation utilized nopaline-type strains of Agrobacterium. Since we have had success with the nopaline-type strain C58C1(pGV3850), we will continue to use this strain for transformations. We have requested from the INRA group, the wild-type Agrobacterium strain 82-139 that was hypervirulent on poplar. This strain will be used in the protocol developed by Brasileiro.

We have an *A. rhizogenes* strain A4 that was isolated by David Tepfer at INRA for its naturally-increased virulence (Tepfer, unpublished). We have successfully used this strain to transform *Atropa belladonna* and tobacco.

Zhan and co-workers demonstrated that the plant cytokinin gene, *tzs*, from Agrobacterium increased *A. rhizogenes* mediated transformation efficiencies of flax when the two strains were co-inoculated with the plant. We have likewise subcloned the *tzs* gene for overexpression in a disarmed Agrobacterium strain for co-inoculation with A4.

The virulence of *A. rhizogenes* has not yet been optimized, so we have initiated a study into which compounds activate the virulence genes of this species to the greatest extent. Virulence genes are induced by both phenolics and monosaccharides to varying levels. We have introduced a reporter construct (*virB-lacZ*) into *A. rhizogenes* and have tested a range of these compounds to determine the most effective inducers to add to the co-cultivation media. We discovered that galactose, rather than the more common inducer, glucose, induced the virulence genes to the greatest extent. We will therefore use this sugar instead of glucose in the pre-induction media of *A. rhizogenes*.

Transformation of Populus tremula x P. alba INRA clone N717 1-B4. We will use established protocols for this easily transformed and regenerated clone.

Transformation of P. trichocarpa x P. deltoides clone 184-402. Clone 184-402 is a hardy, fast-growing hybrid that is currently used in plantations for pulp production. It is an essentially sterile female triploid, which makes it ideal for transgenic research. We will use a variety of transformation techniques for this more recalcitrant hybrid. In addition to using the method we have already used to obtain our transgenic poplars, we will introduce the supervirulent plasmids with the binary vector into C58C1(pGV3850) and try the co-inoculation method of Brasileiro with the wild-type Agrobacterium 82-139 or with our disarmed Agrobacterium overexpressing the plant cytokinin gene *tzs*, combined with the disarmed strain carrying the binary vector.

We used *A. rhizogenes* to transform poplar using the methods of Pythoud and Zhan. An advantage of using *A. rhizogenes* is that a large number of independent transformants could be analyzed without risking damage to the transgenic line since the hairy roots are easily divided. Those transgenic root lines demonstrating the best metabolism of TNT or RDX will be transferred to shoot-inducing media.

IV. Materials and Methods

Transformation of TNT by *Enterobacter cloacae* nitroreductase

Purified *E. cloacae* nitroreductase (NR) activity was determined using a HPLC-based, time-course assay. The assay comprised NADH and TNT in 50mM phosphate buffer, pH 7.0, at 25 ^{0}C. Aliquots of the assay were removed every 30 minutes and analyzed on HPLC. A C8 column was used to distinguish the 2-HADNT and 4-HADNT isomers and a C18 column was used to identify ADNT and HADNT. These results were then confirmed by UV/visible spectral (UV/vis) analysis of the products.

Production of *35S-nfsI* tobacco

The *nfsI* expressing tobacco lines were produced prior to this study [40]. The *nfsI* gene was cloned into the binary vector pART27 [41] under the control of the CaMV35S promoter and ocs terminator, and transformed into untransformed wild type tobacco (*Nicotiana tabacum* variety Xanthi). The pART27-*nfsI* vector confers resistance to kanamycin and the transformants were selected on media containing kanamycin.

Tobacco germination studies

In order to determine which of the transgenic plants possessed tolerance to TNT, a germination study on TNT-amended agar was undertaken. For each plant line, 30 mg of T1 generation seeds were germinated on agar plates amended with a range of TNT concentrations including 0.025 mM, 0.05 mM, 0.1 mM and 0.25 mM. Germination was defined as the emergence of the radicle (embryonic root) and/ or the cotyledons (embryonic leaves).

Liquid culture studies with NR tobacco

Wild-type and transgenic seeds (50 per flask) were surface-sterilized, germinated in 100 ml Murashige Skoog complete medium [42], and grown for 18 days in constant light at 25 °C with rotary shaking at 121 r.p.m. TNT (solubilized in DMF) was added to the flasks under aseptic conditions. Identical plant weights were established for each flask (in duplicate or individual flasks) and seedlings were then aseptically transferred to TNT-containing media (controls remained in flasks without TNT) where they remained for a further five days. The concentrations used were 0.1 mM (23mg/L) TNT, 0.25 mM TNT (56mg/L), 0.38 mM (86mg/L), 0.5 mM TNT

(113mg/L). TNT was added to the plant growth media and incubated with the plantss for five days at which time weight measurements were taken.

Amendment of soil with TNT

Rowland Series soil was chosen due to its low organic and clay content, as TNT is known to bind to clay [43] and organic matter [44]. Rowland Series soil was obtained from Dr Martin Wood at the University of Reading. The composition of the soil had previously been characterized as 85 % sand, 5 % silt, 10 % clay and 2.25 ± 0.75 % organic matter by Dr Wood at University of Reading, UK. This soil was air-dried and sieved to 2 mm size particles. Soil was then weighed and equal amounts placed in large plastic bags. TNT was solubilized in 100 ml of acetone and added to soil at final concentrations of 25, 50, 150, 500, 1,500 and 4,000 mg TNT/ kg soil. The solubilized TNT, or the same volume of pure acetone, was added a drop at a time, with continual mixing, to half of the soil required. One portion of the soil was not amended. The contaminated soil was left for three days to evaporate the acetone. The other half of the soil (pristine) was then added to the contaminated soil, prior to a thorough mixing of the soil. This method of contaminating only half of the soil initially was performed in order to maintain microbial populations in the pristine soil. Solvent addition can prove toxic to microbes.

Rowland Series was characterized and found to contain 10 % water content. In order to slowly re-introduce the same amount of moisture to the dried soil, half of this water (v/w) was added to soil four days before the start of the experiment, followed by the remaining water on the day before plants were introduced into the soil.

Ageing of TNT-Amended Soil

Bags of soil were sealed, placed into plastic boxes and stored in the dark at 4 °C for three months in order to age the soil. Soil was aged to allow the microbial community to adapt to the TNT-contamination. Soil was kept under these conditions to keep microbial transformation and opportunistic fungal contamination to a minimum before the experiment was performed. Aged soil was then analyzed and termed the Pre-planted Soil.

Growth of Plants in TNT-Amended Soil

Wild type and transgenic tobacco seeds were planted in a mixture of three parts Rowland Series soil to one part of sand and germinated in the greenhouse. Germination trays were filled with this soil mixture and individual seeds were sewn into small hollows on top of the soil. The trays were then covered with a propagator lid to encourage germination. Growth conditions in the greenhouse were 23 °C with an 18-hour photoperiod. After five weeks of growth, uniform sized plantlets were selected and transferred to the soil columns containing TNT-amended soil. (These columns were constructed by cutting Marley rainwater drainpipes, diameter 88 mm, into 15 cm lengths.) The soil was added in two layers. A permeable barrier (Scotsdale's weed cloth) was used to separate layers of amended and non-amended soil in the columns, while still allowing free draining (Figure 3). Unplanted soil columns were also constructed as a control to measure non plant-based transformation of TNT. Both planted and unplanted soil columns were watered with the same volumes of water. To begin with, 40 ml volume of water was added every few days , this was increased to 60 ml as the plants grew. Trays of plants were frequently rotated to ensure even growth of all plants. An overview of experimental design is presented in Figure 4. At the end of the 12-week growth study, rhizosphere soil was separated from the bulk soil. Rhizosphere soil was defined as the soil that remained attached to the roots after shaking. Bulk

soil was defined as soil without roots present. Soil pH was assessed directly by dissolving 5 g soil in 5 ml distilled water for 30 min prior to measurement using an EDT instruments (Kent, UK) pH reader. Soil water content was determined by weighing the samples fresh, drying the samples at 100 °C overnight and then reweighing the samples.

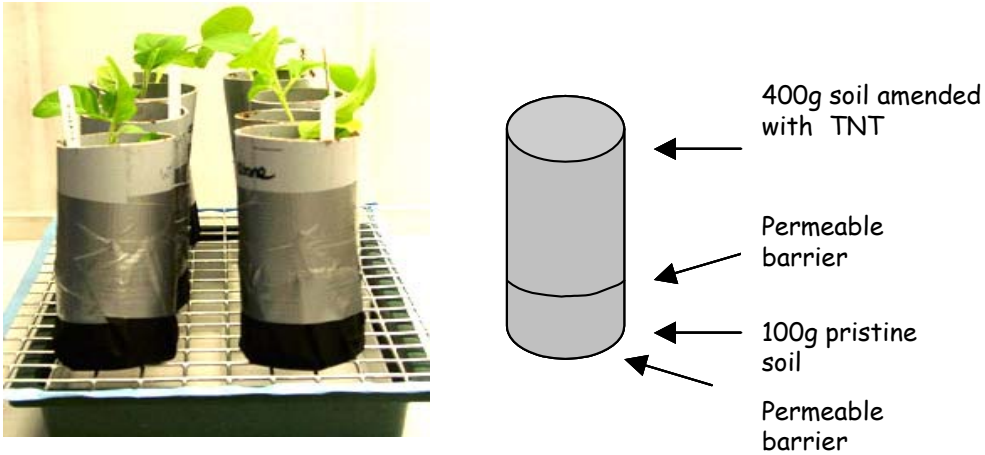


Figure 3. Schematic of the soil columns for growth of tobacco in TNT-amended soil

Soil contaminated with TNT (solubilized in acetone) at 25, 50, 150, 500, 1,500, 4,000 mg/kg and acetone for growth control. Soil left to age at 4 ^{0}C for 3 months

↓

Wild type tobacco and transgenic tobacco (NR 3-2) seeds germinated in pristine Rowland series soil and grown for five weeks (all seedlings at the six leaf stage)

↓

Plantlets transferred to columns containing TNT-contaminated soil and unplanted columns also set up

↓

Transgenic and wild type plants to be grown in TNT-contaminated soil for 12-weeks in total

↓

Samples continually taken from soil run-off to observe disappearance of TNT and production of transformation products → Samples to HPLC

↓

At week four of the experiment, one of each plant line at each level of TNT will be sacrificed for extraction of TNT and transformation products from soil and plant tissue

↓

Remainder of plants grown for 12-weeks in total and measurements of plant root and shoot growth to be taken

↓

TNT and its transformation products extracted from soil and plant tissue

↓

HPLC analysis and quantitation of TNT and its transformation products in soil and plant tissue

Figure 4. Outline of TNT-amended soil experiment design

Colony forming unit count

For counting the number of colony forming units (CFU) 1 g soil was diluted in 10 ml of 50 mM phosphate buffered saline pH 8.0 (PBS) and homogenized by vortexing on maximum speed for 1 min. Serial dilutions were performed (from 10^{-4} to 10^{-7}) and spread onto tryptic soya agar plates (Oxoid, Basingstoke, Hampshire, UK) which were incubated at 25 °C. CFU were counted every 48 hr for six days. Univariate analysis of variance (ANOVA) was employed to test for significant associations.

Biolog Eco plates

Each 1 g soil sample was diluted in 10 ml PBS and homogenized by vortexing on maximum speed for 1 min. A 10^{-3} dilution was prepared in 10 ml PBS and vortexed to ensure thorough mixing. 150 µl was inoculated into each well of the Biolog EcoPlate™ (Biolog. Inc., Hayward, California, USA) prior to incubation at 25 ˚C. Absorbance at 595 nm was assessed by an EL800 Universal Microplate Reader (Bio-Tek Instruments, Inc. Winooski, Vermont, USA) every 48 hr for six days. Well color density was corrected compared to the control well containing water, with negative values discounted for further analysis.

Statistical analysis of data from bacterial soil communities

Absorbance versus incubation time curves were plotted to illustrate utilization of substrate in an individual well. An integrative approach [45] was also used to provide a method of analysis that was independent of incubation time. The area under the absorbance versus time curve (AAT) was therefore calculated using a trapezoidal approximation (Equation 1), with the end time point determined as when the majority of substrates had reached Vmax. This AAT is considered to reflect differences in lag phases, rates of increase and maximal optical density achieved. The average area under the curve for all substrates (average AAT) was also calculated for each sample to provide a measure of overall metabolic activity. ANOVA was employed to test for significant associations. The relative AAT compared to average AAT was calculated for each substrate to minimize bias in the data set due to varying inoculum densities between samples [46]. The multivariate analytical procedure, principal component analysis (PCA) was then applied to the relative AATs.

$$\sum_{i=1}^{4}((v_i + v_{i-1})/2)*(t_i + t_{i-1})$$

Equation 1 Formula for calculation of the trapezoidal area under the absorbance versus time curve (AAT)
Where vi = value and ti = time.

For the data set of the relative AATs, an un-rotated factor matrix was computed (using SPSS for Windows, Rel. 11.5.1. 2001. Chicago: SPSS Inc.), and factor loadings displayed. Factor loadings are the correlation of each variable and its factor, with higher loadings making the variable more representative of its factor. Substrates with significant loadings for each factor were determined.

Based on this analysis orthogonal plots of principal component 1 (PC1) versus principal component 2 (PC2) were constructed.

Genomic DNA extraction from soil

Genomic DNA (gDNA) was extracted directly from soil using the Ultra Clean™ Soil DNA Isolation Kit (Mo Bio Laboratories, Inc., Solana beach, California, USA) according to the manufacturer's instructions

Amplification of 16S rDNA by PCR

The PCR amplification of the gDNA was performed in 50 µl volumes using 1 µl gDNA as template with universal primers spanning the V3 region of 16S rDNA and incorporating a GC-clamped primer [47]. The primers (as synthesized by the PNAC facility, University of Cambridge,UK) were: GC338F, 5' – CGC CCG CCG CGC CCC CGC CCC GGC CCG CCG CCC CCG CCC ACT CCT ACG GGA GGC AGC –3' and 530R, 5' – GTA TTA CCG CGG CTG CTG – 3'. N.B. The forward primer was modified to target to the exact location of the EUB338 probe binding site[47]. PCR amplification was as described [47] with cycle modifications: 2 min at 95 °C, then 35 cycles of 1 min at 95 °C, 0.75 min at 60 °C and 1 min at 72 °C. Then 30 min at 72 °C.

Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) analysis was performed as described in (Whiteley and Bailey, 2000) [47]. 1 µg of PCR-amplified DNA product was loaded onto a 10 % (w/v) acrylamide gel containing a denaturing gradient of 30 % to 60 % (100% denaturant consisted of 7 M urea and 40 % (v/v) formamide) parallel to the direction of electrophoresis by using the INGENYPhorU-2x2 system (Ingeny International, Goes, The Netherlands). Gels were electrophoresed at 60 °C at a constant voltage of 100 for 16 hr prior to staining with Sybr Gold (Molecular Probe, Eugene, Oregon, USA). Gels were visualized under UV illumination using UVItec transilluminator. The image was recorded and subsequently analyzed using Scion Imaging for Windows Software (Scion Corporation, Maryland, USA). Band profiling was performed and dendrograms constructed for comparison of DGGE samples by the Pearson Correlation method using SPSS. Individual bands were excised, extracted and PCR amplified using the forward primer 338F 5' – ACT CCT ACG GGA GGC AGC –3' and 530R (described above). PCR amplification products were verified using gel electrophoresis and sequenced.

TNT extraction from soil

The method was adapted from EPA 8330. Soil was air-dried, homogenized and sieved to 2 mm sized particles. Soil was then ground to a fine powder using a mortar and pestle. A 2 g sample of the ground soil was added to a glass universal and 10 ml of acetonitrile was added. Samples were placed in a sonicating water bath in a 4 °C cold room for one hour. The extractions were then removed from the bath and allowed to settle for 30 min and the top 5 ml was removed to a clean universal. Next, 5 ml of 5 g/l (w/v) calcium chloride solution was added to the soil extraction and allowed to settle for 30 min. The clear supernatant was removed and filtered (0.22 µM) and 20 µl of the extraction injected onto the high performance liquid chromatography (HPLC) column. The quantitation limit for TNT extraction from soil using this method has been established as 0.25 mg/kg.

HPLC for detection of TNT and metabolites

Samples were analyzed for TNT and its metabolites by reverse-phase HPLC using a Waters 4 µm C8 Nova Pak column on a Waters 2690 separations module with a Waters photodiode array detector (Waters Corporation, Milford, MA, USA). The mobile phase consisted of 82 % (v/v) water and 18 % (v/v) isopropanol. A flow rate of 1ml/min was used and chromatograms were extracted for quantification at 230 nm [24]. Analysis was performed using the Millenium software (Waters Corporation, Milford, MA, USA). Peak identification was based on a comparison to authentic standards (AccuStandards, New Haven, USA) using UV spectra and retention times. TNT and metabolites were quantified by comparison to a standard curve generated from authentic standards. The detection limit for TNT with this method was determined to be 0.2 mg/l [24].

Transforming Arabidopsis with *xplA, xplB, nfsI* and *3A4*

The *xplA* and *3A4* genes were amplified using the modified 5' primers: *xplA* 5' AAGCTT AAT ATG GCC GAC GTA ACT GTC CTG TTC GGA and 3A4 5' ATCGAT ATT ATG GCT CTG TTA TTA GCA GTT TTT CTG to increase expression in plants [48]and 3' primers with homology to the 3' end of the gene. The PCR products were cloned into the TOPO vector (Invitrogen). Following sequencing, the genes were cloned via pART7 into the binary vector system, pMLBart[49] under the control of the CaMV35S promoter and ocs terminator to produce the vectors pMLBart-*xplA* and pMLBart-*3A4*. *Agrobacterium tumefaciens*-mediated floral dipping was used to transform the constructs into. The pMLBart-*xplA* vector confers resistance to the herbicide Basta and transformants were selected on media containing Basta.

The *xplB* gene was cloned in a similar manner into the binary vector pART27 [50] under the control of the CaMV35S promoter and ocs terminator, and transformed into Arabidopsis[51]. The pART27-*nfsI* vector was as used for the *nfsI* expressing tobacco lines [40]. The pART27-*xplB* and pART27-*nfsI* vectors confer resistance and transformants were selected on media containing kanamycin.

T3 transformants for each transgene, homozygous for the selectable markers were selected for further analysis.

Liquid culture analysis of Arabidopsis expressing XplA and NR

Arabidopsis seeds were germinated on agar plates containing ½MS media for 24 hours then 200 seedlings transferred to sterile, 100ml conical flasks containing 20 mls of ½MS liquid medium, with 20 mM sucrose, and grown on a rotary shaker at 60 rpm, in a growth room with 50 $\mu moles.m^{-2}.s^{-2}$ white light. When the seedlings were ten days old, the media was replaced with 20 mM sucrose containing 0, 75, 100, 150, 175 or 250 µM TNT and 180µM RDX. Three replicate flasks were prepared for each line alongside three, no plant control (NPC) flasks. The rate of uptake was determined using HPLC analysis on aliquots of media taken over seven days. All steps were carried out using aseptic technique in a laminar flow hood.

Inhibition of XplA by TNT

Purified XplA was obtained using the expression vector pET-16b containing *xplA* expressed in *Escherichia coli* (Rosetta gami B, Novagen). The cells were grown in Luria Broth at 20 °C, 180 r.p.m. to $O.D_{600}$. of 1.0 then 100 µM IPTG, 5 µg / L riboflavin, 1 mM α-aminolevuleic acid and 0.5 mM $FeCl_3$ added. Cells were harvested after 24 h, resuspended in 50 mM sodium phosphate buffer pH 8.0, 200 µM phenylmethylsulphonylfluoride and lyzed at 1500 p.s.i. in a

French Press (Thermo IEC). Following centrifugation at 10,000 g the supernatant was loaded onto HIS-Select resin (Sigma) in sodium phosphate buffer pH 8.0, 0.3 M NaCl (buffer A) and incubated batch wise for 2h. Following extensive washing with buffer A, XplA was eluted with 250 mM imidazole in buffer A and then dialyzed at 4 °C against several changes of 50 mM potassium phosphate pH 7.0. The protein was then stored in aliquots in 50% glycerol at -80 °C. XplA activity was assayed in a 500 µl volume containing 100 mM potassium phosphate pH 6.8, 300 µM NADPH, 0.1 units of ferredoxin reductase (Sigma) and 48 µg XplA. The reaction was initiated with 100 µM RDX and terminated with 10% 1.5 M trichloracetic acid. Samples were analyzed using HPLC

Soil leachate studies on Arabidopsis expressing XplA and NR

Soil leachate studies were carried out on six-week-old Arabidopsis plants grown under 180 $\mu m.m^2.s^{-1}$ light and 12 h photoperiod. Plants were grown in pots containing 30 g of uncontaminated soil (Levingtons F2 compost), five plants per pot. Plants were grown in a controlled environment cabinet with 12 hour photoperiod to maximize rosette leaf biomass, with 18 ºC and 21 ºC night and day temperatures respectively. Each pot was flooded with 50 ml of a saturating (180 µM) solution of RDX (1 x total pot volume), then seven days later, flushed through with 50 ml water. Leachate was measured from five replicate pots per line and leachate from control pots containing soil only (no plant controls, NPC) were also measured. The collected soil leachates were analyzed for RDX content using HPLC as described above.

Transformation of Aspen

Transgenes were introduced into the aspen hybrid, *P. tremula* x *P. alba* (INRA 717-1B4) using co-transformation with *Agrobacterium rhizogenes* strain A4. Bacterial cultures containing either CYP3A4, CPR (cytochrome P450 oxidoreductase), or NR (nitroreductase) were grown overnight, washed in Induction Medium and diluted to OD600 of 0.3. The strains containing the 3A4, NR, and CPR genes were then mixed together and used to inoculate the poplar leaves. After 10-12 days, transgenic hairy roots were visible on the leaves. These roots were subcultured on MS medium with antibiotics and kanamycin for selection until they reached a sufficient size for DNA sampling and PCR analysis. Root lines that were positive for the genes of interest were propagated on MS medium with kanamycin, and used in TNT tolerance assays and TNT and RDX uptake assays.

TNT and RDX tolerance assays

Individual roots from independent transgenic lines were placed on MS plates containing levels of TNT ranging from 0.6mM, 0.5mM, 0.25mM, 0.15mM, 0.1mM to 0.02mM. Root length was monitored after 2 weeks. RDX toxicity was tested in a similar manner using concentrations of 60,65 and 70 ug/ml (0.270 mM, 0.295 mM, and 0.318 mM) .

Regeneration of transgenic Aspen

Root lines were cultured on agar media with plant hormones (thidiazuron) and under strong lighting for induction of shoot growth. Once plants were growing well on agar media, we used the following procedure for transfer to soils and propagation: knife and pots were sterilized in 10% bleach solution and rinsed well. Root cuttings were dipped in rooting powder and placed with at least 2.5 cm stem in pot and no leaves touching soil in pots containing soil and watered well with fungicide solution (1 g Benlate per liter of water). The cuttings were misted with

Foliar 20-20-20 fertilizer (1 g/liter) every 3-4 days. If any blackening appeared, the plants were sprayed with fungicide solution.

TNT and RDX uptake by transgenic aspen in nitrogen free media

Cuttings from tissue-culture grown transgenic plants were transferred in triplicates into 40-ml VOA vials containing 12 ml of MS medium with TNT (0.05 mM) and RDX (40µg/ml; 0.1810mM) in nitrogen free media and allowed to grow. Time 0, 2d, 9d and 16d samples of 1.5 ml were taken and analyzed by HPLC. Media control blanks were also maintained. Wet weights of the samples were taken at the end of each experiment in order to calculate removal per gram of plant tissue.

Nitrogen Analysis

Nitrogen analysis was done as a measure of RDX metabolism. 40-ml VOA vials were acid-washed, rinsed well with purified water, and autoclaved with 15 ml of nitrogen-free MS medium (Caisson Labs) containing 3% sucrose. RDX from a 50 mg/ml stock in acetone was added to a final concentration of 40 µg/ml (0.181 mM). Photos and wet-weight measurements were taken at the beginning and end of the experiment. Tissues were dried, and the CHN content was analyzed (CFR Soils Lab).

TNT metabolism in *nfsI*-transformed aspen

TNT and reduced amino metabolites were analyzed by reverse-phase HPLC using a Waters NovaPak C18 4.6X250mm on a photodiode array detector with mobile phase that consisted of 1:1 (v/v) water and acetonitrile at a flow rate of 1ml/min. Peak identification was based on comparison to standards using retention times.

TNT uptake by *nfsI*-transformed plants and control cuttings was determined after a 24 h incubation of line 6 aspen cuttings in 15ml 0.5x strength Hoaglands solution dosed with 32µl of 50mM TNT stock solution. The cuttings were ground in liquid nitrogen and oven-dried overnight. TNT and metabolites in the plants were extracted in acetonitrile using EPA Method 8330.

Hardening off of Transgenic Aspen Line 10

Before new plant species can be used in the field they must be hardened off (i.e., acclimatized to open and outdoor conditions) and propagated (Figure 1). The protocol for hardening off the transformed INRA 717 was as follows:

<u>Protocol to root INRA tip cuttings in soil</u>

1) Cuttings were take from the tips of branches, 1.5 to 2 inches long, with about four leaves. Entire cuttings were submerged in fungicide (Benomyl), diluted according to package directions.

2) Two inch pots were labeled and filled firmly with potting soil (e.g., Pro-Mix). It was not necessary to sterilize the soil. Saturate the pots with water.

3) For each cutting, the lowest leaf was removed, the bare stem coated with rooting powder (e.g., Rootone with fungicide), and planted in soil up to first leaf.

4) The fungicide that was used to soak the cuttings was applied to the pots, wetting the leaves and soil.

5) The pots were placed in a clear, airtight container(s), completely covering the pots on all sides. There was very little standing water beneath the pots. Plastic bags (Ziplock) worked well for this.

6) The containers were sealed and placed directly under florescent lights, so that the tips of the cuttings were about an inch away from the light source.

7) The cuttings were left undisturbed for at least two weeks. In two weeks, we observed new growth from most of the cuttings. In any case the plants were not left undisturbed for more than four weeks.

Protocol to transfer INRA plants from tissue culture to soil

1) Once the plants were well rooted in the tissue culture media, but not crowded (about four weeks from transferring a cutting to new media), they were transferred to soil.

2) All materials were made ready before the tissue culture container was opened: gloves, clean and labeled two inch pots, sterile potting soil, a bowl of E-pure water for rinsing the roots, E-pure water for watering, a clean tray, fungicide (e.g., Benomyl) diluted to package directions, and clean clear plastic quart-size ziplock bags.

3) Two inch pots were filled halfway with sterile potting soil (I used Pro-Mix), firming it with gloved fingers.

4) With gloved fingers, the plants were carefully lifted from the tissue culture container, keeping roots intact. All of the agar was carefully rinsed off in the bowl of E-pure water.

5) Holding the plant in position in the pot, soil was added to fill the pot. The soil was gently firmed around the plant. The soil was saturated with E-pure water.

6) The fungicide was poured over each plant, wetting the leaves and soil.

7) Each pot was placed in a clear quart-size Ziplock bag. Very little standing water was allowed beneath the pots. A tray was used to stabilize the pots.

8) The containers were sealed and placed directly under florescent lights, so that the tips of the cuttings were about an inch away from the light source.

9) We left the plants undisturbed until we observed new growth, which occured in about four weeks.

Protocol to harden off INRA plants to room conditions

1) Each of the plants in 2 inch pots was transferred to its own 4 inch pot, and the labels were transferred as well, with a record of the transplant date.

2) The soil of each pot was saturated with water, fertilized with a 20-20-20 liquid fertilizer, and sprayed with fungicide to coat the entire plant and soil.

3) Each pot was covered with a quart sized ziplock bag. The bottoms of the pots were not covered.

4) After a few days to a week, the plants adjusted to their new environment, two top corners off the bags were cut off to begin ventilation of the plant/pot headspace.

5) After two to four weeks, when the plants were growing well and had reached through the holes of the bag, they were transplanted them to 6 inch pots and labelled.

6) The soil in each pot was saturated with water, fertilized with a 20-20-20 liquid fertilizer, and sprayed with fungicide to coat the entire plant.

7) The pots were placed in trays or saucers which were filled with water. With good light and air circulation, the water was gone in 2-3 days. When water remained after 2-3 days, it was poured off the water and the plants allowed to sit in dry trays for a few hours. Then the trays were refilled with water.

Eventually five intact Line 10 transgenic aspens were hardened off to growth room conditions. Aspen Line 10 proved very sensitive to drying out in the plant growth room. The leaf and plant morphologies differed from Line 10 compared to the wild-type INRA 717 aspen. As shown in Figure 51, Line 10 was stunted and the leaves were elongated and highly wrinkled at the edges. Line 10 also had smaller internodes and its growth was significantly slower than the wild-type. Due to its poor growth and sensitivity, we concluded that Line 10 was unsuited to mass propagation. Since the vector control, pART27, also transformed with *A. rhizogenes*, had similar morphology to Line 10, we suspect that transformation of INRA 717 aspen with *A. rhizogenes* is not practical.

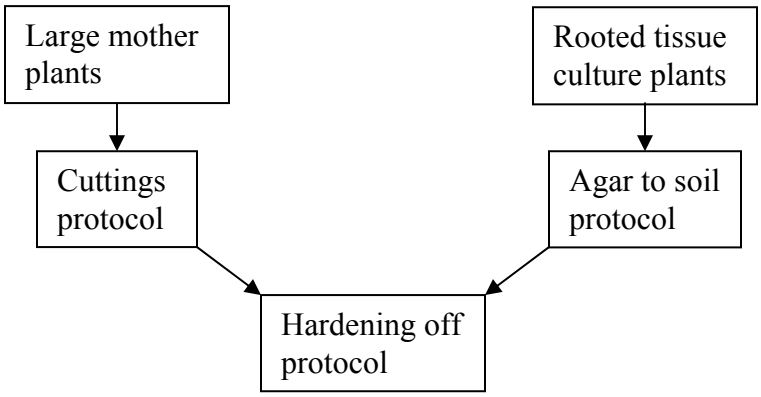


Figure 5. Hardening off protocol

Transformation of aspen using *A. tumefaciens*

Since the *A. rhizogenes*-transformed plants failed to perform well in soil, the transformation experiments were recently repeated using disarmed *A. tumefaciens*. Transgenic lines were verified by PCR and are currently being propagated for evaluation.

V. Results and Accomplishments

Reduction products of *Enterobacter cloacae* NR

The products of TNT reduction by *E. cloacae* NR, strain NCIMB10101, were studied using a HPLC-based, time-course assay. A C8 column was used for HADNT isomer identification, as 2-HADNT and 4-HADNT ran at different times, and the C18 column was used for reduction product identification, as ADNT and HADNT ran at different times Figure 6 and Figure 7. These results were then confirmed by UV/visible spectral (UV/vis) analysis of the products (Figure 8).

Initially, TNT appeared to be reduced stoichiometrically with a 1:1 conversion to 4-HADNT (Figure 9). Concentrations of 4-HADNT decreased slightly as another minor product peak was formed. Properties of this peak were consistent with the retention time and UV/vis spectra characteristics of 2,4-dihydroxylamino-6-nitrotoluene (2,4-HADNT) [52] indicating that a small proportion of 4-HADNT products were further reduced at the 2 position to 2,4-dihydroxylamino-6-nitrotoluene. Establishing the nature of the TNT reduction products from *E. cloacae* NR activity enables use to examine their production in transgenic plants, compared to transformation of TNT by untransformed wild type plants.

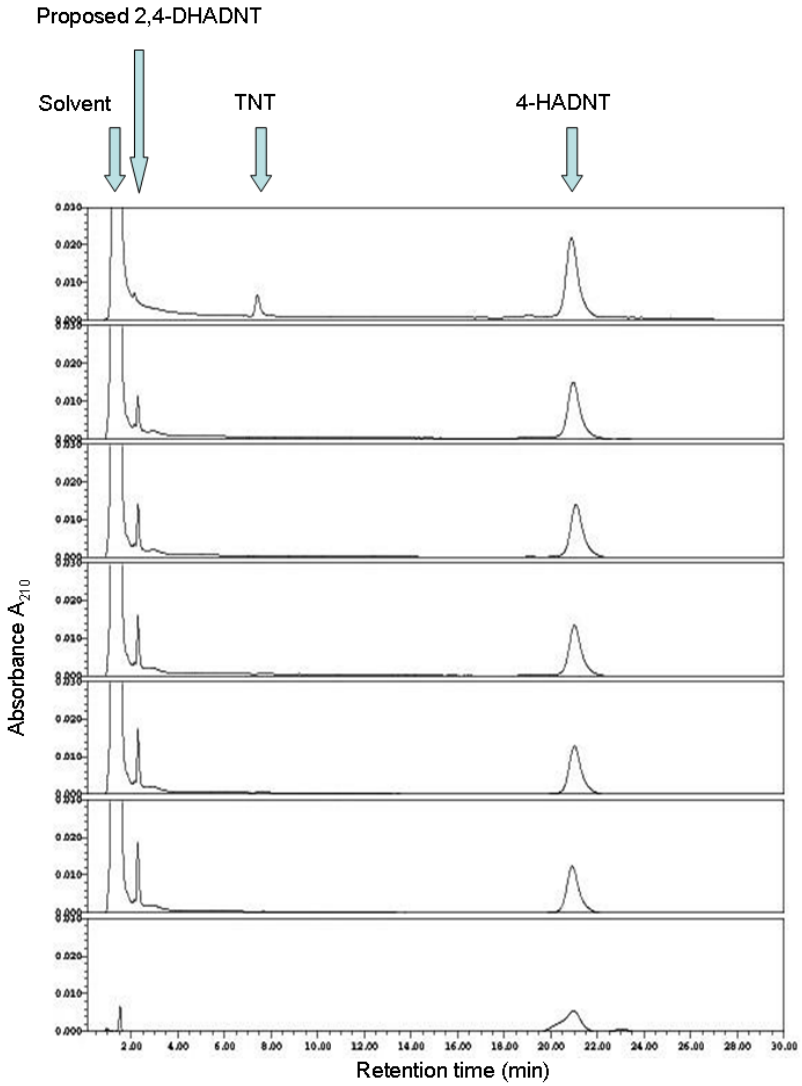


Figure 6. Transformation of TNT by *Enterobacter cloacae* nitroreductase

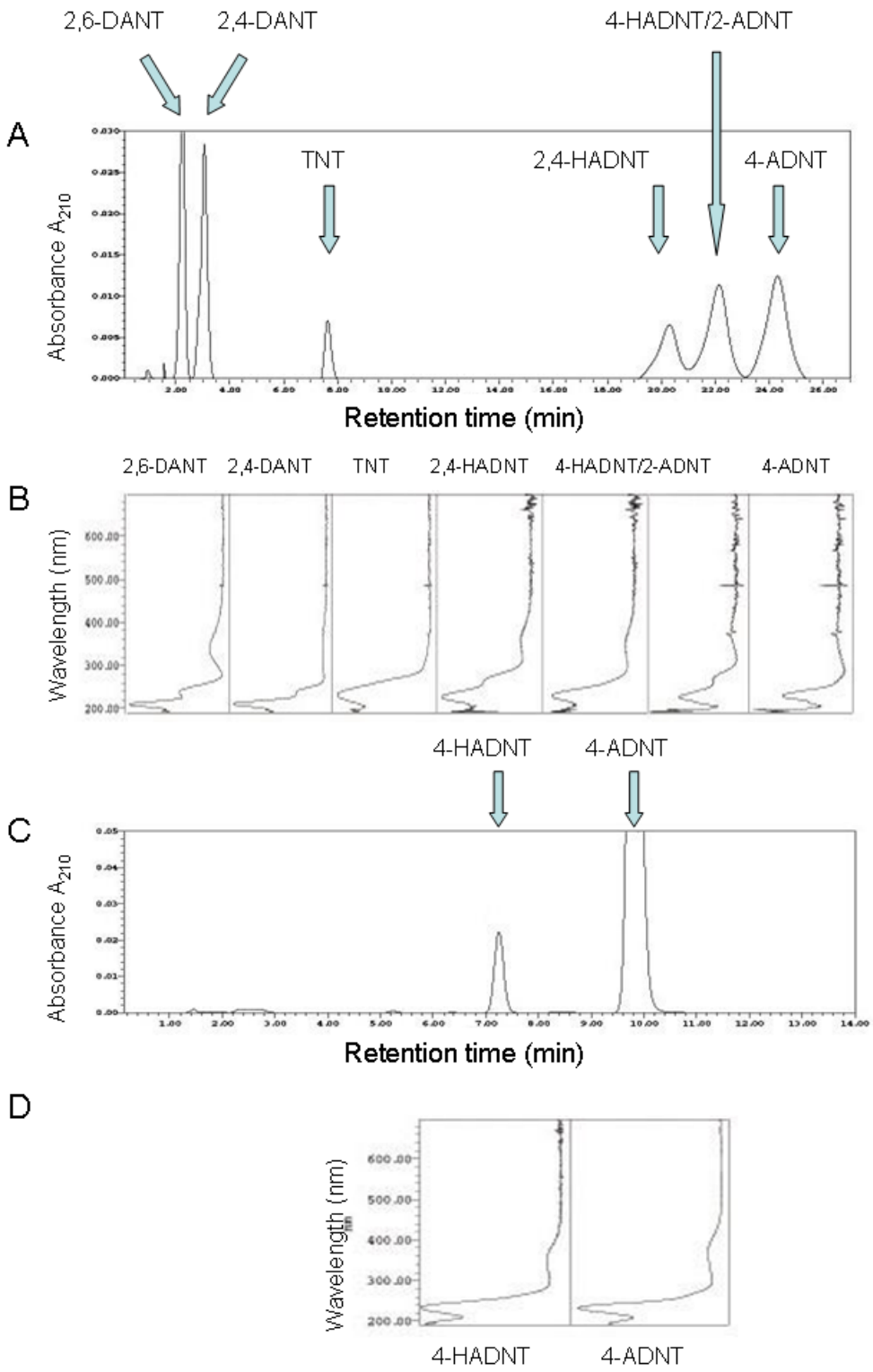


Figure 7. Analysis of TNT transformation products from *Enterobacter cloacae* nitroreductase HPLC chromatograms and UV/vis spectra of authentic standards of TNT and selected reduction products. A) Chromatograms and B) characteristic UV/vis spectra of TNT and transformation products. C) chromatograms and D) characteristic UV/vis spectra of 4-HADNT and 4-ADNT.

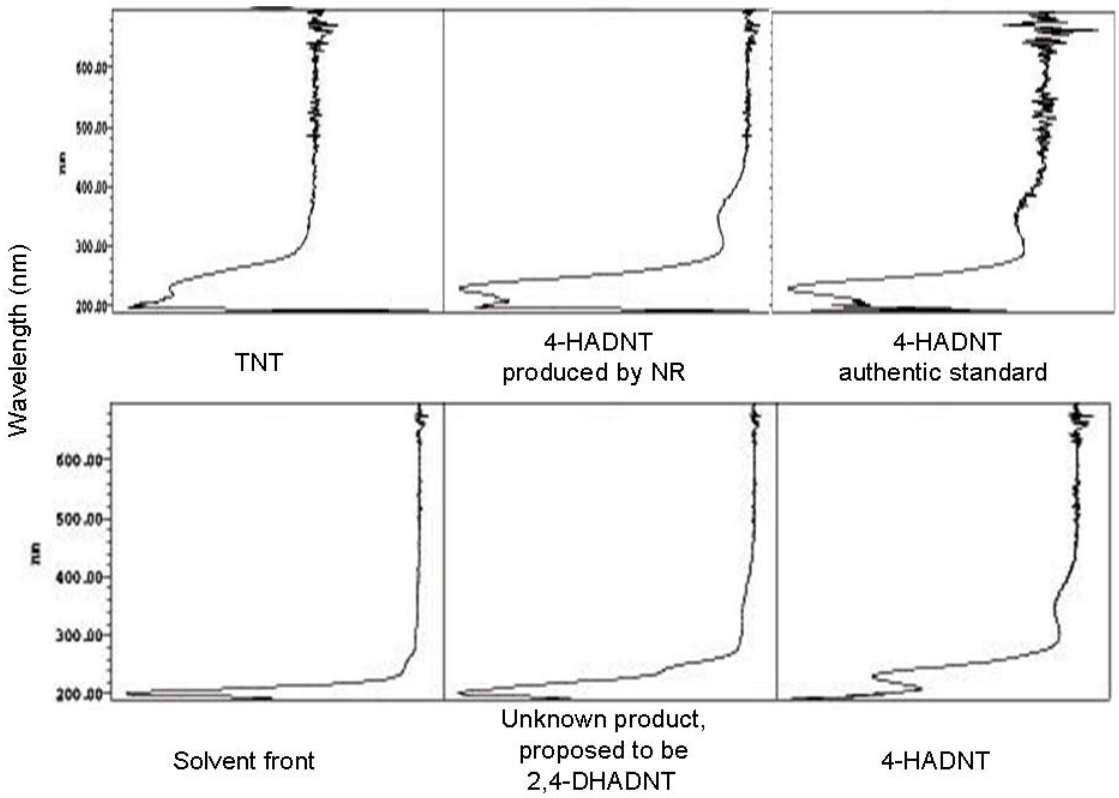


Figure 8. Characteristic UV/vis spectra of 4-HADNT produced by *Enterobacter cloacae* nitroreductase

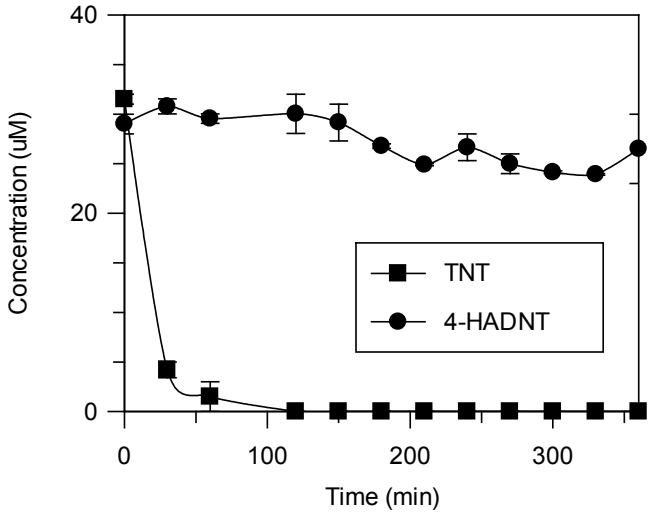


Figure 9. Reduction of TNT to 4-HADNT by *E. cloacae* nitroreductase
Results are the mean and standard error of the mean of duplicated results. Results were quantitated using authentic standards.

Quantitative RT- PCR analysis of *nfsI* expression in tobacco

The primers used for quantitative RT-PCR were designed from the bacterial sequence and did not cross react with endogenous tobacco nitroreductase gene expression. Interestingly, the transgenic line NR 15-1 was found, like untransformed wild type, to have no *nfsI* expression. Levels of *nfsI* transcript expression were expressed relative to the lowest *nfsI* expressing transgenic line, NR 20-3 (Figure 10).

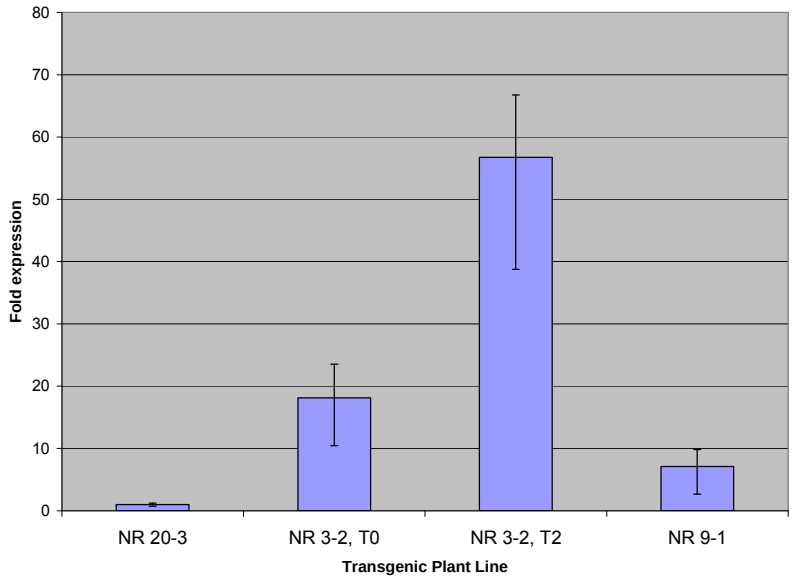


Figure 10. Relative quantitative levels of *nfsI* expression in transgenic plant lines
Results are the mean relative expression of *nfsI* ± sd from triplicate studies

Results of quantitative RT-PCR demonstrate that NR 9-1 has on average, seven-fold expression compared to NR 20-3. A large increase (18 fold) in *nfsI* expression was found in T0 generation results of NR 3-2. Analysis of homozygous NR 3-2 (T2) showed an even greater increase of 57-fold as these plants contain overall more copies of *nfsI*, then the heterozygous T0 generation..

Root morphology of seedlings exposed to TNT

Studies of germinating seeds in the presence of TNT show that it is mainly the radicle that is affected by TNT phytotoxicity [1]. To examine the effect of this toxicity on roots, seeds of homozygous line NR 3-2 were sown in a horizontal array, germinated and grown vertically to enable easier observation of root morphology. Photographs were taken to display differences in plant morphology (Figure 11) and details of lateral root formation were examined and presented in Table 1. Roots normally develop outward from the seminal or primary root (previously the radicle from the seed). From this forms a first order lateral root which in turn produces second order laterals and so on. Reduced lateral root formation was examined here as it was found to be a sign of phytotoxicity in germination studies with TNT.

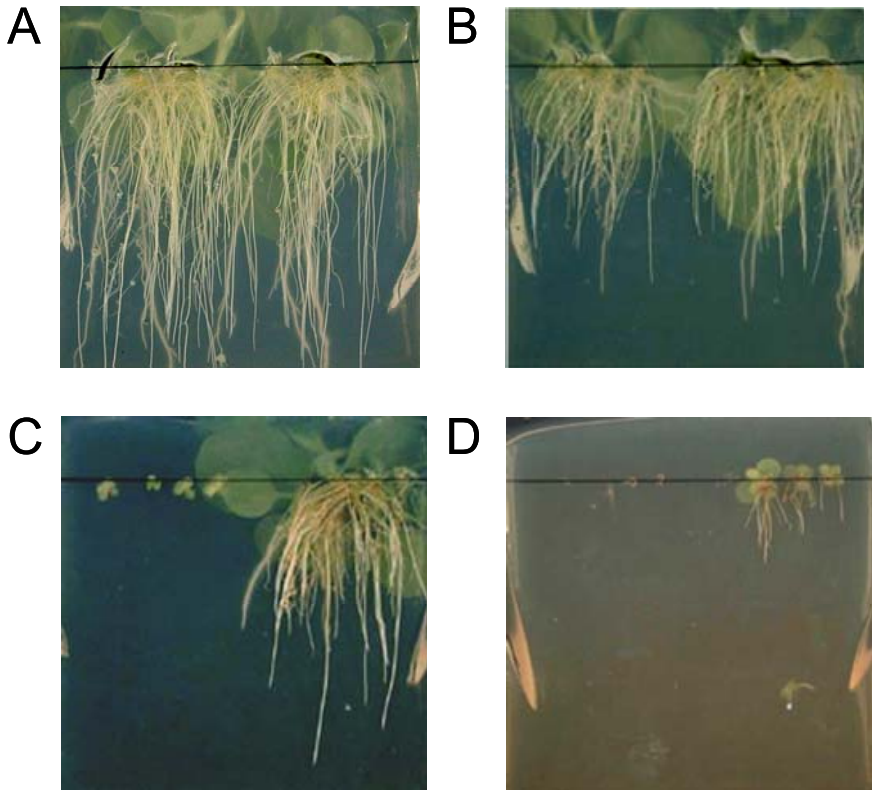


Figure 11. Root morphology of seedlings germinated on TNT
Four seeds of untransformed wild type tobacco (left on all plates) and four seeds of transgenic line NR 3-2 (right on all plates) were planted in media amended with TNT and grown vertically for 20 days. A: Media without TNT amendment (media amended with DMF which was used to solubilize TNT) was indistinguishable from this Figure, data not shown); B: media amended with 0.05 mM TNT; C: media amended with 0.1 mM TNT and D: media amended with 0.25 mM TNT.

Table 1. Development of lateral roots of untransformed wild type and transgenic seedlings germinated on TNT.

Lateral Root Development	No TNT		DMF		0.05 mM (11mg/L)		0.1 mM (23mg/L)		0.25 mM (56mg/L)	
	WT	NR	WT	NR	WT	NR	WT	NR	WT	NR
Seminal root	+	+	+	+	+	+	+	+	+ *	+
1st order	+	+	+	+	+	+	+	+	-	+
2nd order	+	+	+	+	+	+	+	+	-	+
3rd order	+	+	+	+	+	+	-	+	-	-

Results are representative of observations from 12 individual seedlings for all except 0.25mM TNT which was of six seedlings for untransformed wild type and nine for NR 3-2, taken after 21 days exposure to TNT. * Seminal root was the original radicle.

In the absence of TNT, both plant lines began germination four days after vernalization. Both lines produced leaves to the eight-leaf stage, which appeared healthy and were green in color. Both untransformed wild type and transgenic plants developed similar length roots with extensive root branching (see Figure 11 and Table 1). TNT was delivered to the growth media after solubilization in dimethylformamide (DMF). As this solvent is toxic, control plates containing the highest concentration of DMF used (25 μL) were included. Neither untransformed wild type nor transgenic plants displayed signs of phytotoxicity when germinated on media amended with DMF (Table 1).

At 0.05 mM TNT, there was an observable difference in growth between the two lines. Untransformed wild type plants produced shorter roots than those of the transgenic but still developed normal lateral roots as did seedlings without exposure TNT (Table 1). The transgenic seedlings displayed some root stunting in comparison to growth without TNT, however it was not as significant as that for untransformed wild type.

Results at 0.1 mM TNT displayed a stark contrast in TNT tolerance between the two plant lines. Untransformed wild type growth was severely retarded reaching only the two-true leaf stage and roots did not develop beyond second order lateral roots (Table 1) and were severely stunted (Figure 13). Leaves were also chlorotic in appearance. BY comparison, the NR 3-2 seedlings germinated on 0.1 mM almost as well as on the 0.05 mM TNT media producing eight healthy green leaves. Roots did not appear any more stunted than at 0.05 mM TNT and were not affected in lateral root development. However, there was a reduction in root density in NR 3-2 root growth at this concentration.

The root morphology study was also conducted at 0.25 mM TNT; however the untransformed wild type plants remained at the cotyledon stage, producing a severely stunted radicle. Nitroreductase plants were also stunted at this concentration but not as severely as untransformed wild type. The NR seedlings produced from four to six true leaves and only second order lateral roots were formed (Table 1).

A further sign of TNT phytotoxicity has been observed as lack of root hairs in grass seedlings exposed to TNT [2, 22]. Root hairs are extensions of the root epidermis. They increase the surface area of roots, increasing the capacity of the plant to absorb water and nutrients. It is therefore detrimental for a plant if a toxic environment decreases root hair production. Seedlings were examined under a light microscope to examine root formation in more detail (Figure 12 and Figure 13).

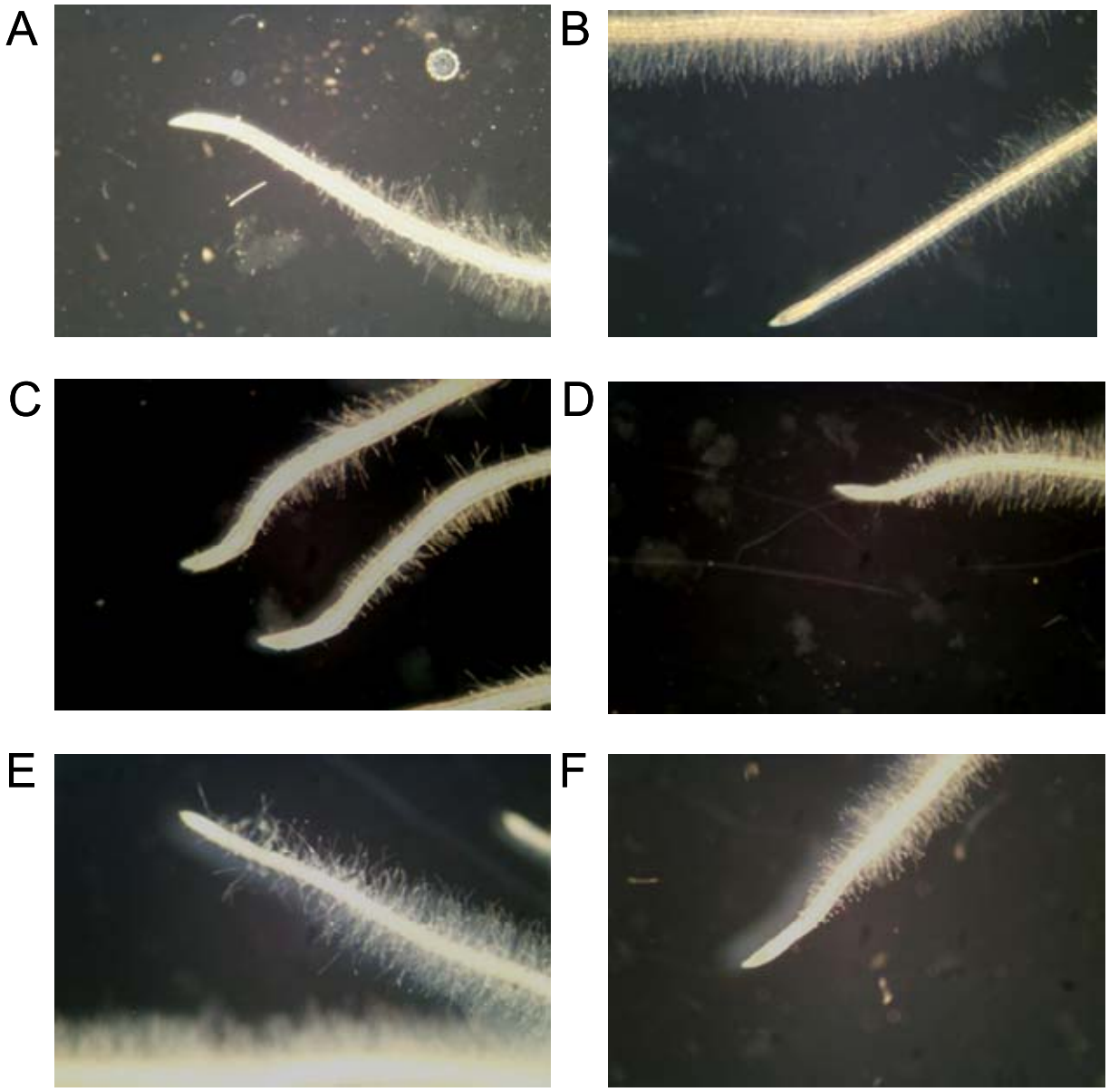


Figure 12. Morphology of transgenic NR 3-2 tobacco roots grown on TNT.
A, C and E: Roots of untransformed wild type seedling germinated on GM media, media amended with DMF, 0.05mM TNT respectively. Pictures B, D and F: Roots of transgenic seedlings (NR 3-2) germinated on GM media, media amended with DMF, 0.05mM TNT respectively. Seedlings all photographed at 7-fold magnification. Scale bar in picture A represents 1 mm.

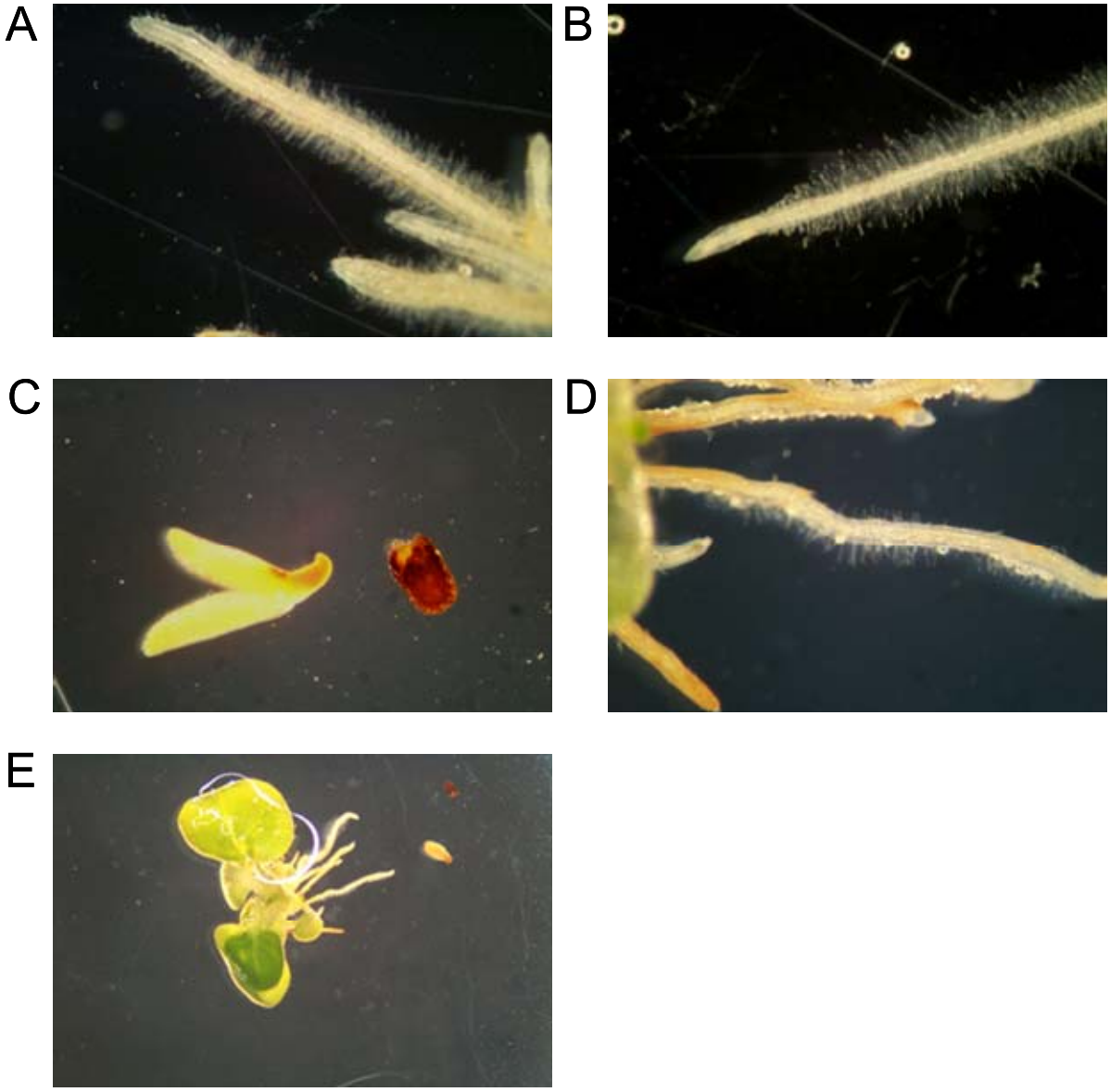


Figure 13. Morphology of transgenic NR 3-2 tobacco seedlings germinated on TNT.
A and C: Roots of untransformed wild type seedling germinated on media amended with 0.1mM TNT and 0.25mM TNT respectively. Pictures B and D: Roots of transgenic seedlings (NR 3-2) germinated on media amended with 0.1mM TNT and 0.25mM TNT respectively. Seedlings all photographed at 7- fold magnification. Scale bar in picture A represents 1 mm; E: Nitroreductase (left) and untransformed wild type (right) seedlings germinated on 0.25mM TNT. Photographed at 1- fold magnification, scale bar represents 5 mm.

Our studies illustrate that, even at very high concentrations of TNT (up to 0.1 mM TNT), untransformed wild type and NR 3-2 transgenic tobacco seedlings produce roots with apparently normal root hairs (compared to growth without TNT). Interestingly, even when roots were severely stunted at 0.1 mM TNT, untransformed wild type seminal and lateral roots still

produced root hairs. The difference in root morphology at 0.25 mM TNT is, however, very different when comparing untransformed wild type and transgenic plants. As can be seen in Figure 13 C and E, untransformed wild type seedlings were not able to develop beyond early post-germinative growth; the cotyledons exhibited severe chlorosis (a change in color from green to yellow, or in extreme cases brown), radicle extension was retarded and root hairs were absent. NR 3-2 germination was also severely affected by 0.25 mM TNT. In contrast to untransformed wild type, however, roots and shoots were produced after germination and the seedlings had developed root hairs. Root length was stunted and the root hairs present did not appear as numerous as growth without TNT amendment or at lower TNT concentrations.

Root Tolerance Index to TNT

Our studies revealed that tobacco roots were particularly affected by TNT phytotoxicity, thus, a tolerance index to TNT was performed to quantitate this (Table 2).

Table 2. Root tolerance index of tobacco seedlings after exposure to TNT
The tolerance index was calculated as root length of TNT-treated seedlings/root length of untreated control x 100. Results are an average and standard deviation of the measurements made on 12 individual seedlings for all except 0.25mM TNT which was an average of six seedlings for untransformed wild type and nine for NR 3-2. Seedlings were exposed to TNT for 21 days.

Concentration [mM]	Wild Type Plants		NR 3-2 Plants	
	Primary Root Length (cm)	Tolerance Index (%)	Primary Root Length (cm)	Tolerance Index (%)
0	8.1 ± 0.1	100	8.1 ± 0.1	100
DMF	8.1 ± 0.1	100	8.0 ± 0.2	99
0.05 (11.5mg/L)	5.5 ± 0.6	68	6.2 ± 0.9	77
0.1 (23 mg/L)	0.3 ± 0.2	4	5.5 ± 0.9	68
0.25 (56 mg/L)	0.05 ± 0.05	0.6	1.5 ± 0.7	19

The results presented in Table 2 demonstrate the more severe root stunting of untransformed wild type plants compared to NR 3-2 plants germinated on TNT-amended media. For both untransformed wild type and transgenic NR 3-2 seedlings, the primary roots (and many lateral roots) grew to approximately 8.1 cm in length. Neither untransformed wild type nor transgenic seedlings demonstrated root stunting in the presence of DMF. At 0.05 mM TNT, both untransformed wild type and transgenic seedlings were stunted in root length. At 0.1 mM TNT there was a greater difference in seedling root development between the two lines with NR 3-2 producing a 64 % greater tolerance than untransformed wild type. At 0.25 mM, untransformed wild type plants had a tolerance of less than 1 % with NR 3-2 seedlings successfully germinating but severely stunted at 19 % tolerance.

Phytotoxicity of TNT during vegetative growth

The ability of a plant to grow in a toxic environment is an indication that the plant has some tolerance to the compounds it is exposed to. As plant growth can be measured through an

increase in biomass, a study was undertaken to measure the change in biomass of untransformed wild type and transgenic plants in the presence of TNT. Untransformed wild type tobacco and tobacco NR 3-2 seeds were grown in liquid culture and dosed with 0.1 mM, 0.25 mM, 0.38 mM or 0.5 mM TNT. 0.5 mM TNT is above the aqueous solubility limit of TNT and it was important to establish if plants could tolerate this level of TNT. A higher biomass of the NR transgenic line at 0.5mM TNT was used as it was thought necessary to increase biomass at such a high TNT concentration (above the solubility limit of TNT) in order to enhance TNT tolerance and give the plants a better opportunity to metabolize TNT. However, there was not enough untransformed wild type seedling biomass to also increase the starting biomass of untransformed wild type seedlings here. Results are shown in Table 3.

Table 3. Effects of TNT toxicity on the vegetative growth of tobacco
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 at concentrations 0.38 and 0.5mM, which are individual results. * As TNT was not added to these flasks, weight measurements were taken at the same time as the addition of TNT to the other flasks. NA, not available.

TNT Concentration [mM]	Wild Type Plants			NR 3-2 Plants		
	Weight before TNT (g)	Weight after TNT (g)	Weight gain/ loss (g)	Weight before TNT (g)	Weight after TNT (g)	Weight gain/ loss (g)
0	2.3 ±NA*	4.3 ±NA*	+2.0 ±NA*	3.3 ±NA*	6.0 ±NA*	+2.7 ±NA*
0.1 (23 mg/L)	3.7 ±0	4.6 ±2.26	+1.15 ±1.9	3.9 ±0.28	6.85 ±1.2	+2.95 ±0.91
0.25 (56 mg/L)	3.4 ±0.14	3.35 ±0.49	-0.5 ±0.28	3.75 ±0.07	6.5 ±0.7	+2.75 ±0.63
0.38 (86mg/L)	3.5 ±NA	1.6 ± NA	-1.9 ±NA	4.1 ±NA	5.7 ±NA	+1.6 ±NA
0.5 (113mg/L)	3.2 ±NA	1.5 ±NA	-2.4 ±NA	8.9 ±NA	13.6 ±NA	4.7 ±NA

There was no significant difference in growth of between either plant line when grown without TNT amendment; untransformed wild type plants gained 86 % and the NR 3-2 line gained 81 % in biomass over the five-day study. Untransformed wild type plants did increase in biomass when grown at 0.1 mM TNT (24 %) but the transgenic line gained approximately three times as much biomass (75 %). Untransformed wild type plants lost biomass when incubated with 0.25 mM TNT (14 % loss) and were necrotic at the end of the five-day study (Figure 6). Untransformed wild type plants lost biomass (14 % loss at 0.25 mM, a 54 % loss at 0.38 mM and a 75 % loss at 0.5 mM TNT) and were necrotic at all concentrations of TNT after five days. In

contrast, the transgenic line, NR 3-2 gained weight at both 0.38mM and 0.5mM TNT concentrations (39 % and 52 % respectively). NR 3-2 produced more biomass than untransformed wild type at 0.5 mM TNT however; NR 3-2 had a higher starting weight than at 0.38mM TNT (Table 3).

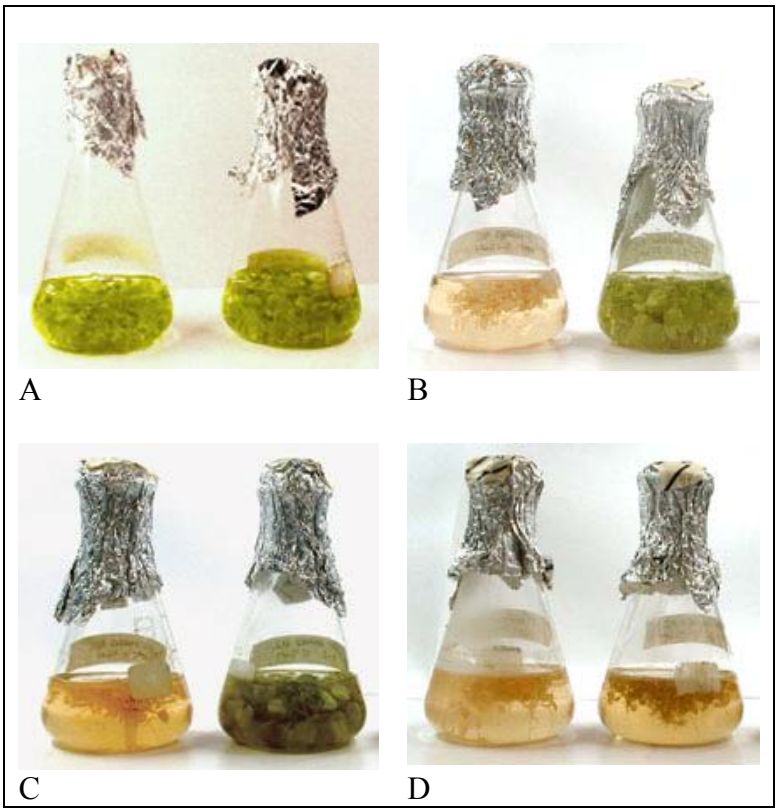


Figure 14. Toxicity of TNT on liquid culture grown tobacco
Fifty seedlings of each plant line were grown for 14 days, at which time TNT was added and remained in growth medium for a further five days. Pictures are untransformed wild type seedlings (all left hand flasks) and transgenic NR 3-2 seedlings (all right hand flasks) at A: Without TNT amendment; B: 0.25 mM TNT; C: 0.5 mM TNT and D: 0.75 mM TNT.

Figure 14 illustrates the phytotoxic effects of TNT towards the plant lines at increasing concentrations of TNT. Without TNT both plant lines appeared normal indicating that submersion in growth media was not inducing toxic effects. At 0.25 mM TNT, NR 3-2 plants appeared normal; however, untransformed wild type plants appeared severely chlorotic and necrotic. Untransformed wild type plants exhibited these same phytotoxic effects at 0.5 mM TNT (the aqueous solubility limit of TNT) whereas NR 3-2 plants were not chlorotic but did appear to have changed color to a darker green. At the saturating TNT concentration of 0.75 mM both untransformed wild type and transgenic plants appeared to be chlorotic and necrotic.

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.

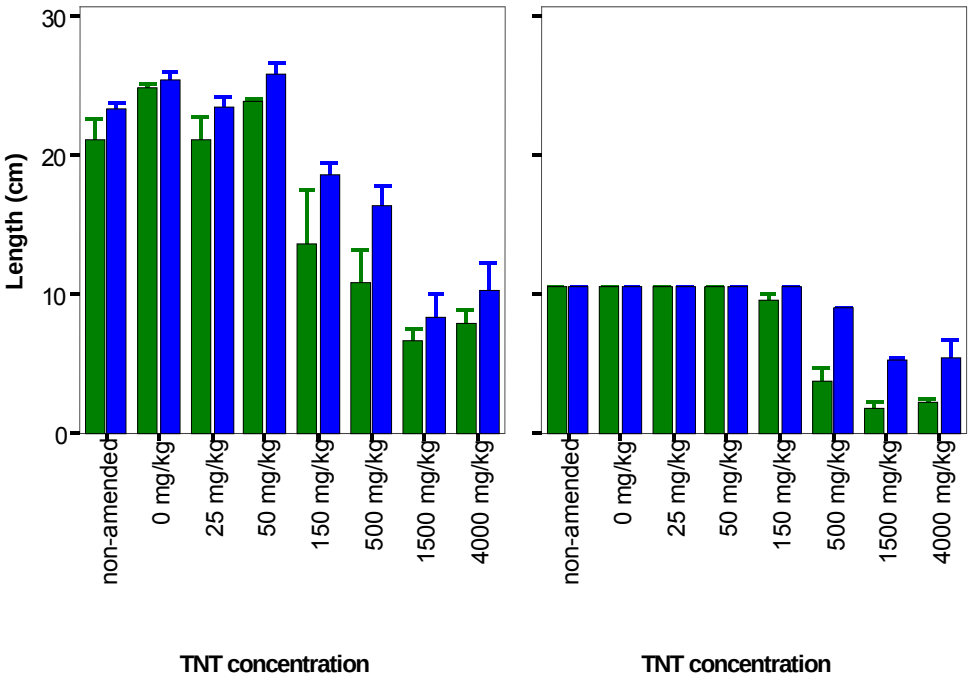
Soil studies on NR-expressing tobacco

As the ultimate application for our transgenic plants is to phytoremediate TNT contaminated soil and ground water, it was important to gain an understanding of the detoxification capabilities of the plants in soil. The tolerance, uptake and transformation of TNT from soil by untransformed wild type and transgenic tobacco were therefore investigated. The soil was amended with a range of concentrations of TNT then aged for three months in order to more accurately represent sites requiring remediation. Soil columns were packed with a layer of pristine soil was packed beneath the layer of TNT amended soil, in the soil columns, in order to establish the effects of the plants on TNT migration. The soil run-off was also collected to establish if TNT and/or its transformation products were disappearing from the soil in the water run-off.

The transgenic NR-expressing tobacco plants were found to tolerate TNT significantly better than the untransformed wild type plants. In the case of the untransformed wild type plants, TNT exerted a clear phytotoxic effect after 12 weeks growth Figure 15. Severe stunting of root and shoot growth, was observed at high concentrations of TNT (greater than 500 mg TNT/kg soil). A decrease in lateral root production was also seen at these concentrations. Chlorosis of leaves also occurred at TNT-amended soil of concentrations of 500 mg TNT/kg soil and above. Transgenic NR-expressing tobacco exhibited a marked improvement in growth and health, compared to the untransformed wild type plants, when grown in high (> 500 mg TNT/kg soil) concentrations of TNT-amended soil (Figure 15). All plants grown in soil contaminated with 500 mg/kg or higher concentrations of TNT exhibited considerable stunting, compared with those grown in control soil with no TNT contamination. But the severity of the stunting and chlorosis in the NR plants was much less than that observed for untransformed wild type plants. Lengths of shoots and roots were measured (Figure 16) and the differences in root length between untransformed wild type and transgenic tobacco were found to be more dramatic than the changes in shoot length. In the case of untransformed wild type plants, high concentrations of TNT in soil prevented root formation, whereas in the case of NR tobacco, roots grew even at the highest level of TNT-amendment.

A **B**

Figure 15 Tobacco plants grown in TNT-amended soils for 12 weeks
A: untransformed wild type tobacco plants; B: transgenic NR-expressing tobacco plants. In both A and B TNT concentration increased from left to right as follows: 0 mg/kg, 50 mg/kg, 150 mg/kg, 500 mg/kg, 1500 mg/kg and 4000 mg/kg TNT.



A. B.

Figure 16. Root and shoot lengths of NR expressing tobacco grown in TNT contaminated soil
Comparison of shoot (A) and root (B) length for untransformed, wild type (■) and transgenic NR-expressing (■) tobacco. It should be noted that the experimental design prevented roots growing longer than 10.5 cm. Bars represent the mean of triplicate samples and error bars show + 1 S.E. of the mean.

Fate of TNT and transformation products following plant uptake

The fate of TNT and its transformation products was also investigated. Soil which had been planted with either untransformed wild type or NR-expressing tobacco had slightly lower levels of TNT than unplanted soil, suggesting that the tobacco plants had taken up TNT. At low levels of TNT-amendment, the monoamino derivatives (2-ADNT and 4-ADNT) were observed in the unplanted soil, but were absent in the planted soil, again suggesting uptake by the plants. In unplanted and planted soil 2,4-diaminonitrotoluene (2,4-DANT) appeared at low levels, which was assumed to be the product of anaerobic bacterial transformation. Variations in the concentrations of TNT and transformation products in soil planted with untransformed wild type compared to NR-expressing tobacco were also observed. In general the concentrations of TNT and its monoamino derivatives (2ADNT and 4ADNT) were lower in NR-expressing tobacco-

planted soil compared with wild-type tobacco-planted soil, suggesting that the NR-expressing tobacco had greater rates of TNT uptake.

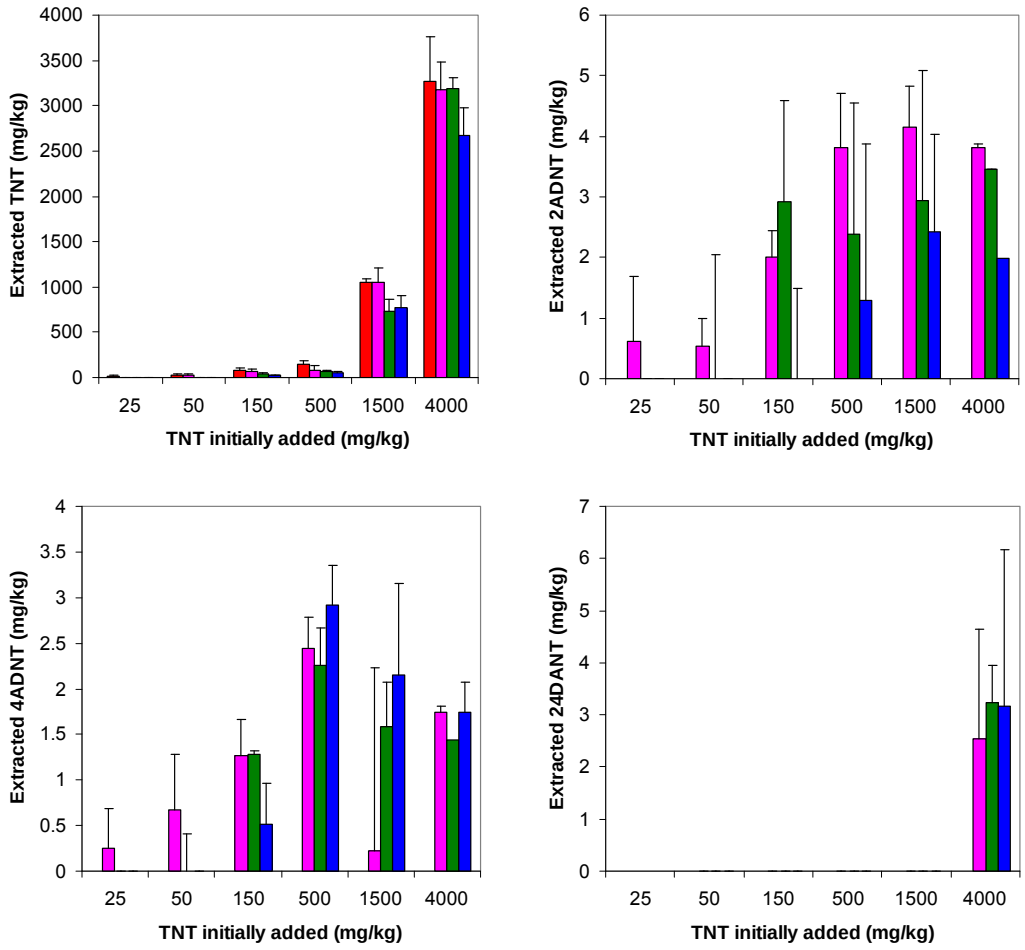


Figure 17. Concentrations of TNT and transformation products within soil
Soil samples are distinguished by color: pre-planted soil (■), unplanted soil (■), untransformed wild type soil(■) and NR-expressing transgenic soil (■). Bars represent the mean of triplicate samples and error bars show + 1 S.D. of the mean.

Soil characterization

The pH of soil samples was determined (Figure 18). There was a significant difference between the pH of the soil of soil taken from unplanted, untransformed wild type tobacco bulk soil, NR-expressing tobacco bulk soil and pre-planted soil, as judged by a one-way ANOVA ($p<0.01$). The most noticeable difference was that the pre-planted soil samples were more acidic than the other soil samples. The NR-expressing tobacco bulk soil was slightly more acidic on average than the unplanted soil, which was in turn more acidic than the untransformed wild type tobacco bulk soil. There was no significant difference ($p<0.05$), between soil samples contaminated across a range of concentrations of TNT.

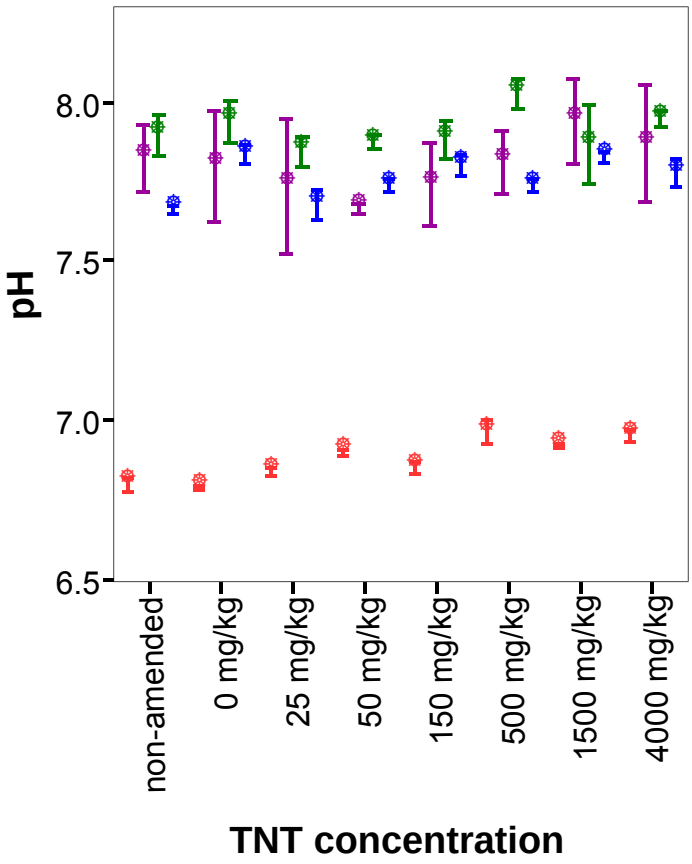


Figure 18. Soil pH across a range of TNT concentrations for different soil samples
Soil samples are distinguished by colour: pre-planted soil (■), unplanted soil (■), untransformed wild type bulk soil (■) and NR-expressing transgenic bulk soil (■). The means of triplicate samples are displayed and error bars show ± 1 S.E. of the mean.

Colony forming unit count

Colony forming unit (CFU) data provided information about the culturable portion of the soil microbial community of TNT-contaminated soil planted with NR-expressing transgenic or untransformed wild type tobacco compared to unplanted soil (Figure 19).

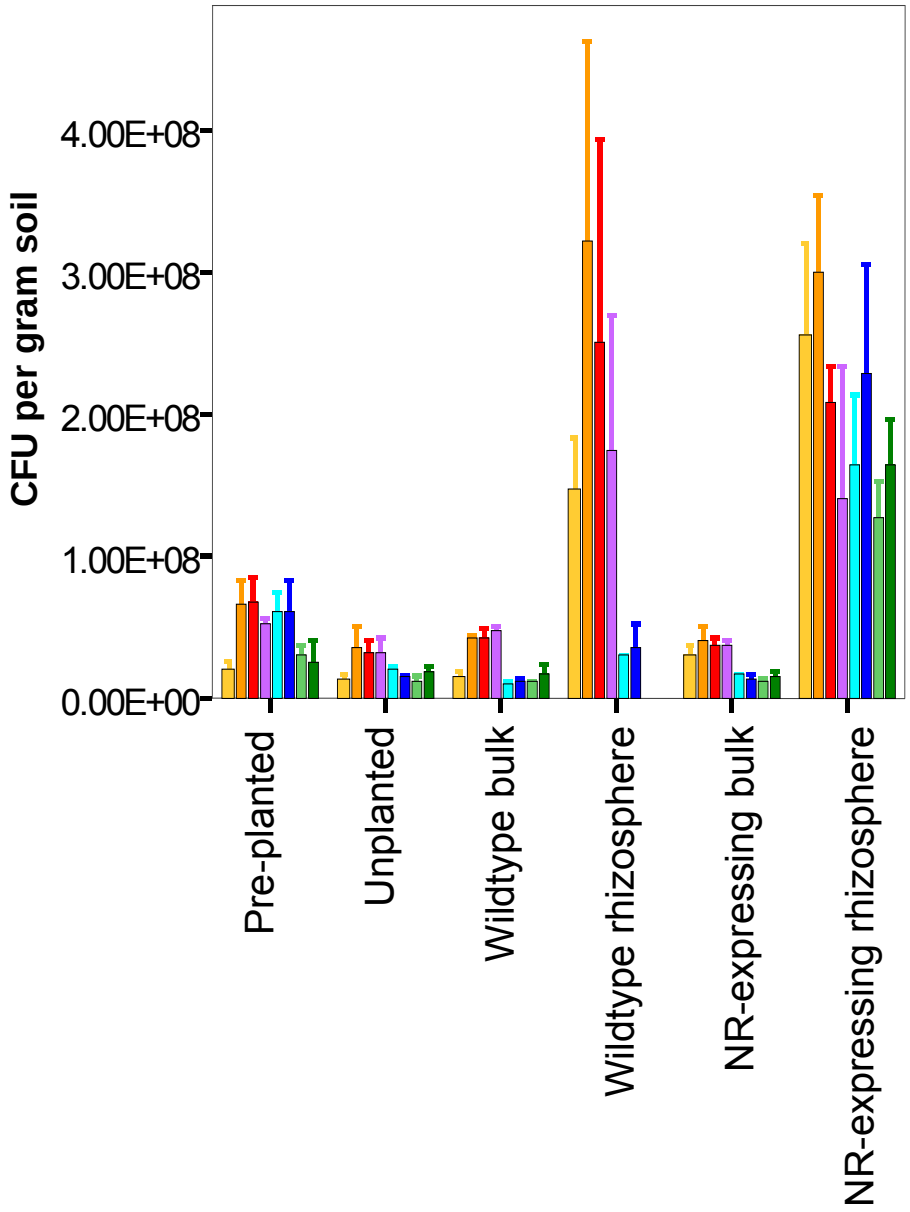


Figure 19. Colony forming unit count per gram of soil after six days incubation
Soil was taken from pre-planted soil, unplanted soil, untransformed wild type bulk soil, untransformed wild type rhizosphere soil, transgenic NR-expressing bulk soil, or transgenic NR-expressing rhizosphere soil. TNT concentration is represented by the following colored bars: non-amended (■), acetone-amended (■), 25 mg/kg TNT (■), 50 mg/kg TNT (■), 150 mg/kg TNT (■), 500 mg/kg TNT (■), 1500 mg/kg TNT (■) and 4000 mg/kg TNT (■) Bars represent the mean of triplicate samples and error bars show + 1 S.E. of the mean.

Univariate analysis of variance (ANOVA) (Table 4) allowed statistical determination of associations between type of soil, TNT concentration and CFU per gram of soil. It showed that there was a main effect due to the factor of type of soil: $F(5,137) = 17$; $p<0.01$. There was also a

main effect due to the factor of TNT concentration: $F(7,137) = 37$; $p<0.01$. An interaction effect between the factors soil type and TNT concentration: $F(33,137) = 8$; $p<0.01$ was also seen.

The acetone-amended soil was considered the control soil, as there was an increase in CFU compared to the non-amended soil. Soil from before the experiment started (pre-planted soil) exhibited higher CFU count than the unplanted or the bulk soil from either untransformed wild type or transgenic tobacco. For pre-planted soil, the CFU counts at low and intermediate levels of TNT (up to 500 mg/kg) were not significantly different to the control soil. As concentrations of TNT increased (1500 and 4000 mg/kg) the CFU count of the pre-planted soil dropped approximately two-fold. There was no apparent difference between the CFU count profile for bulk soil taken from both untransformed wild type and transgenic tobacco planted soil, nor was there a difference compared to the unplanted soil. The CFU counts for unplanted and the two bulk soils at low concentrations of TNT (25, 50 mg/kg) were not significantly different from the control soil. At concentrations of 150 mg/kg and above there was a marked decrease in CFU counts for all three soils. The highest CFU count was observed in the rhizosphere soil samples. Both untransformed wild type and NR-expressing transgenic tobacco rhizosphere samples resulted in high CFU counts at 0, 25 and 50 mg TNT/ kg soil. There was a marked difference between untransformed wild type and transgenic rhizosphere samples at higher concentrations of TNT. For untransformed wild type rhizosphere samples the CFU count dropped dramatically to levels seen in bulk or unplanted soil at equivalent concentrations of TNT (150, 500 mg/kg). No roots were present for untransformed wild type tobacco when grown in highly TNT-contaminated soil (1500 and 4000 mg/kg) and therefore there were no rhizosphere samples. In sharp contrast, for the rhizosphere samples from NR-expressing tobacco, the CFU count was high across the range of TNT concentrations.

Table 4. ANOVA testing for association of CFU per gram soil, with TNT concentration and soil type. An interaction effect was also tested for between the two factors (TNT concentration*soil type). N=135; r^2=0.859; Adjusted r^2=0.790.

Source	Sum of Squares	d.f.	Mean Square	F	Prob>F
Corrected Model	22049	45	490	12	0.000
Intercept	319426	1	319426	8057	0.000
TNT concentration	4584	7	655	17	0.000
Soil type	7365	5	1473	37	0.000
TNT concentration*soil type	10373	33	314	8	0.000
Error	3608	91	40		
Total	348088	137			
Corrected Total	25657	136			

Substrate utilization profiles

The functional potential of the culturable portion of the microbial community was assessed by examination of sole carbon utilization patterns from Biolog EcoPlates. The patterns of substrate utilization varied, with differences observed in the rate of color development, the length

of the lag phase and the maximum optical density achieved. Typical time courses of substrate utilization are shown in Figure 20. It was apparent that the time course of substrate utilization varies dependent on both the origin of the soil and the TNT concentration of the soil.

The area under the absorbance versus time curve (AAT) was calculated for each substrate and sample using a trapezoidal approximation with 96 hours as the end-point. This value provided information on colour development for each well and incorporates aspects of the curve such as variations in lag phase and rate of colour development [45]. The average AAT (a measure of the metabolic activity of the culturable portion of the soil microbial community) was calculated for each sample (Figure 21). The average AAT was dependent on both the origin of the soil and the TNT concentration of the soil. Univariate analysis of variance (ANOVA) (Table 5) allowed statistical determination of associations between type of soil, TNT concentration and average AAT. It showed that there was a main effect due to the factor of type of soil: $F(5,137) = 14$; $p<0.01$. There was also a main effect due to the factor of TNT concentration: $F(7,137) = 18$; $p<0.01$. An interaction effect between the factors soil type and TNT concentration: $F(33,137) = 6$; $p<0.01$ was also seen. The CFU per gram of soil was considered as a covariate but had no significant main effect on average AAT.

The pre-planted sample exhibited the largest variation in average AAT across the range of TNT concentrations. The average AAT of the control soil was low, an increase in average AAT was observed as TNT concentrations increased to 150 mg/kg TNT. As the concentrations of TNT increased further, the average AAT remained constant. The unplanted and untransformed wild type bulk soil exhibited similar trends compared with each other, although for all concentrations of TNT the average AAT of untransformed wild type planted bulk soil was slightly higher than that of unplanted soil. For unplanted and untransformed wild type bulk soil the average AAT was slightly lower with either very low (0, 25 mg/kg) or high (1500, 4000 mg/kg) concentrations of TNT than at intermediate concentrations. The average AAT profile for transgenic bulk soil was similar to the untransformed wild type bulk samples with the exception that the 25 mg/kg samples were higher and comparable in magnitude to the intermediate soil TNT concentrations. The rhizosphere samples for both untransformed wild type and transgenic tobacco displayed a different pattern compared with the bulk or unplanted soil. For the rhizosphere samples, the low and high concentrations exhibited a higher average AAT than the intermediate TNT concentrations (50, 150 mg/kg). This variation across the range of TNT concentrations was more pronounced in untransformed wild type samples compared with transgenic samples. It was in the transgenic rhizosphere samples at high TNT concentrations (500, 1500 and 4000 mg/kg TNT) that the highest average AAT from any soil origin or concentration of TNT was observed.

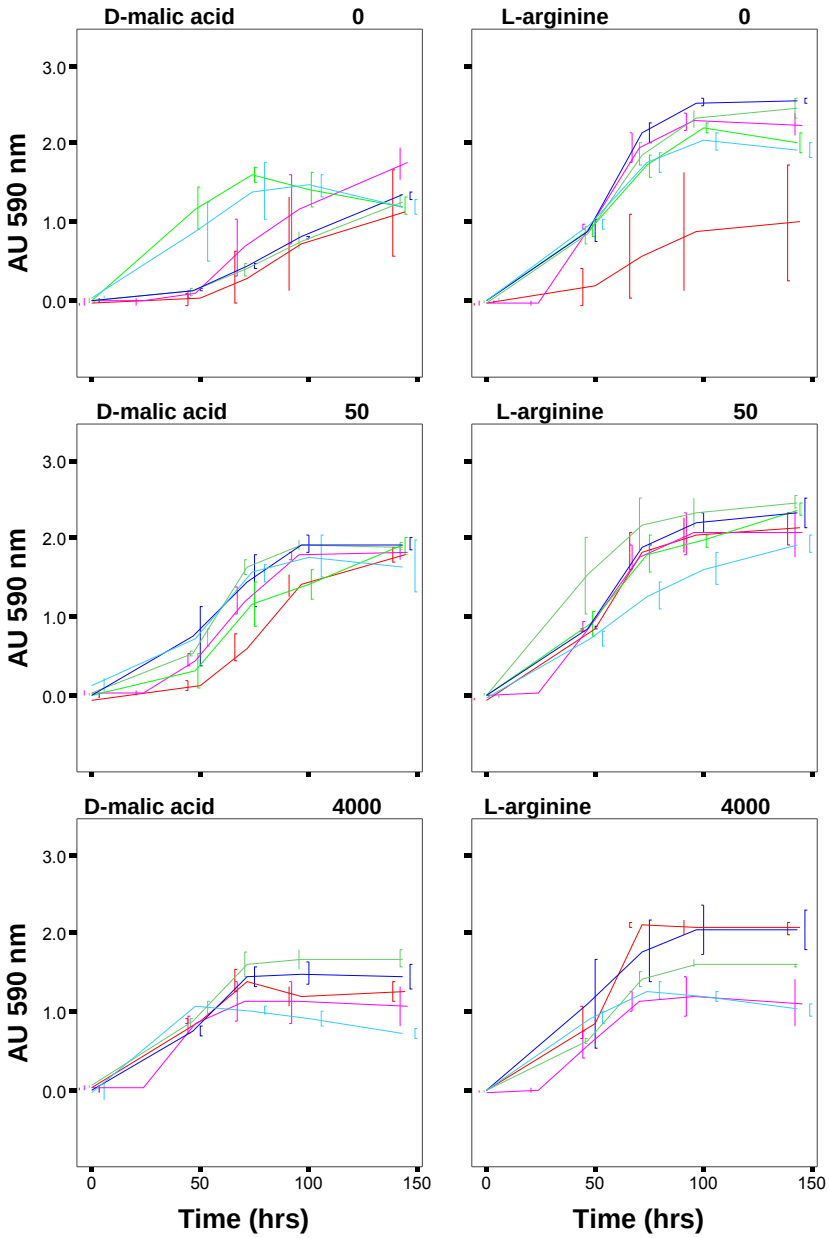


Figure 20. Time courses of substrate utilization in TNT contaminated soil
Levels of D-malic acid (left) and L-arginine (right) for soil contaminated with 0 (top), 50 (middle) and 4000 (bottom) mg.TNT/kg soil. Pre-planted soil (■), unplanted soil (■), untransformed wild type bulk soil (■), NR-expressing transgenic bulk soil (■), untransformed wild type rhizosphere soil (■) and NR-expressing transgenic rhizosphere TNT (■) are shown. Each time point represents the mean of triplicate samples and the error bar shows ± 1 S.E. of the mean.

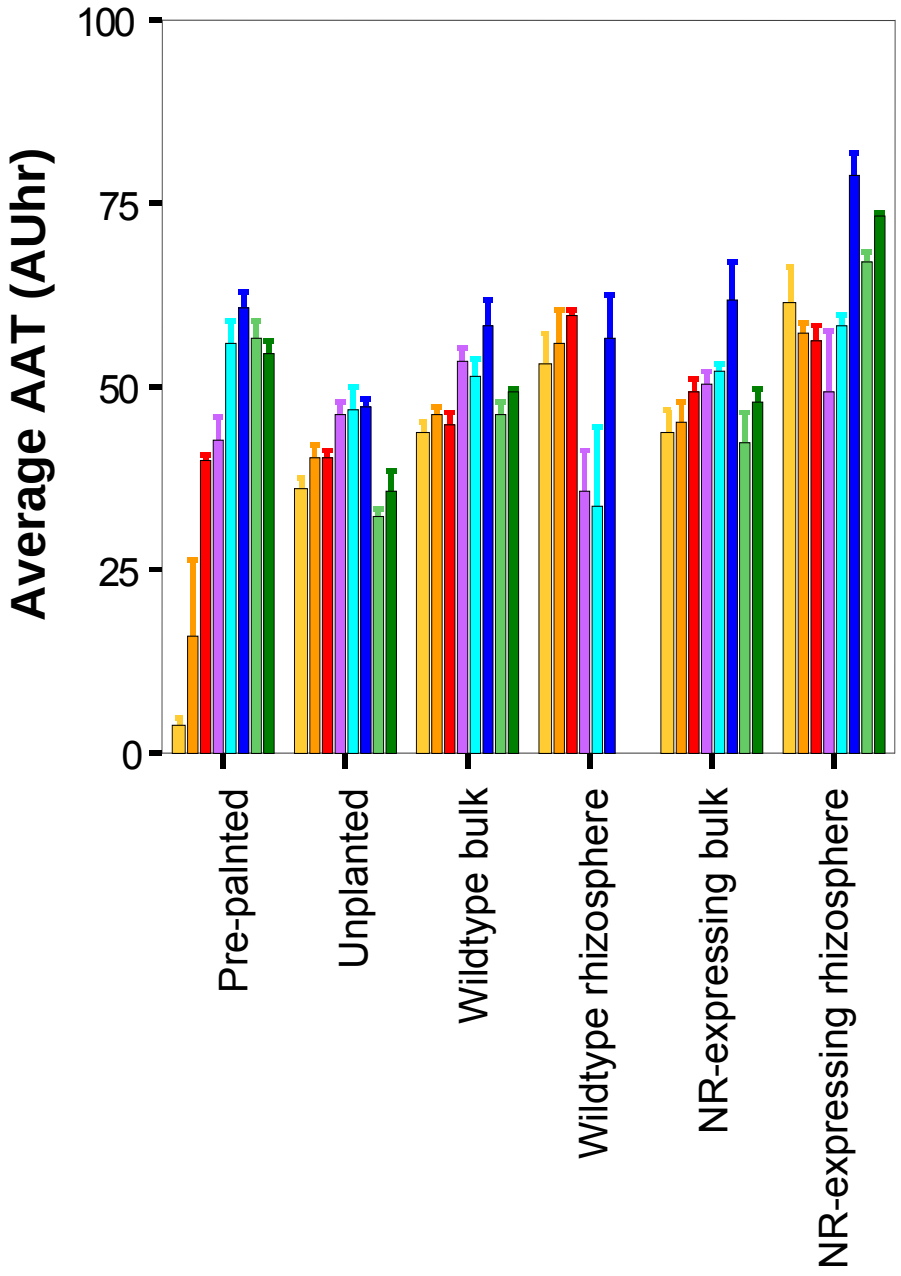


Figure 21. Average AAT for EcoPlate incubations from soil samples
TNT concentration is represented by the following colored bars: non-amended (■), acetone-amended (■), 25 mg/kg TNT (■), 50 mg/kg TNT (■), 150 mg/kg TNT (■), 500 mg/kg TNT (■), 1500 mg/kg TNT (■) and 4000 mg/kg TNT (■) Bars represent the mean of triplicate samples and error bars show + 1 S.E. of the mean.

Table 5. ANOVA testing for association of average AAT with TNT concentration and soil type An interaction effect was also tested for between the two factors (TNT concentration*soil type). CFU per gram of soil was also considered as a covariate. N=137; r^2=0.865; Adjusted r^2=0.786.

Source	Sum of Squares	d.f.	Mean Square	F	Prob>F
Corrected Model	21242	46	462	11	0.000
Intercept	91486	1	91486	2178	0.000
CFU per gram soil	91	1	91	2	0.145
TNT concentration	4171	7	596	14	0.000
Soil type	3701	5	740	18	0.000
TNT concentration*soil type	8626	33	261	6	0.000
Error	3319	79	42		
Total	317741	126			
Corrected Total	24561	125			

Principal component analysis was performed to obtain a more detailed insight into the patterns of substrate utilization. In order to correct for variations in inoculum density, the ratio of the individual substrate to the average AAT was compared [53]. Variables with low overall variance and therefore low individual measure of sampling adequacy (L-serine, tween 80), were excluded from the PCA to increase the overall sampling adequacy of the analysis. The Bartlett's test of sphericity showed that the non-zero correlations were significant at the 0.001 level. The Kaiser-Meyer-Olkin measure of sampling adequacy was determined to be 0.769, which exceeds the threshold value, thereby indicating that the fundamental requirements for PCA were met.

Seven components were extracted and retained by applying the latent root criterion (Eigenvalue < 1) the results for each component are shown in Table 6

The seven components account for 71.6 % of the total variance. For 137 samples, loadings above 0.5 were significant ($p<0.05$). The component matrix is shown in Table 7.

Table 6. Eigenvalues, percentage variance and number of loadings

Component	Eigenvalue	Percentage of Variance	Cumulative Percentage	Number of loadings
1	6.897	23.783	23.783	15
2	4.341	14.967	38.750	8
3	2.703	9.320	48.070	4
4	2.230	7.689	55.759	3
5	1.854	6.392	62.151	2
6	1.590	5.481	67.632	1
7	1.143	3.942	71.574	0

Table 7. The factor solution is presented with significant loadings for each factor emboldened.

Substrate	**Component**						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Pyruvic acid methyl ester	.019	-.290	.136	**-.754**	.195	-.060	.350
Tween 40	.373	**-.611**	-.481	.124	-.124	-.204	-.171
Cyclodextrin	**.641**	-.152	.284	-.363	.055	.060	.257
Glycogen	**.735**	-.425	.021	.319	.187	.000	.198
D-cellulose	**.814**	.115	.453	.080	-.063	-.026	-.153
D-lactose	**.706**	-.359	-.089	**.523**	.020	-.125	.072
Methyl-D-glucoside	**.503**	.377	**.526**	-.213	-.230	.090	-.322
D-xylose	.197	**.700**	-.229	-.035	.349	-.031	-.259
Erythritol	.259	.293	-.285	.053	**.659**	-.145	-.233
D-mannitol	.242	**-.512**	**-.505**	-.211	-.245	.016	-.299
N-Acetyl-D-glucosamine	.467	-.262	**.503**	.095	.335	.265	-.115
D-glucosaminic acid	**-.649**	.035	.029	.247	.290	.174	-.132
Glucose-1-phosphate	**.531**	.461	.432	-.075	-.233	.189	-.206
D,L-Glycerol Phosphate	.149	.190	**-.725**	-.033	-.262	.318	-.070
D-galactonic acid -lactone	**-.675**	-.022	.177	.065	-.106	-.285	-.023
D-galacturonic acid	**-.750**	-.125	.230	.201	.083	-.024	-.073
2-Hydroxy Benzoic Acid	.265	**.547**	.202	.077	-.005	-.119	.021
4-Hydroxy Benzoic Acid	**-.502**	.232	.055	.361	.018	-.021	-.204
Hydroxybutyric acid	-.268	-.153	.016	-.009	**.639**	.365	.151
Racinic acid	.113	**.667**	-.165	-.049	.354	-.330	.081
Ketobutyric acid	.180	.302	-.337	.104	-.026	**.615**	.345
D-malic acid	-.060	**.617**	-.208	-.020	.042	-.373	.166
L-arginine	**-.639**	-.036	.206	.301	-.083	.277	.123
L-asparagine	**-.576**	.007	.200	.086	-.302	-.347	.234
L-phenylalanine	.178	**.655**	-.040	.031	-.153	-.125	.327
L-threonine	**.535**	-.307	.085	**.606**	.105	-.304	.167
Glycyl-L-Glutamic Acid	**.644**	.285	.015	.421	-.178	.116	.170
Phenylethylamine	.016	**.566**	-.249	.180	-.216	.203	.081
Putrescine	**-.594**	.068	.247	.301	-.066	.201	-.019

Principle component analysis of the Biolog EcoPlate data indicated that the patterns of carbon utilisation varied across the TNT concentration in a fashion dependent on the origin of soil. Figure 22 shows PC1 plotted against PC2 for each origin of soil across a range of concentrations of TNT. A bar chart showing PC1 and PC2 against TNT concentration is presented in Figure 23 to enable a clearer visualisation of the trends.

The variation in PC1 was most strongly associated with the type of soil tested. In general, rhizosphere soil samples exhibited a positive PC1, whilst soil samples from non-rhizosphere origins tended to be negative. The main exceptions to this were the non-amended and 0 mg/kg pre-planted soil samples that were positive, but exhibited a large standard error of the mean. A trend also existed for PC1 across the range of concentrations of TNT-contaminated soils, with the more highly contaminated soil showing a more positive PC1 than soils with low levels of contamination. The variation in PC2 could mainly be attributed to changes in TNT concentration. For the pre-planted, unplanted and bulk soil from both tobacco strains, the trend across the range of TNT was the same. Uncontaminated soil, or soil that was only contaminated with low concentrations of TNT (25, 50 mg/kg), exhibited a negative PC2, whereas more highly contaminated soil showed a positive PC2. For rhizosphere samples from both tobacco genotypes, the PC2 value became more positive as TNT concentration increased. This trend was less pronounced than for bulk soil.

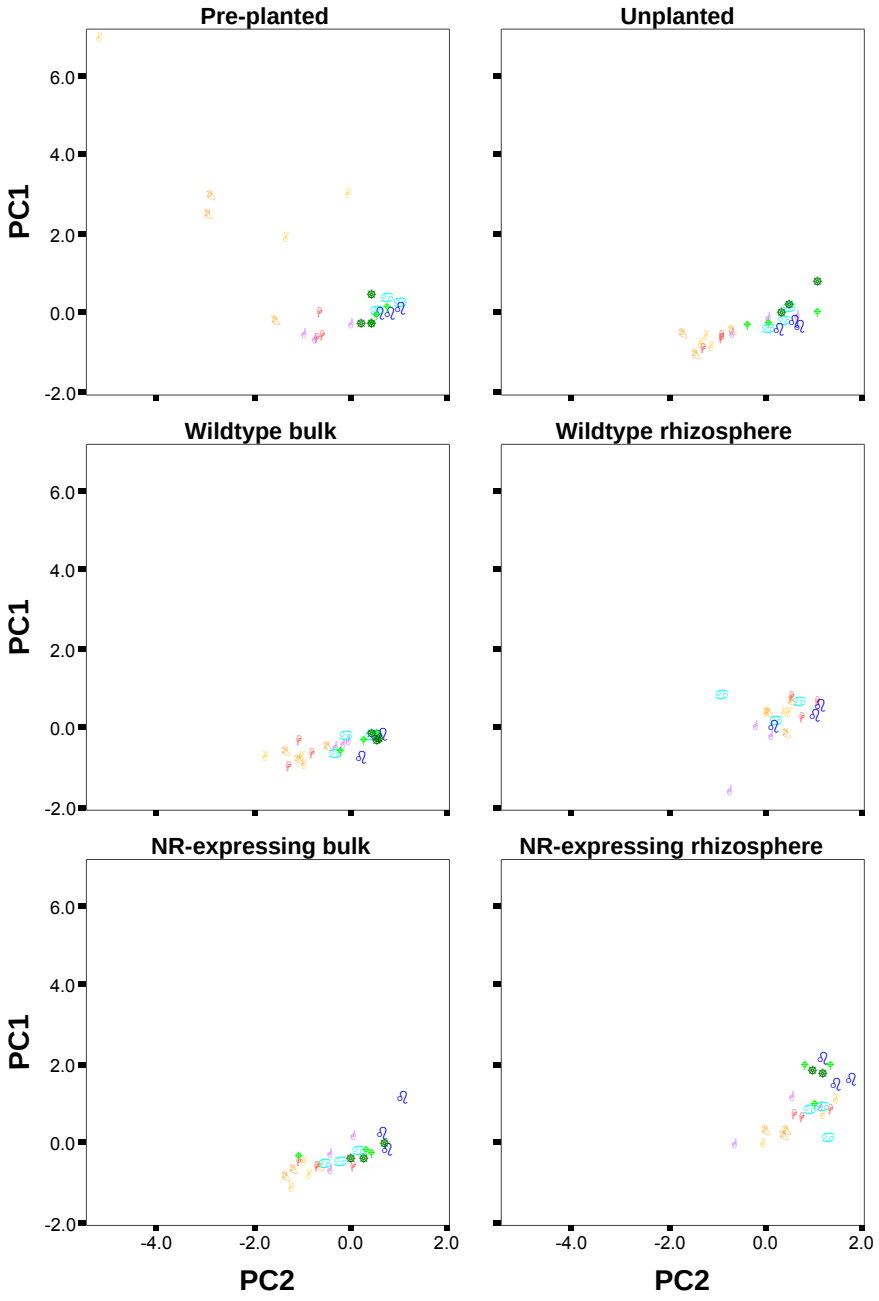


Figure 22. Principle component analysis of the Biolog EcoPlate data Principal component 1 (PC1) plotted against principal component 2 (PC2). Panels represent origin of the soil. TNT concentration is represented by the following colours: non-amended (♈), acetone-amended (♉), 25 mg/kg TNT (♊), 50 mg/kg TNT (♋), 150 mg/kg TNT (♌), 500 mg/kg TNT (♍), 1500 mg/kg TNT (♎) and 4000 mg/kg TNT (♏).

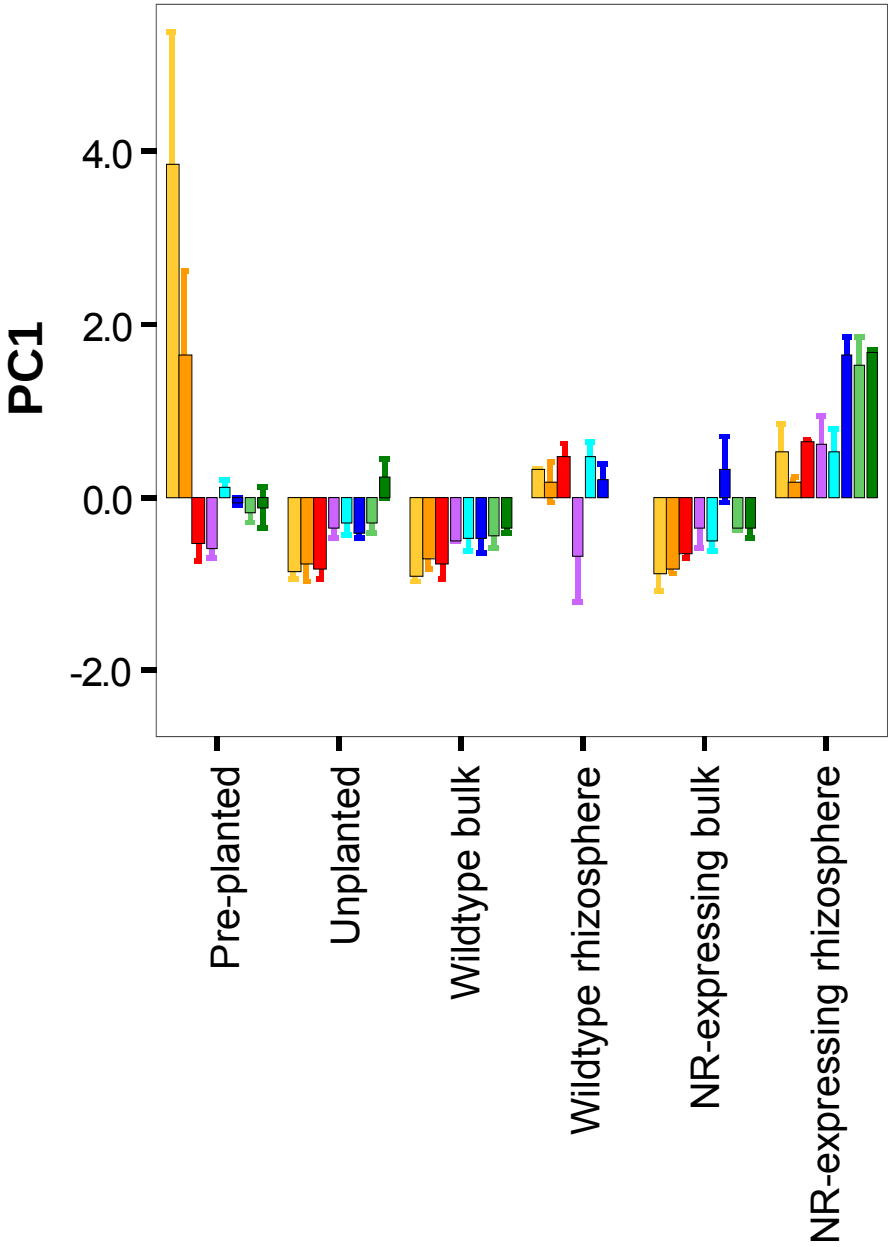


Figure 23. Principal component 1 scores for soil amended with TNT. Samples taken from bulk and rhizosphere soil from untransformed wild type and transgenic tobacco and unplanted soil. TNT concentration is represented by the following colored bars: non-amended (■), acetone-amended (■), 25 mg/kg TNT (■), 50 mg/kg TNT (■), 150 mg/kg TNT (■), 500 mg/kg TNT (■), 1500 mg/kg TNT (■) and 4000 mg/kg TNT (■) Bars represent the mean of triplicate samples and error bars show ± 1 S.E. of the mean.

Patterns of substrate utilization were discerned by PCA analysis; those substrates that were strongly loaded on PC1 or PC2 are shown in Table 8. It was of interest that five of the eight substrates that were strongly loaded in a positive fashion on PC1 were carbohydrates, whilst none of the six substrates negatively loaded on PC1 were carbohydrates. No clear pattern of substrate type emerged as being strongly loaded either negatively or positively on the second principal component.

Table 8. Substrates with significant loadings for PC1 and PC2.

Principal Component	Substrate	Loading	Type of substrate
PC 1	D-cellulose	0.814	carbohydrate
	Glycogen	0.735	carbohydrate
	D-lactose	0.706	carbohydrate
	Glycyl-L-Glutamic Acid	0.644	amino acid
	Cyclodextrin	0.641	carbohydrate
	L-threonine	0.535	amino acid
	Glucose-1-phosphate	0.531	carboxylic acid
	Methyl-D-glucoside	0.503	carbohydrate
	4-Hydroxy Benzoic Acid	-0.502	carboxylic acid
	L-asparagine	-0.576	amino acid
	Putrescine	-0.594	amine
	L-arginine	-0.639	amino acid
	D-glucosaminic acid	-0.649	carboxylic acid
	D-galactonic acid	-0.675	carboxylic acid
	D-galacturonic acid	-0.75	carboxylic acid
PC 2	D-xylose	0.700	carbohydrate
	Racinic acid	0.667	carboxylic acid
	L-phenylalanine	0.655	amino acid
	D-malic acid	0.617	carboxylic acid
	Phenylethylamine	0.566	amine
	2-Hydroxy Benzoic Acid	0.547	carboxylic acid
	D-mannitol	-0.512	carbohydrate
	Tween 40	-0.611	carbohydrate

Genetic diversity

Genetic diversity was investigated by extraction of the genomic DNA from the soil, PCR amplification of 16S rDNA fragments, followed by separation by denaturing gradient gel electrophoresis (DGGE). Genomic DNA of, predominantly, between 5 and 12 kb was extracted from all samples, as confirmed by gel electrophoresis (Figure 24). Typical yields were 4 ± 1 μg/g soil.

Denaturing gradient gel electrophoresis (DGGE) revealed that the genetic diversity of the soil altered in a manner dependent on both the origin of the soil and the concentration of TNT at which the soil had been amended. Typical DGGE images of samples from across the range of TNT concentrations and derived from each soil origin are shown in Figure 26. Triplicate samples of soil for each condition yielded similar banding patterns.The patterns of banding for unplanted soil and bulk soil, from both tobacco genotypes, across the range of TNT concentrations, shared a great degree of similarity. As the concentration of TNT increased, additional bands became prominent. Sequencing and alignment of the emergent bands revealed the similarity to pseudomonads and a *Burkholderia* sp. The most dominant band arose from a 16 S rDNA sequence that was 100% identical to a *Pseudomonas putida*. The banding patterns of unplanted and bulk soil across the range of TNT concentrations were akin to the patterns observed after 30 days of amendment with TNT. The pre-planted soil had a comparable profile, although an additional, unidentified, band became prominent at high concentrations of TNT.

The banding patterns of the rhizosphere soil samples exhibited a less clear pattern across the range of TNT concentrations. Additional bands did appear as TNT concentrations increased, but the positions of the extra bands were not consistent across the TNT concentrations, nor indeed between replicate samples of soil. Pearson correlation (Figure 27) of the digitised gel lanes showed that samples contaminated with low concentrations of TNT (<50 mg/kg TNT) had a tendency to cluster together, indicating similarity. Soils contaminated with the higher concentrations of TNT were also observed to cluster together. There was no clear clustering of bands patterns from NR-expressing rhizosphere samples separate to untransformed wild type tobacco rhizosphere samples, indicating that the banding profiles were similar for each. A selection of the additional bands observed with increased TNT concentration was sequenced and the resultant sequences compared with databases. All of the emergent bands were most closely related to the *Bacteriodetes* bacterial division, which encompasses the genera *Cytophaga* and *Flavobacteria*.

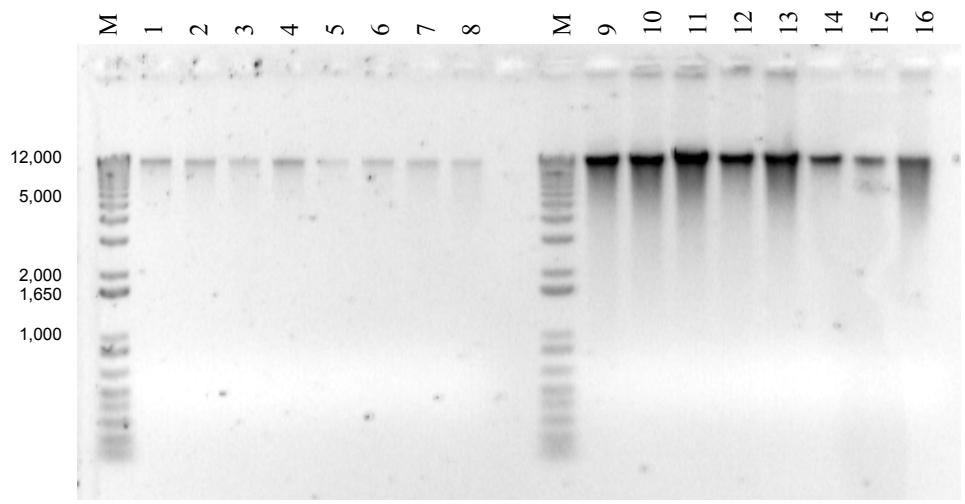


Figure 24. Agarose gel of community genomic DNA extracted from soil samples.
The marker (M) was the 1 kb plus ladder. Lanes 1-8 represent genomic DNA derived from pre-planted soil. Lanes 9-16 represent genomic DNA derived from NR-expressing tobacco rhizosphere soil. Lanes 1 and 9: no amendment; Lanes 2 and 10: 0 mg/kg; Lanes 3 and 11: 25 mg/kg; Lanes 4 and 12: 50 mg/kg; Lanes 5 and 13: 150 mg/kg; Lanes 6 and 14: 500 mg/kg; Lanes 7 and 15: 1500 mg/kg; Lanes 8 and 16: 4000 mg/kg.
PCR amplification of genomic DNA resulted in a PCR product of approximately 225 bp, as expected from examination of 16 S rDNA sequences.

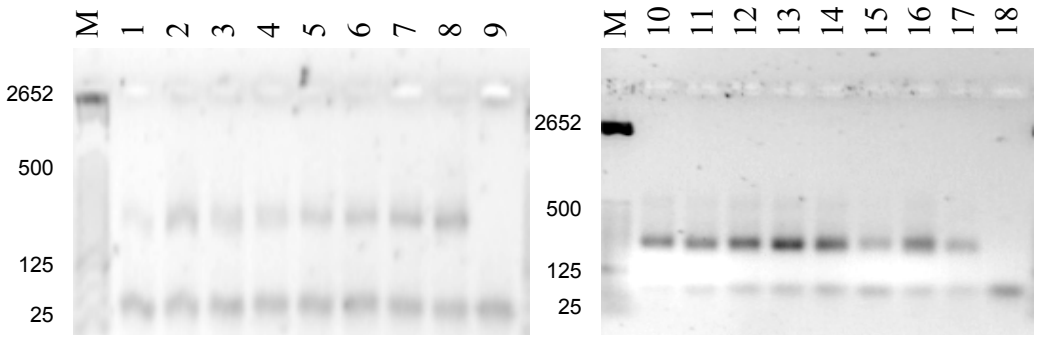


Figure 25. 16S rDNA PCR amplification products. The marker (M) was a 25 bp ladder. Lanes 1-8 represent PCR products derived from pre-planted soil. Lanes 10-17 represent PCR products derived from NR-expressing tobacco rhizosphere soil using 530R and GC338F primers and community DNA. Lanes 9 and 18 display the negative control of no template DNA. Lanes 1 and 10: no amendment; Lanes 2 and 11: 0 mg/kg; Lanes 3 and 12: 25 mg/kg; Lanes 4 and 13: 50 mg/kg; Lanes 5 and 14: 150 mg/kg; Lanes 6 and 15: 500 mg/kg; Lanes 7 and 16: 1500 mg/kg ; Lanes 8 and 17: 4000 mg/kg.

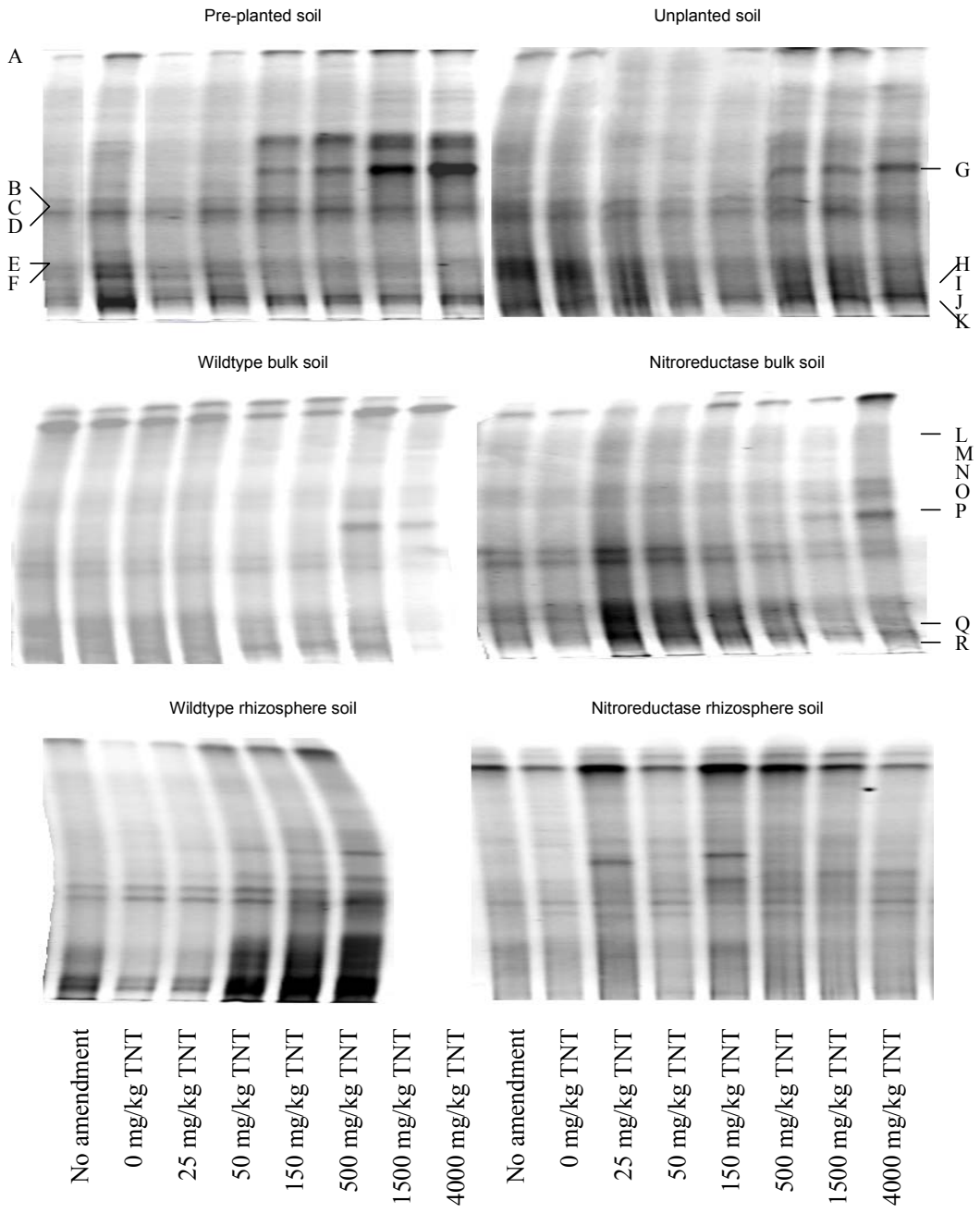


Figure 26. DGGE of 16S rDNA products TNT amended soil. Letters illustrate bands that have been sequenced and characterized.

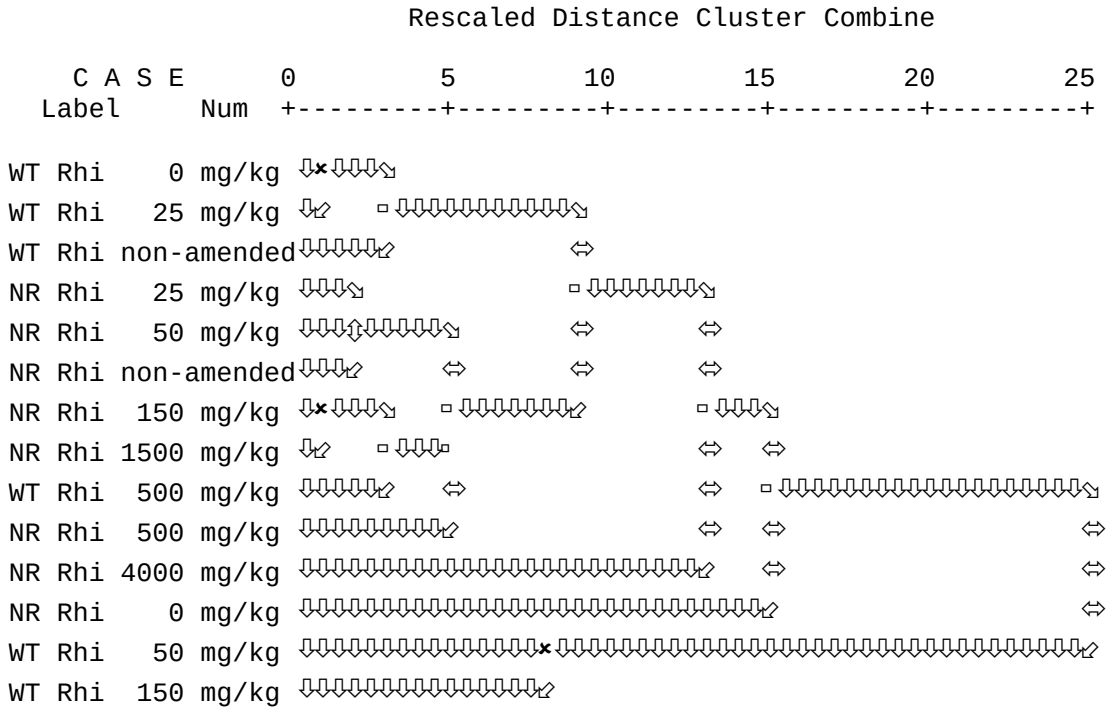


Figure 27. Pearson correlation of lanes from DGGE analysis. Samples from untransformed wild type (WT Rhi) and NR-expressing (NR Rhi) tobacco rhizosphere soil contaminated with a range of TNT concentrations

Cluster analysis was performed using average linkage between groups. A number of bands were excised from the DGGE gels and sequenced. The resultant, aligned sequences are displayed in Figure 28. These sequences were compared with databases of nucleic acid sequences and the most closely related bacteria determined, as shown in Table 9. A phylogenetic tree was constructed to illustrate the relatedness of the sequences, both to each other and to their most closely related sequences from the database (Figure 29).

```
A   1    CAATGGGCGAAAGCCTGATCCAGCCACGCCGCGTGAGTGATGAAGGCCTT-CGGGTTGTA 59
B   1    --ATGGGCGAAAGCCTGATCCAGCCACGCCGCGTGAGTGATGAAGGCCTT-CGGGTTGTA 57
C   1    CAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCTT-CGGGTTGTA 59
D   1    -AATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCTT-AGGGTTGTA 58
E   1    CAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCTT-AGGGTTGTA 59
F   1    CAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTT-CGGGTTGTA 59
G   1    CAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTT-CGGATTGTA 59
H   1    -AATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTT-CGGGTTGTA 58
I   1    -AATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTT-CGGATTGTA 58
J   1    -AATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTT-CGGATTGTA 58
K   1    -AATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTT-CGGATTGTA 58
L   1    --ATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGCAGGATGACGGTCCTATGGATTGTA 58
M   1    CAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGCAGGATGACGGTCCTATGGATTGTA 60
N   1    --ATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGCAGGATGACGGTCCTATGGATTGTA 58
O   1    --ATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGCAGGATGACGGTCCTATGGATTGTA 58
P   1    -AATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGCATGATGACGGTCCTATGGATTGTA 59
Q   1    ----GGGCGCAAGCCTGATCCAGCCATGCCGCGTGCAGGATGACGGTCCTATGGATTGTA 56
R   1    --ATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGCATGATGACGGTCCTATGGATTGTA 58

A   60   AAGCTCTTTCGGCCGGGACGAAAAGGCCCGGGCTAATATCCCGGGGTTGGGCCGGTCCCT 119
B   58   AAGCTCTTTCGGCCGGGAAGATAATGACGGTACCGGAA---------------------- 95
C   60   AAGCTCTTTTACCCGGGAAGATAA------------------------TGACGGTACCG 94
D   58   AAGCTCTTTTACCCGGGAAGATAA------------------------TGACGGTACCG 92
E   60   AAGCTCTTTTACCCGGGAAGAAAA------------------------TGACGGTACCG 94
F   60   AACCTCTTTCAGCAGGGACGAAGCGCACGGGACCGGTA------GAAC------------ 101
G   60   AAGCACTTTAAGTTGGGAGGAAGGGCAGTAAATTAATACTCTGCTGTTTTGACGTTACCG 119
H   59   AAGCACTTTTGTCCGGGAAGAAAACGCTCGGGTTAATACCCTGGGCGGATGACGGTACCG 118
I   59   AAGCACTTTTAGTTGGGAGGAAGGGTTGTAGATTAATACTCTGCAATTTTGACGTTACCG 118
J   59   AAGCACTTTTGTTTGGGAGGAAGGGTTGTGGATTAATACTCTGGGATTTTGACGTTACCG 118
K   59   AAGCACTTTAAGTTGGGAGGAAGGGTTGTAGATTAATACTCTGCAATTTTGACGTTACCG 118
L   59   AACTGCTTTTRTACGAGAAGAAACACTCCTTCGTG-A-----AGGATCTTGACGGTATCG 112
M   61   AACTGCTTTTGTACGGGAAGAAACAATCCTACGTG-T-----AGAGTCTTGACGGTACTG 114
N   59   AACTGCTTTTGTACGARAAGAAACACTGCTTCGTG-A-----AGCAGCTTGACGGNARCG 112
O   59   AACTGCTTTTGTACGGGAAGAAACACTGCTTCTTG-T-----AGGATCTTGACGGT-CCG 111
P   60   AACTGCTTTTGTACGAGAAGAAACACTGCTTCGTG-A-----AGCAGCTTGACGGTATCG 113
Q   57   AACTGCTTTTGCACRRGAAGAAACACCGCTACGTG-T-----AGCGTCTTGACGGTACTG 110
R   59   AACTGCTTTTGCACGGGAAGAAACACCGCTACGTG-T-----AGCGTCTTGACGGTACTG 112

A   120  GAAGAAGAAGCCCGGGCTAACTCTGTGCCAGCAGCCGCGGTAATAC 165
B   95   ---GAATAAGCCCCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC 138
C   95   GAACAATAAGCCCCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC 140
D   93   GGAGAATAAGCCCCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC 138
E   95   GGAGAATAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATAC 140
F   101  --AGAAGAAGC--AGGGTAAGTCCCTGCCAGCAGCCGCGGTAATAC 139
G   120  ACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATAC 165
H   119  GAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATAC 164
I   119  ACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATAC 164
J   119  ACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATAC 164
K   119  ACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATAC 164
L   113  TAAGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC 158
M   115  TAAGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC 160
N   113  TAAGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC 158
O   112  TAAGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC 157
P   114  TAAGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC 159
Q   111  TGAGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC 156
R   113  TGAGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC 158
```

Figure 28. Sequence alignment of excised and sequenced bands from DGGE gels
Consensus bases are indicated by colored blocks. G=guanine; C=cytosine; A=adenine; T=thiamine.

Table 9. Bacterial species with related sequences identified from DGGE bands
In the case of the most closely related sequence being an uncultured bacterium, then the most closely related cultured bacterium are also noted.

Band	Most closely related bacterial sequence	% Identity	Accession no.	Reference
A	Uncultured *Xanthomonas* sp.	89.1	AF467301	[54]
	Azoarcus sp.	86.7	AF011344	[55]
B	*Bradyrhizobium* sp.	95.6	AF352563	[56]
C	Uncultured bacterium clone	97.1	AF358023	[34]
	Sphingomonas sp. ATCC 35159	96.4	AF503283	[57]
D	Uncultured bacterium	99.3	AJ289986	[58]
	Sphingomonas sp.	98.5	AJ313019	[59]
E	Uncultured bacterium clone	99.3	AF358023	[34]
	Methylocapsa acidiphila	97.9	AJ278726	[60]
F	*Mycobacterium rhodesiae*	86.3	AY162027	[61]
G	*Pseudomonas putida*	100.0	D85995	[62]
H	*Burkholderia* sp.	98.2	AJ011508	[63]
I	*Pseudomonas* sp.	99.4	AY151820	[64]
J	Arctic sea ice bacterium	97.4	AF468447	[65]
	Pseudomonas sp.	96.3	AY151820	[64]
K	*Pseudomonas* sp.	100.0	AY151820	[64]
L	*Cytophaga* sp.	98.7	AF427162	[66]
M	Uncultured *Flavobacteriaceae* bacterium	98.1	AF388899	[67]
	Flavobacterium limicola	97.5	AB075232	[68]
N	*Flavobacterium* sp.	98.1	AF493659	[69]
O	Uncultured soil bacterium	93.6	AB080334	[70]
	Flavobacterium sp.	92.4	AF493659	[69]
P	*Flavobacterium* sp.	99.4	AF493659	[69]
Q	*Flavobacterium* sp.	98.1	AF493665	[69]
R	Flavobacterium sp.	98.1	AF493665	[69]

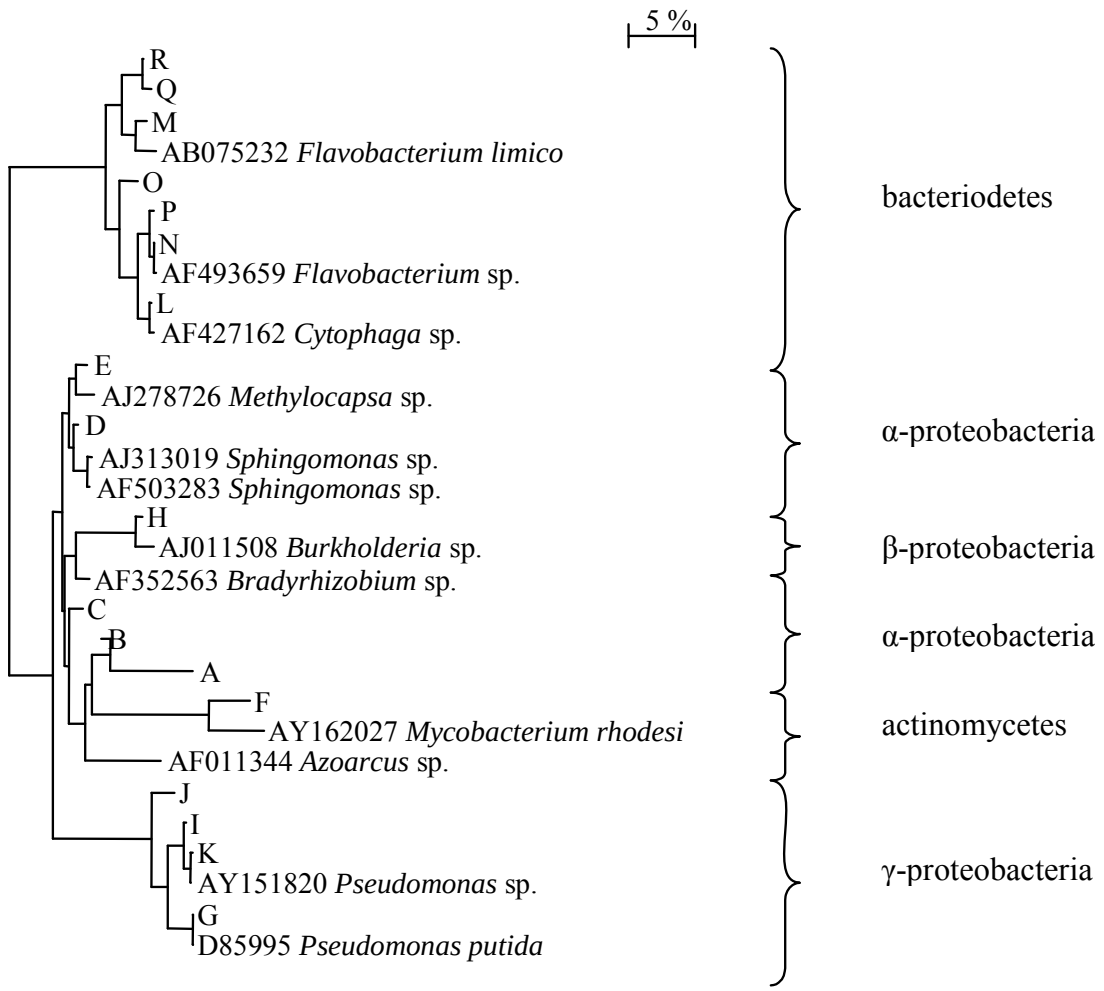


Figure 29. Phylogenetic tree of DGGE-related sequences
Bacterial divisions are indicated. The scale bar indicates a change of 5 %.

Characterization of XplA expressing Arabidopsis

We have engineered Arabidopsis plants expressing *xplA*, under the control of the 35S constitutive promoter and ocs terminator in the binary vector pMLBart-*xplA*. This vector also contains the selectable marker gene bar, which confers resistance to the herbicide Basta. We identified fifty, independently transformed T1 transgenic lines, then and selected five, T3 homozygous lines for further characterization: Lines 1, 6, 7, 10 and 12. Northern analysis shows that all five lines strongly express *xplA* transcript, with line 6 showing the highest level of expression (Figure 30A). We have produced an antibody to purified XplA and Western blot analysis (Figure 30B) shows that XplA is expressed.

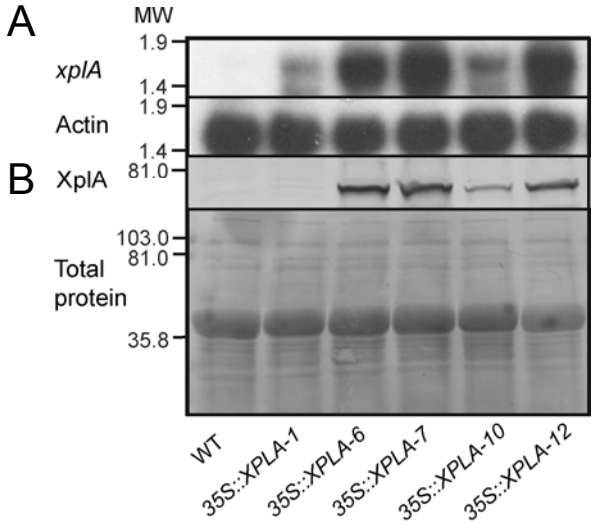


Figure 30. Transcript expression of *xplA* and XplA protein levels. A) Northern blot analysis of *xplA* expression and the constitutively expressed acting gene (ACT2, NCBI accession no. U41998). MW, molecular weight markers in kb. B) Western blot analysis showing levels of XplA and immunoblot membrane stained with Ponceau-S. MW in kDa. RNA and protein from Arabidopsis rosette leaves. WT, untransformed wild type.

Axenic liquid cultures were performed to determine rates of RDX uptake, using ten-day-old plants dosed with 180 μM (40 mg/l) RDX. After five days, the level of RDX in the untransformed wild type flasks was reduced by only 19 %, while the *35S-xplA*-6 and 10 lines had removed all the RDX and lines 1, 7 and 12 reduced the levels of RDX by 32, 42 and 58 % respectively (Figure 31). In the flasks dosed with 100 μM RDX, the rates of uptake were similar to those at 180 μM RDX, indicating that even at the solubility limits of RDX, the maximum exposure levels likely to be encountered in contaminated soils, the rate of RDX uptake in the transgenic lines is not reduced.

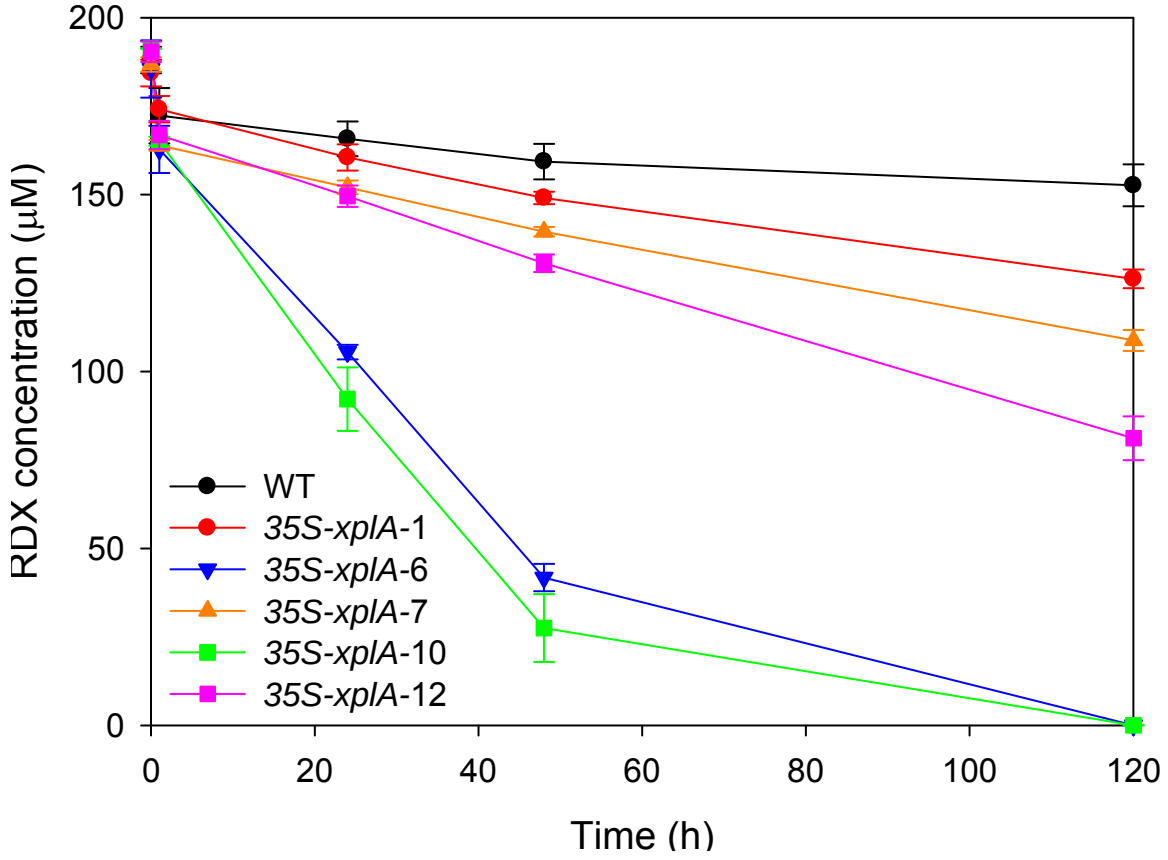


Figure 31. Uptake of RDX from liquid medium by Arabidopsis seedlings.
Results are means ± s.e.m. of five replicate measurements.

To detect RDX degradation metabolites, we have developed methods to measure formaldehyde and nitrite in the media. However, we have been unable to detect significant levels of either formaldehyde or nitrite in the media from any of our liquid culture experiments. It is likely that the plant's endogenous enzymes, formaldehyde dehydrogenase and nitrite reductase, are metabolizing both formaldehyde and nitrite. We were also unable to follow XplA activity in assays on crude plant extracts monitoring both the decrease in RDX using HPLC, and the RDX-specific oxidation of NADH spectrophotometrically at A_{340}. This may be due to the instability of the XplA protein.

Characterization of Arabidopsis expressing 3A4

We have engineered Arabidopsis plants expressing the human cytochrome P450 gene, *3A4*, complete with an ER targeting signal (ER-*3A4*). Eight homozygous T3 lines were produced and 3A4 expression confirmed by RT-PCR. Liquid culture experiments performed to monitor rates of RDX uptake. After seven days, the control, *35S-xplA*-10 line plants had removed 90 % of the RDX; however, there was no difference in the uptake rates of any of the ER-*3A4* lines when compared to untransformed wild type plants and subsequent studies were focused on the *35S-xplA* lines.

Arabidopsis expressing NR

The *nfsI* gene was transformed into Arabidopsis and five, independently transformed, T3 homozygous lines exhibiting tolerance to TNT, as judged by root lengths on media containing TNT, selected (Figure 32). To determine the optimum TNT concentration to use in our liquid culture experiments, the line exhibiting the highest tolerance towards TNT was further studied in liquid culture with three concentrations of TNT; 100, 250 and 500 µM (Figure 33). At 100 µM TNT, both untransformed wild type and NR lines remained green, appeared healthy and removed all the TNT from the media within three days. At 250 µM TNT, the NR lines remained healthy and removed all the TNT from the media whilst the untransformed wild type plants became brown and necrotic, and failed to remove all the TNT from the media. At 500 µM TNT both untransformed wild type and NR plants exhibited phototoxic symptoms. We concluded that 250 µM TNT is the optimal TNT concentration for future liquid culture experiments.

.

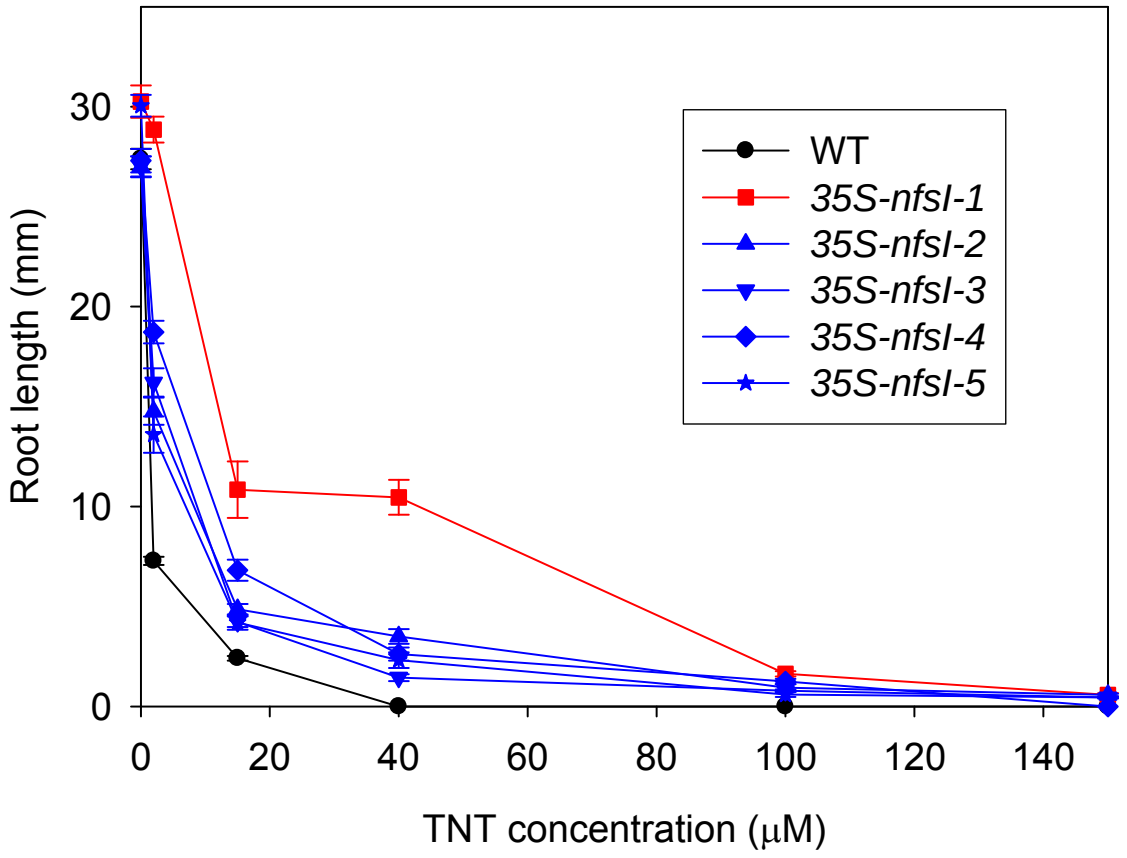


Figure 32. Root lengths of NR expressing Arabidopsis grown on TNT
Seedlings were grown for ten days on agar plates containing ½ MS and increasing concentrations of TNT. WT, untransformed wild type seedlings.

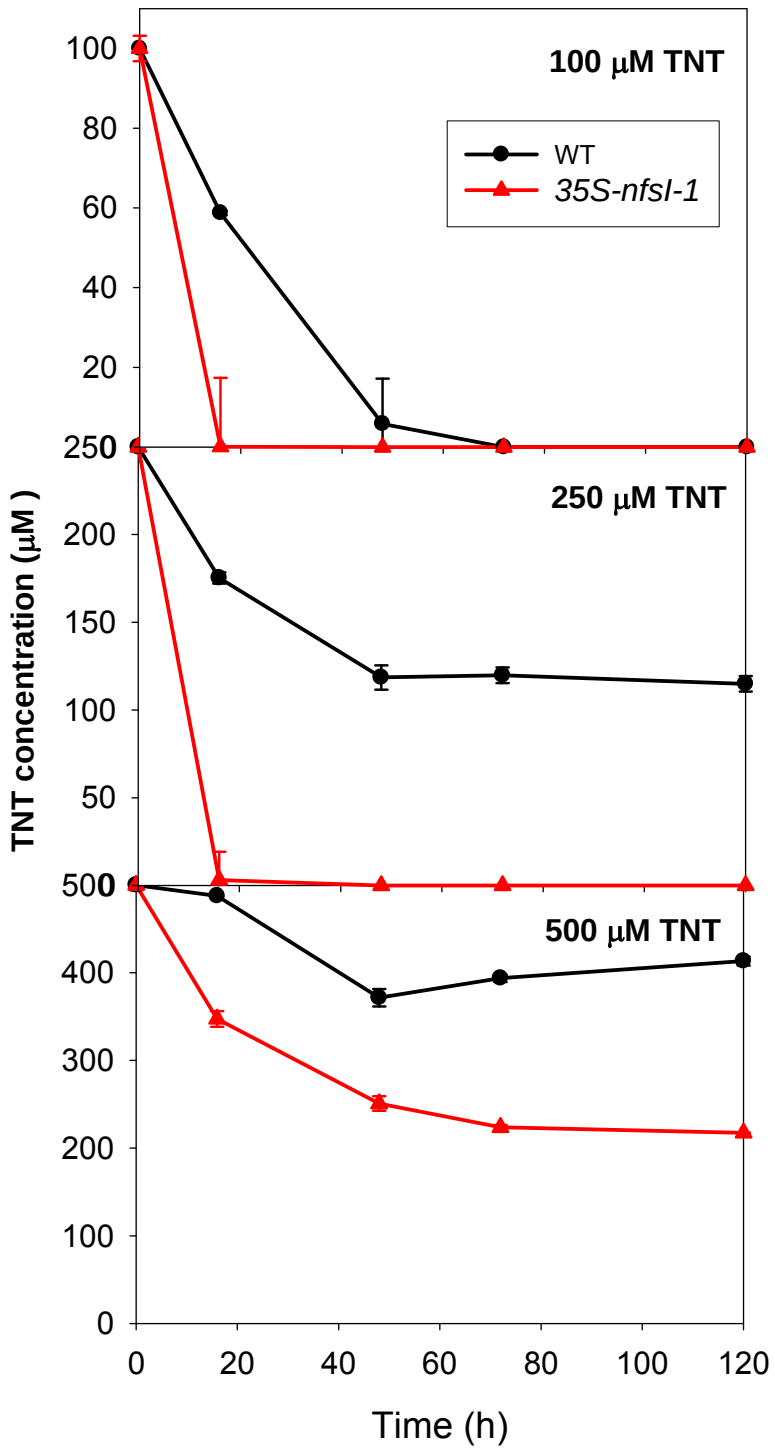


Figure 33. TNT Uptake from liquid culture by NR expressing Arabidopsis
WT, untransformed wild type. Results are means ± s.e.m. of five replicate measurements.

Arabidopsis lines expressing XplA and NR

The most resistant line, *35S-nfsI*-1, was crossed with lines *35S-xplA*-6 and 10 to produce transgenic lines expressing both XplA and NR activities. The segregation ratios of seed from heterozygous *35S-nfsI*-1, *35S-xplA*-6 and 10 were all 3:1 for resistance to kanamycin or Basta, respectively. This is consistent with single insertion events for these lines

The presence of both *xplA* and *nfsI* transgenes in the T1 progeny was verified using PCR and homozygous, T3 *35S-xplA-nfsI* lines identified. These lines show 100 % resistance to Basta herbicide, conferred by the selectable marker linked with *xplA* and 100 % resistance to kanamycin, conferred by the selectable marker gene linked to *nfsI*. Liquid culture experiments on these lines were performed using 180 μM RDX and 250 μM TNT (Figure 34A). The results showed that the *35S-xplA-nfsI* line was able to take up RDX from the media, removing nearly all 180 μM RDX after seven days. Both *35S-xplA-nfsI* and the *35S-nfsI* lines removed all of the TNT from the media within three days, whereas the untransformed wild type and *35S-xplA* lines removed only 125 μM of TNT in the first three days and thereafter no more TNT was removed from the media (Figure 34B). After 10 days, untransformed wild type and *35S-xplA* lines were brown and necrotic, while the *35S-xplA-nfsI* and the *35S-nfsI* lines remained green and appeared healthy (Figure 34C).

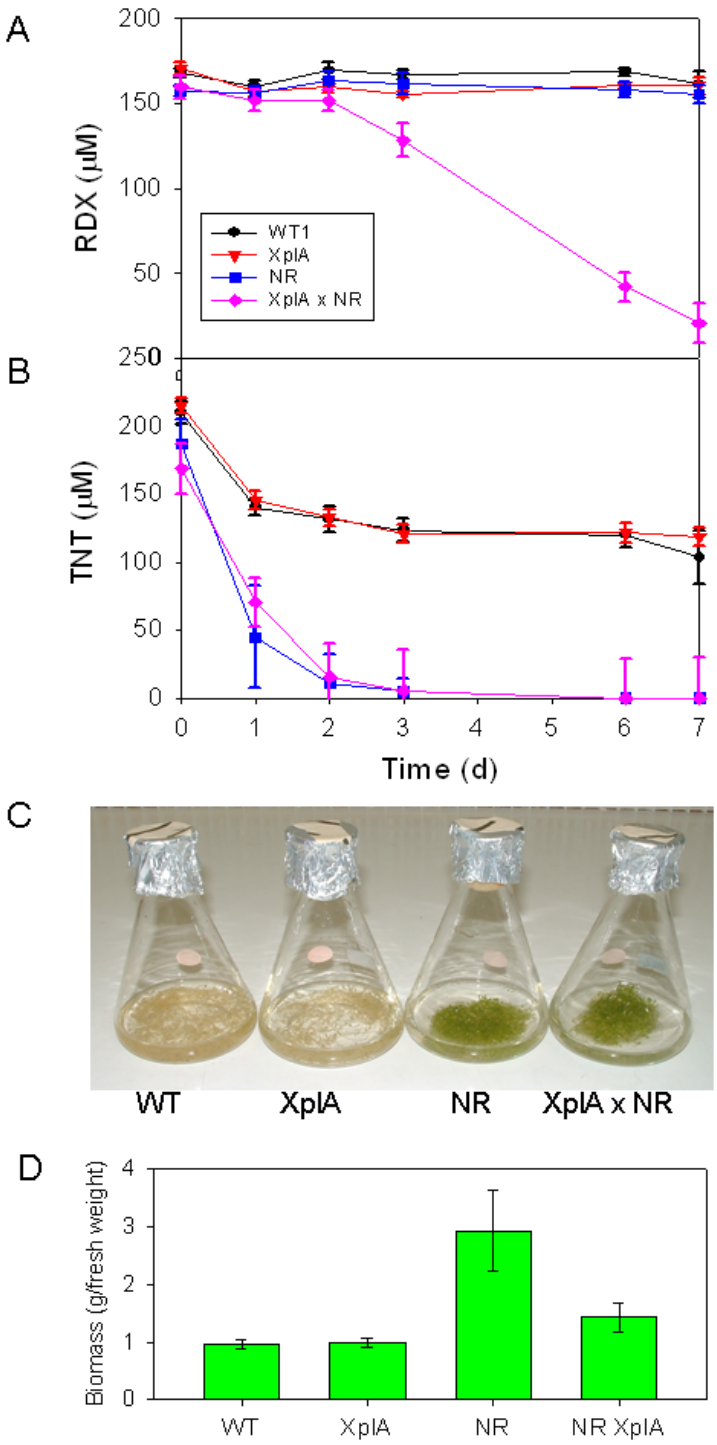


Figure 34 Uptake of RDX and TNT by Arabidopsis expressing both XplA and NR from liquid culture. Ten-day-old seedlings were dosed with 180 □M RDX, 250 □M TNT. A) Level of RDX in media, B) Level of TNT in the media, C) Appearance of the seedlings ten days after dosing. D) Biomass of plants after ten days WT, untransformed wild type. Results are means □ s.e.m. of five replicate measurements.

Prior to any RDX removal from the media by the *35S-xplA-nfsI* line (Figure 34A), there was a two-day lag; this corresponded to the time taken by the *35S-xplA-nfsI* line to remove all the TNT from the media. After ten days, the NR plants were still green and healthy, but some of the *35S-xplA-nfsI* plants showed symptoms of phytotoxicity, with lower biomasses than the NR lines (Figure 34). We interpreted this result to indicate that the uptake or degradation of RDX is inhibited in the presence of TNT. Thus we repeated this experiment dosing with 180 µM RDX only, to investigate whether the *35S-xplA-nfsI* lines could take up RDX at the same rate as the *35S-xplA* parental line (Figure 35). The results showed that in the absence of TNT there is no lag in the rate of RDX removal from the media by the *35S-xplA-nfsI* line, and these plants remove RDX from the media at rates similar to *35S-xplA* plants. We concluded that the lag seen in RDX uptake was due to the presence of TNT.

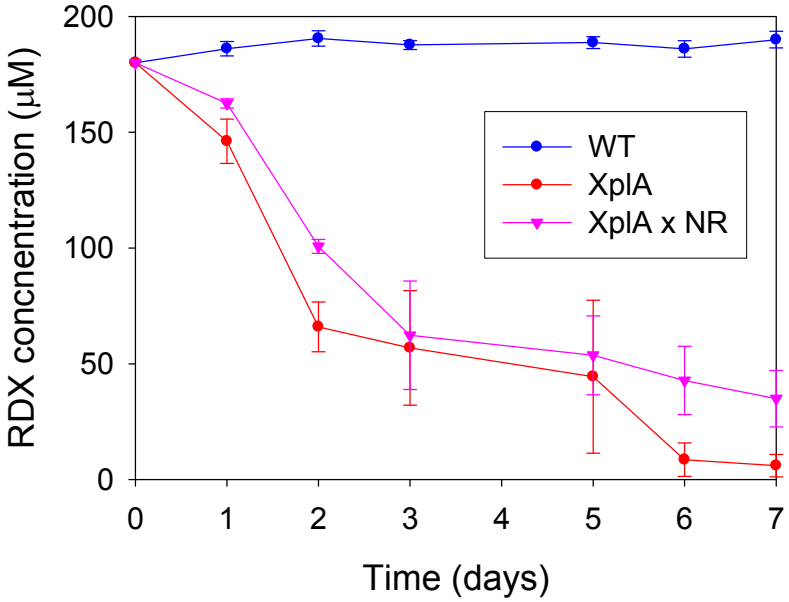


Figure 35. Uptake of RDX by Arabidopsis expressing both XplA and NR from liquid culture Ten-day-old seedlings were doused with 180 µM RDX. WT, untransformed wild type. Results are means ± s.e.m. of five replicate measurements.

Inhibition of XplA by TNT

Liquid culture studies were performed on the *35S-xplA-nfsI* line to measure RDX uptake in the presence of increasing amounts of TNT. The results (Figure 36) showed that a two-day lag in RDX uptake was present at the lowest (75 µM) TNT concentration, and that this delay increased with increasing TNT concentration. There was a corresponding decrease in rate of RDX uptake. While the plants remained green and appeared healthy at the 0- 150 µM TNT, at the two highest concentrations of TNT (175 and 250 µM), the plants expressing both XplA and NR activities were bleached and necrotic after ten days.

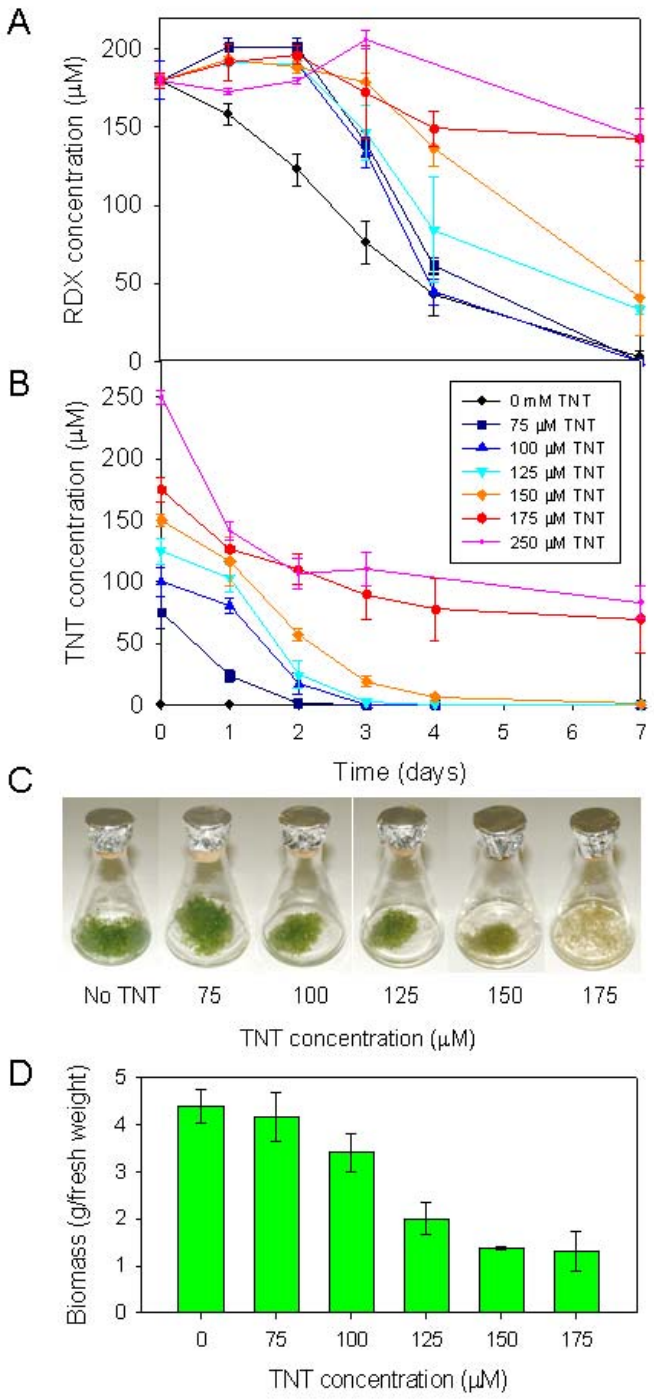


Figure 36. Uptake of RDX and increasing concentrations of TNT from liquid culture by Arabidopsis expressing both XplA and NR. A) Uptake of TNT and B) RDX by *35S-xplA-nfsI* R lines dosed with 180 M RDX and increasing concentrations of TNT. C) Appearance of plants, and D) Biomass ten days after dosing. WT, untransformed wild type. Results are means ± s.e.m. of five replicate measurements.

The activity of purified NR is not altered by the presence of RDX, thus we investigated the effect of TNT on purified XplA protein, measuring RDX degradation with increasing TNT concentration. The results presented in Figure 37 show that XplA activity is inhibited by the presence of TNT, and at levels comparable to those tested in the liquid culture experiments presented in Figure 36.

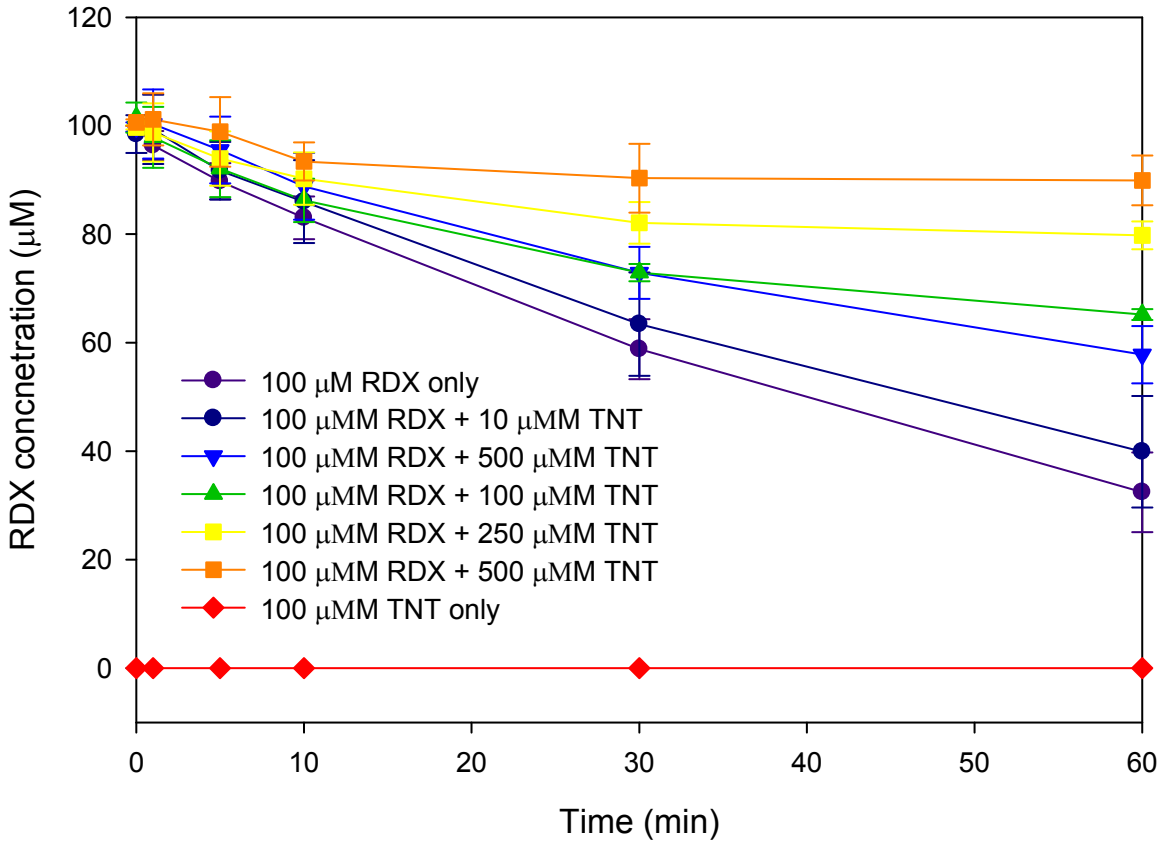


Figure 37. Inhibition of purified XplA activity by TNT

Soil studies on XplA expressing Arabidopsis

We conducted soil studies to compare plant growth and RDX uptake in untransformed wild type with *35S-xplA*-10 plants grown in RDX-contaminated soil. Plants were grown for eight weeks in John Innes No1 soil contaminated with 50, 250, 500 and 2000 mg/kg RDX. At the end of the growth period, the *35S-xplA* plants were significantly larger than the untransformed wild type grown on RDX-contaminated soil (Figure 38A). The untransformed wild type plants had less shoot and root biomass (Figure 38B) and up to three times more RDX in the shoot tissue (Figure 38C). Interestingly, the phytotoxic effects of RDX were not more severe beyond 250 mg/kg, suggesting that the toxicity is limited by the rate of RDX uptake.

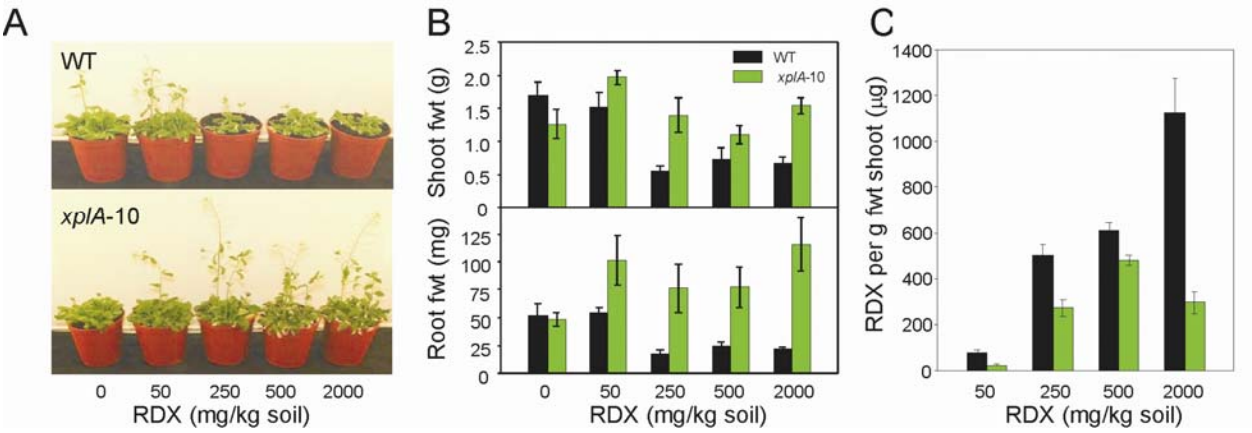


Figure 38 Arabidopsis expressing XplA grown on RDX-contaminated soil
A) Appearance of plants after eight weeks growing on RDX contaminated soil, B) Shoot and root biomass, C) Levels of RDX in the shoots of untransformed wild type and Arabidopsis plants. WT, untransformed wild type. Results are means ± s.e.m. of five replicate measurements.

The RDX levels in the soil were measured and although some RDX had been removed, these levels of removal were low. We do not consider this surprising since the root biomass to soil ratio was low (600: 1 soil: root). To overcome this, we modified our experimental design by increasing plant density (to 10 plants per pot), increasing pot diameter and reducing pot volume. These conditions were used in combination with: a) a closed pot system using pots without drainage holes to increase exposure time of the roots to RDX and b) a run-through system using plants grown in uncontaminated soil to high biomass in pots with drainage holes. RDX was then watered in and the levels of RDX in the soil leachate monitored in the following days.

Wild type and *35S-xplA* plants were grown for eight weeks in pots without drainage holes. The results, presented in Figure 39, show that soil in which the *35S-xplA* plants were grown contained between 7.2-fold and 2.4-fold less RDX than soil in which untransformed wild type plants had been grown. In our first soil experiment, phytotoxic effects were seen in the untransformed wild type plants grown in soil containing upwards of 250 mg/kg RDX. We did not see any phytotoxic effects in this experiment and presume this is because the increased biomass to soil ratio enabled both the untransformed wild type and *35S-xplA* plants to reduce the RDX to below phytotoxic levels. The experimental parameters: high plant density, low soil volume and lack of drainage holes resulted in high fluctuations in soil water content, and the plants displayed high levels of anthocyanins and bolted prematurely, both signs of environmental stress. We conclude that these particular experimental conditions may be too harsh for subsequent soil studies.

Soil leachate system

Plants were grown for four weeks in uncontaminated soil in pots with drainage holes. After this time, a 180 µM RDX solution was applied to each pot and allowed to drain through. The concentration of the RDX was measured in the soil leachate. After seven days, each pot was dosed with 50 mls of water and the RDX in the subsequent soil leachate measured. Figure 39 shows that, although initially the level of RDX in the soil leachate was similar from all pots, after seven days, the level of RDX in the soil leachate from the pots containing the *35S-xplA* plants was 2.4 fold lower than from pots containing untransformed wild type or no plants.

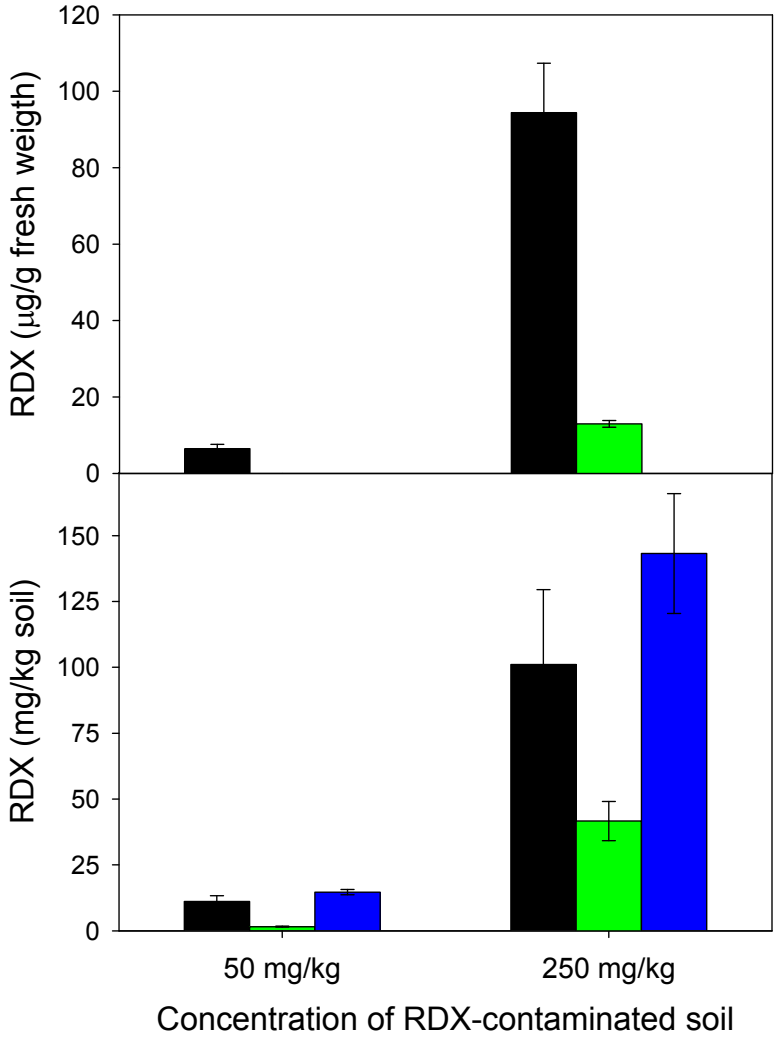


Figure 39. Removal of RDX from contaminated soil by XplA expressing Arabidopsis
A) Levels of RDX in plant shoots, B) levels of RDX in the soil, after eight weeks growth. NPC, no plant control. WT, untransformed wild type; NPC, no plant control.

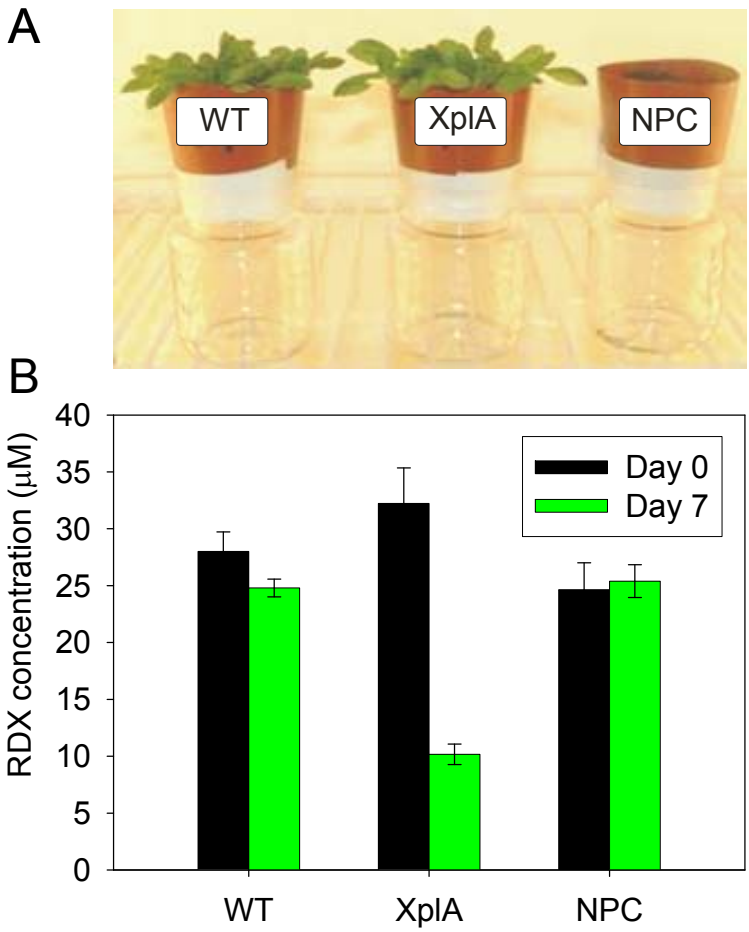


Figure 40. Levels of RDX in soil leachate from Arabidopsis expressing XplA
A) Pots containing five week old untransformed wild type and *35S-xplA* plants were flushed with 180 µM RDX, then after seven days, the pots were flushed with water and B) the level of RDX in the soil leachate measured using HPLC. WT, untransformed wild type; NPC, no plant control.

The first soil experiment (shown in Figure 38) was repeated using all five *35S-xplA* lines in soil contaminated with 2000 mg/kg RDX to investigate if the resistance to RDX correlates with the rates of RDX uptake from liquid media. Figure 41 shows that untransformed wild type and *35S-xplA-1* root biomasses decreased when grown in soil containing RDX. Both untransformed wild type and line 1 had curled leaves with necrotic edges, symptoms of RDX toxicity; these were not observed in the other transgenic lines. Line 1 also exhibited the lowest levels of transgene expression and RDX uptake in liquid culture. Interestingly, the root biomasses of lines

6, 7, 10 and 12 grown in soil containing 2000 mg/kg RDX were all significantly higher than without RDX. This suggests that these lines are utilizing the RDX for growth.

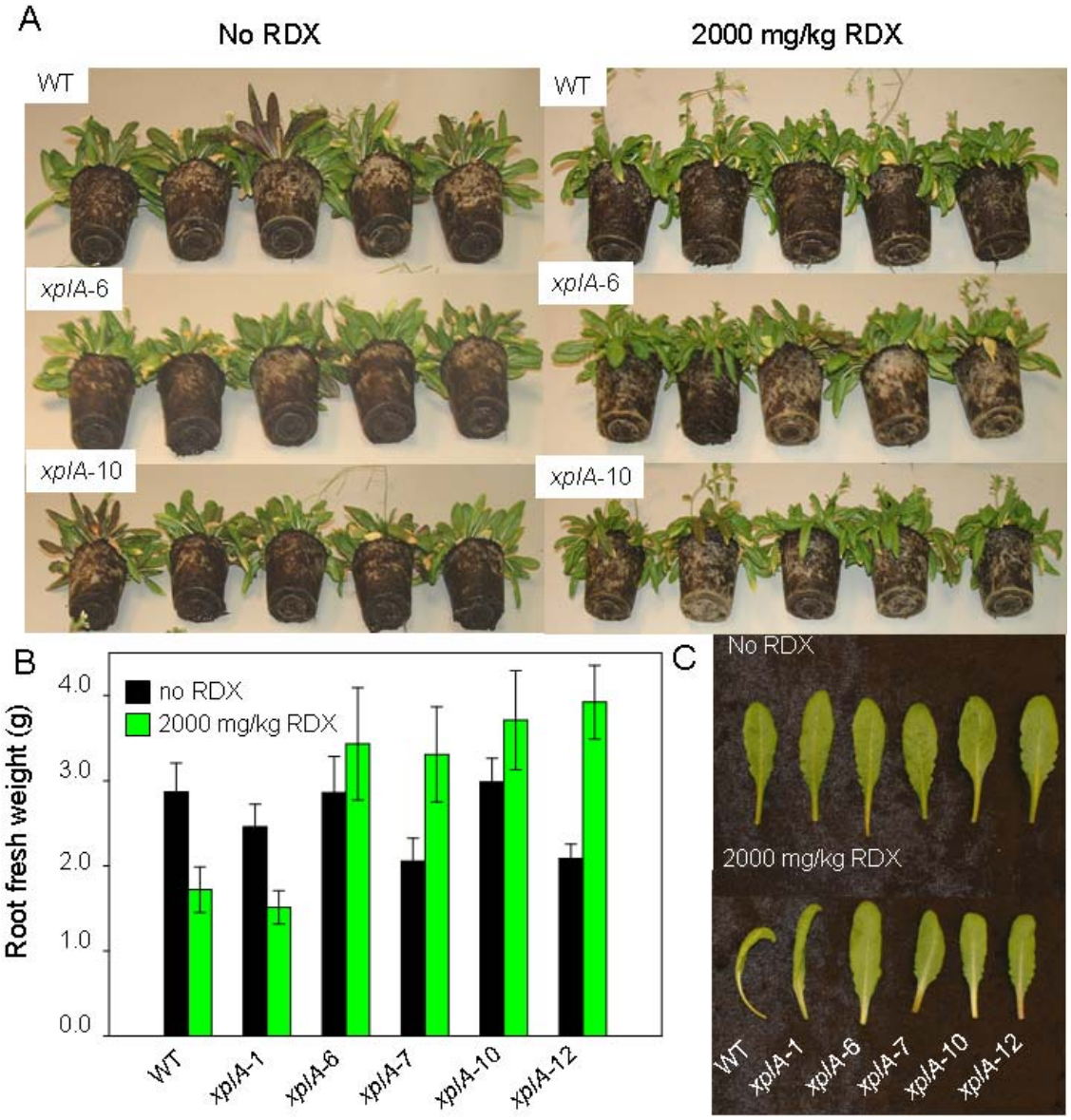


Figure 41. Growth of XplA expressing Arabidopsis lines on RDX contaminated soil
A) Appearance of roots, B) Fresh weight of roots, and curled leaf phonotype of untransformed wild type after eight weeks growth in soil containing 2000 mg/kg RDX.

Soil studies on NR expressing Arabidopsis

TNT is more phytotoxic than RDX, and TNT is particularly toxic to seedlings and young plants, so we performed experiments to determine the tolerance of our Arabidopsis NR lines in TNT-contaminated soil. We used soil contaminated with 25, 50, 100 and 250 mg TNT /kg. After eight weeks, the NR lines developed green, vigorous growth at TNT concentrations up to 250 mg/kg, whilst the untransformed wild type plants showed signs of severe TNT toxicity at 50 mg/kg (Figure 42). We used these data to determine suitable concentration ranges for soil studies using TNT and RDX together to analyze our *35S-xplA-nfsI* lines.

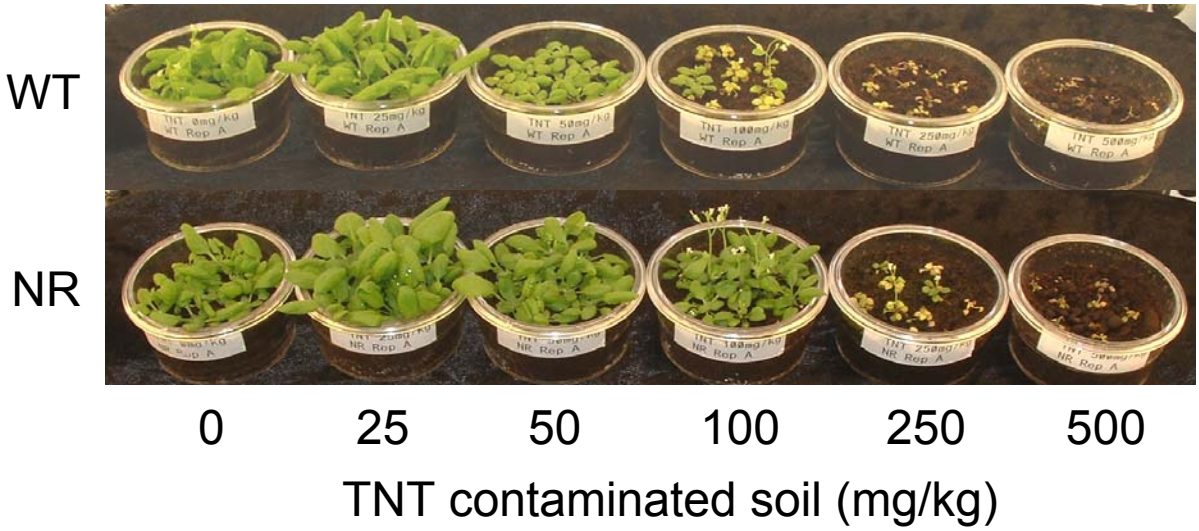


Figure 42. Growth of NR expressing Arabidopsis on TNT contaminated soil
Plants were five weeks old. WT. untransformed wild type; NR, *nfsI*-expressing Arabidopsis

Soil leachate studies on XplA and NR **Arabidopsis**

Following preliminary experiments to optimize measurement of RDX uptake from soil leachate by Arabidopsis, a full-scale experiment was performed to determine RDX uptake in untransformed wild type Arabidopsis alongside plants expressing XplA and NR. Figure 43 shows that the five 35S-xplA-expressing lines removed significantly more (53-80 %) RDX from the soil leachate than either untransformed wild type plants (30 %) or nfsI only-expressing plants (28 %). The *35S-xplA-nfsI* transgenic lines did not perform as well as expected, removing less (20-47 %) than the only-expressing lines. This was not predicted as these lines removed RDX from liquid culture at rates similar to the *35S-xplA*-expressing parental lines. We will use antibodies we have produced to both NR and XplA to check protein expression levels of both transgenes in these lines.

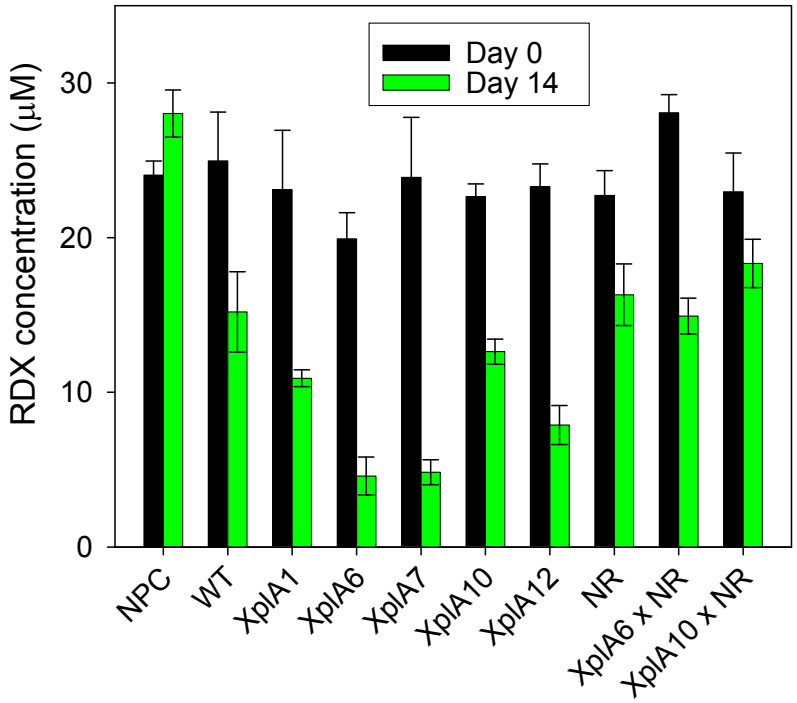


Figure 43. Levels of RDX in soil leachate from *35S-xplA-nfsI* Arabidopsis lines
Pots were flushed with 180 µM RDX (Day 0) then after 14 days, flushed with water (Day 14), and the level of RDX determined by HPLC. NPC, no plant control; WT, untranformed wild type.

Characterization of XplB expressing Arabidopsis.

The reductase, *xplB* from *Rhodococcus rhodochrous* 11Y was transformed into wild type Arabidopsis plants and into *35S-xplA*-10. Forty T1 transgenic plants containing *xplB* in the wild type background and 50 T1 plants in the *35S-xplA*-10 background were generated. The T2 *35S-xplA-xplB* seedlings were scored for resistance to kanamycin, conferred by the selectable marker gene for the *xplB* construct. Forty plants with segregation ratios indicative of single T-DNA insert were identified and five homozygous T3 lines selected for further analysis.

Liquid culture analysis of Arabidopsis expressing XplA and XplB

To investigate if the addition of XplB activity can increase the rate of RDX degradation in *35S-xplA* Arabidopsis, we transformed *35S-xplA*-10, our fastest RDX-degrading line with *xplB* and characterized five lines independent for XplB. Expression of both transgenes was confirmed using RT-PCR (results not shown). The results presented in Figure 44 show that, as expected, RDX levels did not decrease significantly in the flasks containing the untransformed wild type plants while the *35S-xplA*-10 plants removed all the RDX from the media within four days. The rate of RDX uptake in the *35S-xplB* lines only was not significantly different from untransformed wild type plants, while the five lines expressing *xplA* and *xplB* together had significantly increased rates of RDX uptake; all five lines removing all RDX within one day, lines *35S-xplA-xplB*-2 and 27 removing 50 % of the RDX within four hours.

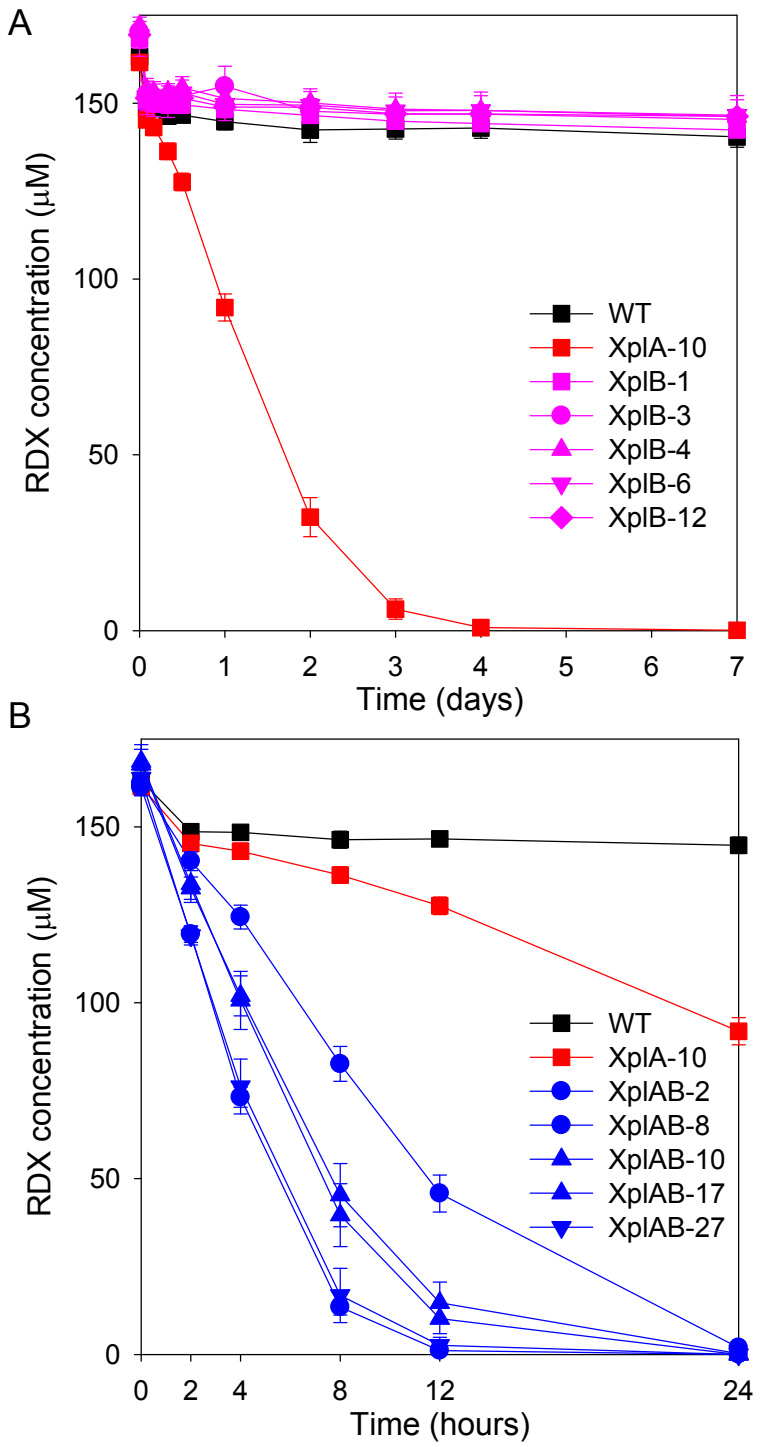


Figure 44. RDX uptake from liquid culture by Arabidopsis expressing XplA and NR A) *35S-xplB* expressing lines, B) *35S-xplA-xplB*-expressing lines. Ten-day-old seedlings were dosed with 180 µM RDX. WT, untransformed wild type. Results are means ± s.e.m. of five replicate measurements.

Production of 3A4, CPR, XplA and NR expressing Aspen

Agrobacterium rhizogenes transformation was used to introduce the transgenes *CYP3A4*, *Cpr1*, *xplA* and *nfsI* into the aspen hybrid, *P. tremula* x *P. alba* (INRA 717-1B4). Co-transformation was also used to generate lines transformed with combinations of these four transgenes. CYP3A4 was chosen because there was some indication that this enzyme had activity on RDX (Bruce, unpublished). CPR1 (provided by Carl Douglas), an oxidoreductase gene from poplar, was included to improve the activity of P450 3A4. *XplA* and NfsI were provided by Neil Bruce. A summary of the transgenic lines and the genes transformed into them is presented in Table 10. Several of the lines had been successfully transformed with multiple transgenes.

Characterization of transgenic aspen expressing *3A4, cpr, xplA* and/or *nfsI*

As an initial screen for expression of the transgenes, aspen lines transformed with *nfsI* were plated on TNT-containing medium and the lines transformed with *xplA* were plated on RDX-containing medium. First, non-transgenic roots were screened on a range of concentrations of TNT and RDX to determine the toxic levels for poplar. Concentrations of 0.02 mM of TNT and 0.318 mM of RDX were chosen because non-transgenic controls were unable to grow at these levels. The initial sizes of the roots were indicated on the plates, and growth was monitored over time. As shown in Table 10, lines transformed with *xplA* or CYP3A4 had improved growth on RDX. Some of the lines transformed with *nfsI* had increased tolerance to TNT.

Table 10. Characterization of transgenic hybrid aspen lines

Hybrid aspen lines, P. tremula x P. alba (INRA 717-1B4) transformed with 3A4, cpr, xplA and nfsI. Transgene presence was determined using PCR. Growth was measured after 2 weeks.

ROOT LINE	GENES	Growth on TNT (0.02 mM)	Growth on RDX (70µg/ml; 0.318 mM)
Vector control-Line 1	*xplA*	0	0
Line 6	*xplA,cpr,nfsI*	1.5 cm	
Line H	*3A4, cpr,nfsI*	0	2.7 cm
Line C	*nfsI*	0.6 cm	
Line F	*3A4, cpr,nfsI*	1.1 cm	1 cm
Line A8	*3A4, cpr*		2.5 cm
Line 9	*xplA*		2.8 cm
Line 10	*xplA*		2.4 cm
Line 5	*xplA*		2.0 cm
Line P	*cpr*		0.8 cm
Line A10	*cpr*		1.8 cm
Line L	*xplA*		++

RDX removal by best aspen root lines in nitrogen free media

Transgenic lines with good growth on RDX plates were selected for further analysis. Root samples were placed in VOA vials and dosed with nitrogen-free medium containing RDX as the sole nitrogen source. RDX uptake was analyzed in these lines, and is presented in Table 11.

Table 10. Uptake of RDX by transgenic hybrid aspen lines, *P. tremula* x *P. alba* (INRA 717-1B4) transformed with *3A4*, *cpr, xplA* and *nfsI*.

Root line	**Transgenes**	**% RDX Removal (40µg/ml; 0.181 mM)**
Media Control	-	0.26
Vector Control Line 1	-	4.8
Vector Control Line 7	-	3.1
Line 6	*xplA, cpr,nfsI*	16.4
Line H	*3A4, cpr,nfsI*	6.1
Line F	*3A4, cpr,nfsI*	13.1
Line A8	*3A4, cpr*	0
Line 10	*xplA*	68.6 (t21d)
Line 5	*xplA*	0
Line A 10	*Cpr*	9.6
Line L	*xplA*	74 (t21d)
Line R	*3A4, cpr,nfsI*	5.6

TNT and RDX uptake by transgenic aspen in nitrogen free media

Cuttings of Vector control Line 1, Line 6 and Line H were transferred in triplicates into vials containing TNT (0.05 mM) and RDX (40µg/ml; 0.181 mM) in nitrogen free media and allowed to grow. Time 0, 2d, 9d and 16d samples were taken and analyzed by HPLC. Media control blanks were also maintained.

Table 11. Uptake of TNT and RDX from hydroponic nitrogen free media by transgenic aspen. Tall cuttings were grown in vials containing TNT(0.05 mM) and RDX(40µg/ml; 0.181 mM) in nitrogen free media and allowed to grow. Experiment was performed in triplicate.

Plant	Genes	2d		9d	
		% TNT Removal	% RDX Removal	% TNT Removal	% RDX Removal
Media Control	-	0	0	0	0
Vector Control Line 1	-	47.6	0	100	0
Line 6	*3A4, cpr,nfsI*	50.2	0	100	0
Line H	*3A4, cpr,nfsI*	64.1	0	100	0

The TNT was removed completely by both the transgenic lines, and the vector control line by day 9. No decrease in RDX by any line was observed. It was later determined that RDX removal does not occur until after TNT is removed.

Experiments were carried out to measure the rate of RDX uptake by rooted cuttings of line 10 and line L in hydroponic conditions. Plants of line 10 grew more vigorously than Line L, so RDX uptake experiments were performed with only Line 10 compared with vector control plants. The plants appeared healthy prior to exposure to 25 ppm (113 mM) RDX, and the concentration of RDX was monitored over 10 days. Rooted Line 10 aspen removed an average of 43% of the RDX compared with only 10% by the vector control plants (Figure 47). There was no removal of RDX from the unplanted vials. The difference in RDX removal between the xplA-expressing line 10 and the vector control was seen after two days.

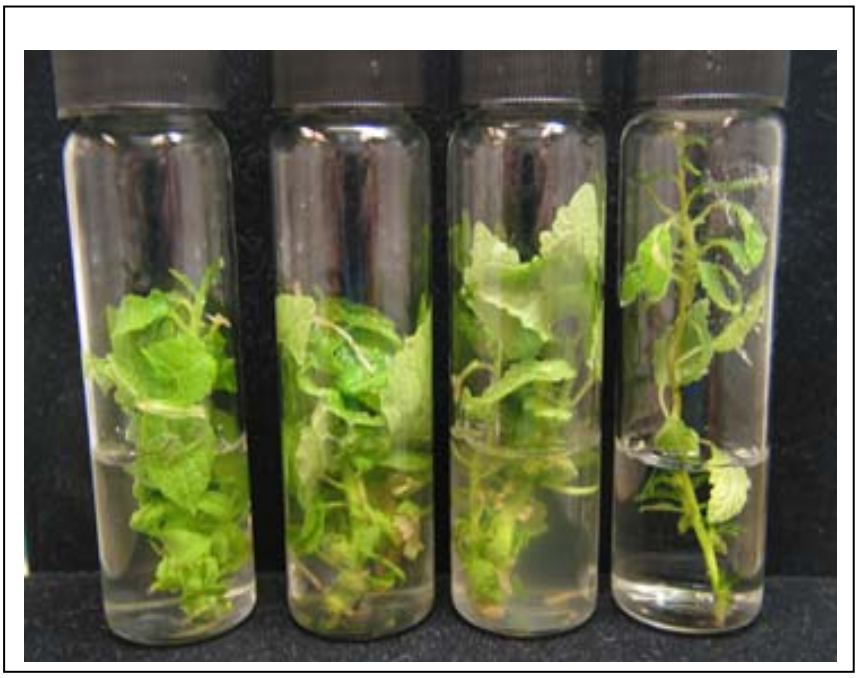


Figure 45. Transgenic aspen expressing XplA (Line 10) plants prior to dosing with RDX

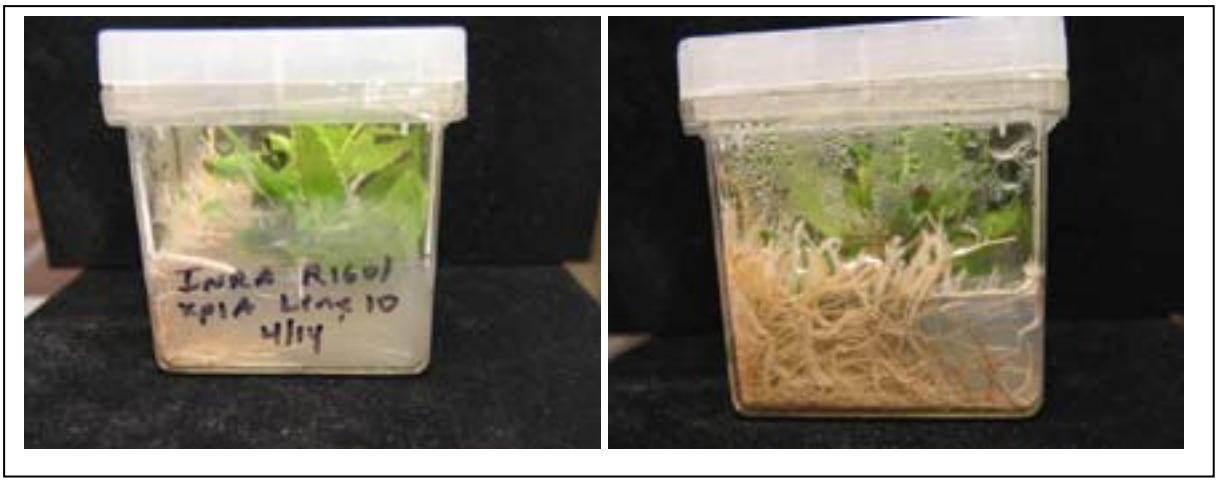


Figure 46. Transgenic aspen expressing *xplA* (Line 10) plants displaying the rooty phenotype.

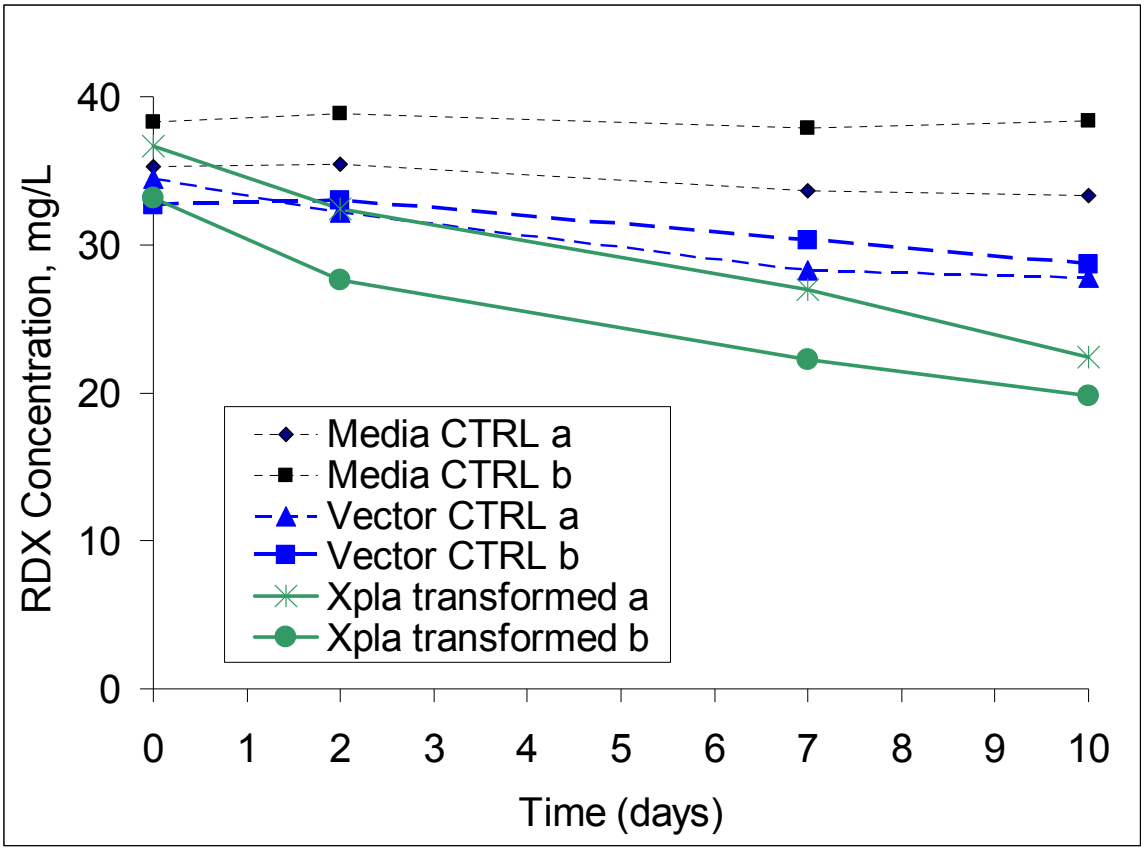


Figure 47. RDX uptake by hydroponic cultures of Line 10 aspens transformed with *xplA* compared to vector control plants and media controls with no plants.

New NR Transgenic Aspen

More aspen lines transformed with *nfsI* and *xplA* were produced. One of the new lines, WK, displayed good growth on plates containing TNT. Genomic preps and PCR revealed that this line was transgenic only for *nfsI* and not for *xplA*. Preliminary experiments to monitor uptake of TNT were performed. Line WK and vector control roots were incubated in medium containing 10 µg/ml (0.044 mM) TNT, and monitored TNT removal by HPLC. After only 24 hours, line WK had removed 98 % of the TNT compared with 59 % for the non-transgenic control even though there was one third less root mass in the WK vials. We began generating plants from this extraordinary root line (Figure 48).

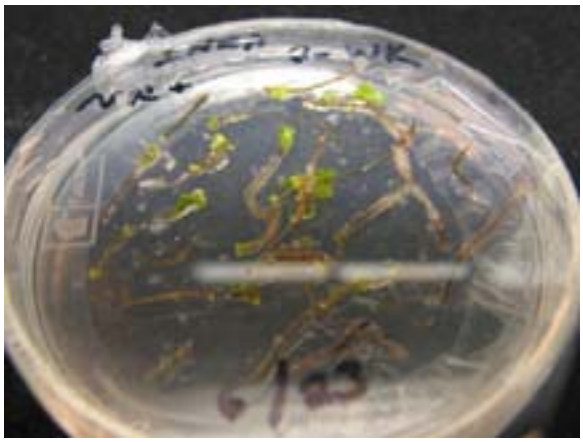


Figure 48. NR transgenic aspen root line WK. Under light and on shoot induction medium, many small plantlets are already forming.

Multigene Transgenics

We previously described two triple lines (Annual Report 2007), Line 6 and Line H have improved TNT and RDX uptake compared to the vector control plants. Line 6 and Line H (Figure 49) were used for metabolite studies. Unfortunately, the TNT and RDX uptake rates of lines 6 and H were lower than the rates of our single *xplA* (Lines 10 and L) or *nfsI* (Line WK) lines. It now seems apparent that it is difficult to get all the genes integrated simultaneously with high expression levels.

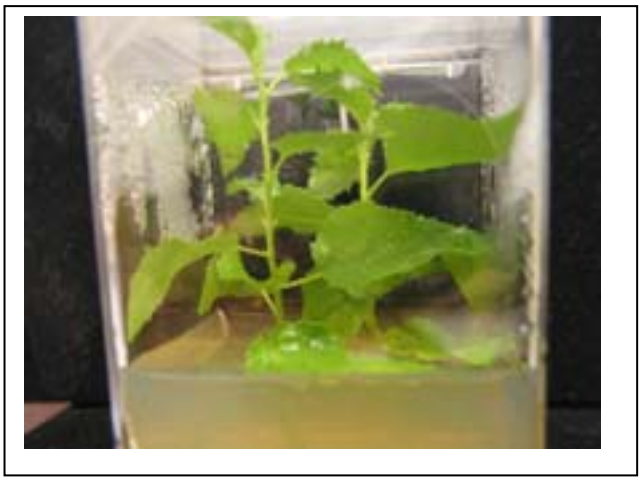
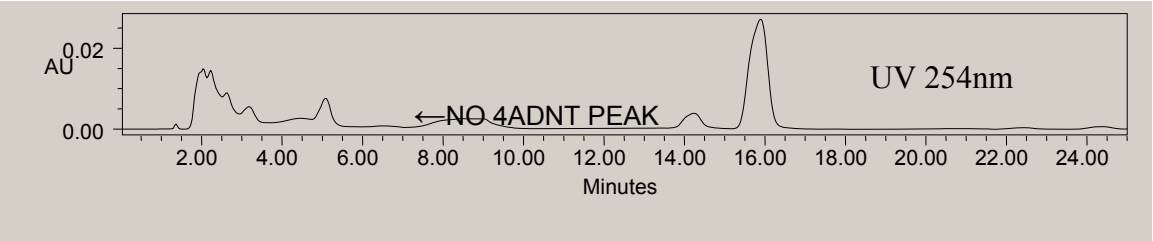


Figure 49. Line H triple transgenic aspen with NR, CPR, and xplA.

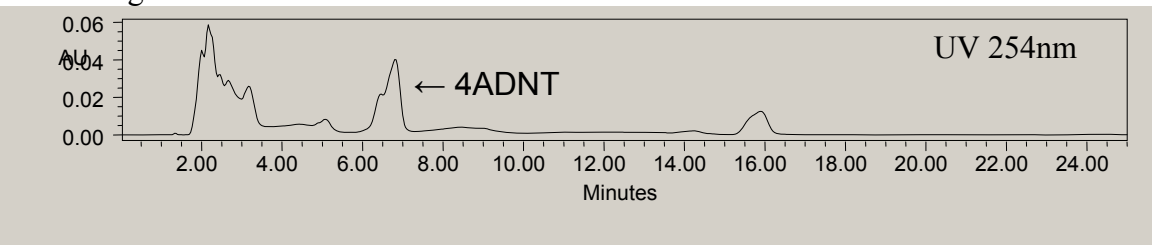
TNT metabolism in nfsI-transformed aspen

The production of ADNT and HADNT metabolites following TNT uptake by *nfsI*-transformed aspen (Line 6 clones) was studied. A peak with a small area count was seen on HPLC with retention time with a retention time correlating to 4 ADNT, based on comparison with a 4 ADNT-spiked control.

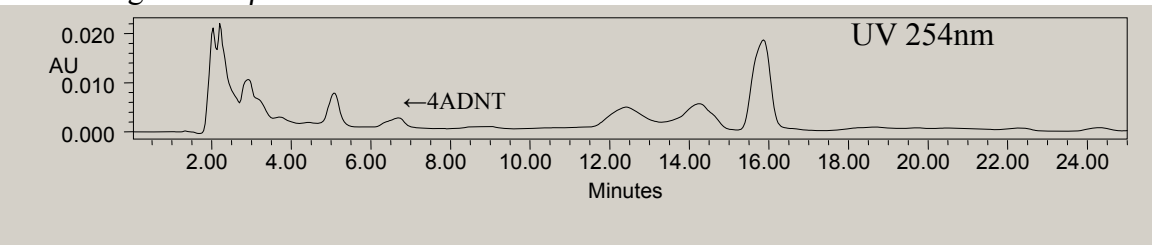
Chromatogram 1. *CONTROL EXTRACT*



Chromatogram 2. *CONTROL EXTRACT SPIKED WITH 10PPM OF 4ADNT*



Chromatogram 3. *nfsI TRANSFORMED PLANT EXTRACT*



Chromatogram 4. *nfsI TRANSFORMED PLANT EXTRACT SPIKED WITH 10PPM OF 4ADNT*

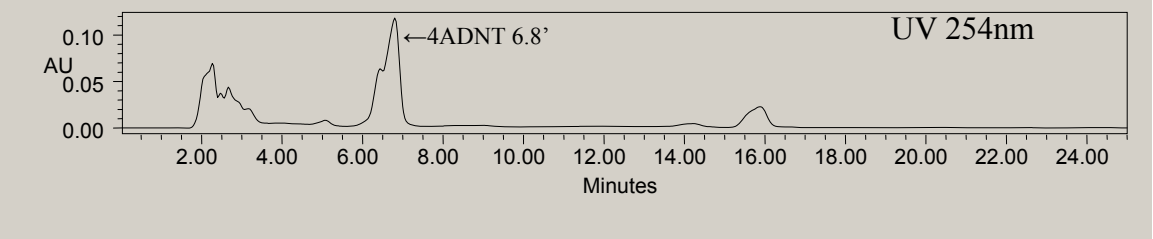


Figure 50. TNT products detected in transgenic aspen expressing NR
TNT peak in acetonitrile:methanol standards is at 8.2 minutes and at 8.5 minutes in aqueous samples, hydroponic solution, and in extracts. In standards retention times were 2ADNT=6.5', 4ADNT=6.8', 2HADNT=6.45', and 4HADNT=6.85'.

RDX Metabolism by Transgenic Aspen Line 10

As an indicator for RDX metabolism, the total nitrogen content of the plants was analyzed following growth in nitrogen-free medium with RDX as the sole nitrogen source. Line 10 (xplA) and vector control plants were incubated in vials for 2 months, with additional RDX added once during the experiment. Wet weights were recorded before the tissue was dried for total nitrogen analysis. The xplA-expressing plants had an average of more than twice the level of total

nitrogen compared to the control plants, with a N% of 2.221 compared to 1.021. The Line 10 plants also grew better in the RDX-containing medium (3.25 g wet weight) compared with medium lacking RDX (1.98 g wet weight). Therefore, it is clear that xplA transgenic aspen plants are able to take up and metabolize RDX, using this pollutant as a source of nitrogen for growth.

Hardening off of Transgenic Aspen Line 10

Eventually five intact Line 10 transgenic aspens were hardened off to growth room conditions. Developing protocols for hardening off the Aspen lines that were transformed with A. rhizogenes took about one year. Aspen Line 10 proved especially sensitive to drying out in the plant growth room. The leaf and plant morphologies differed from Line 10 compared to the wild-type INRA 717 aspen. As shown in Figure 51, Line 10 was stunted and the leaves were elongated and highly wrinkled at the edges. Line 10 also had smaller internodes and its growth was significantly slower than the wild-type.

Due to its poor growth and sensitivity, we concluded that Line 10 was unsuited to mass propagation. Since the vector control, pART27, also transformed with A. rhizogenes, had similar morphology to Line 10, we conclude that transformation of INRA 717 aspen with A. rhizogenes is not practical.

Line 10

Wild-type INRA 717 Aspen

Figure 51. Differences in leaf and plant morphologies between Line 10 (transformed with XplA) and wild-type INRA 717

VI. Concluding Summary

TNT toxicity studies on NR expressing tobacco

The major product of TNT reduction by NR is the 4-HADNT isomer. Under the conditions tested here, the TNT was reduced stoichiometrically with a 1:1 conversion to 4-HADNT, subsequently, small quantities of 2,4-HADNT appeared. In axenic cultures containing NR tobacco, significantly higher levels of 4-HADNT were detected than in wild type cultures. Now that the products of *E. cloacae* TNT reduction have been established, and methods optimized for their detection, Future work will include the analysis of TNT-transformation intermediates produced by the NR and untransformed wild type lines in liquid culture.

The studies of TNT toxicity in NR expressing tobacco plants on solid media revealed that there was a clear difference in TNT tolerance between the two plant lines. On media containing 0.1 mM TNT, untransformed wild type root and shoot growth was severely retarded with chlorotic leaves, while NR expressing plants produced healthy green leaves with only slightly reduced root growth. In liquid culture at the same TNT concentration, both the untransformed wild type plants and NR plants remained green with the NR line producing 3-fold more biomass than untransformed wild type. At the higher concentrations of TNT (0.25 mM and 0.5 mM), only the NR plants remained green and increased in biomass. The untransformed, wild type plants died. As 0.5 mM is the aqueous solubility limit of TNT (at 25 ºC). These data show that plant and grass species transformed with *nfsI* would be able to withstand the maximum level of TNT that would be encountered in the groundwater of military training ranges contaminated with TNT.

Growth of NR-expressing tobacco plants on TNT-contaminated soil

In our soil studies, the NR-expressing tobacco plants exhibited an increased tolerance to TNT compared to untransformed wild type plants. At 150 mg/kg TNT and higher, both transgenic and untransformed wild type plants exhibited signs of phytotoxicity, including stunting of roots, and to a lesser degree leaves, and chlorosis of leaves. This effect was much more dramatic in untransformed wild type plants and was in agreement with the results from the axenic studies [71]. With increasing TNT concentration, the phytotoxic effects on both untransformed wild type and transgenic plants increased and the level of chlorosis also became more severe. At the highest level of TNT (4000 mg/kg) wilting and necrosis was observed in the untransformed wild type plants.

The extent of the increase compared to untransformed wild type plants was not as dramatic as that observed in axenic studies[1]. This could be due to the compounding effects of the complex environment within soil, with both abiotic and biotic effects playing a role. Abiotic effects include the binding of TNT and metabolites to clay particles and humic acid within the soil, altering the bioavailability. Another important factor is the role of the microbial population of the soil. In the hydroponic studies, the tobacco plants were grown under sterile conditions, whereas the soil is a more complex environment. Microbial transformation of TNT is a possibility within the soil, as are interactions between the tobacco plants and microorganisms, particularly within the rhizosphere.

The soil and the microbial community was characterised to gain a more complete understanding of the processed involved. **A** significant change in pH was detected in the pre-planted soil (pH= 6.9 ± 0.1) samples and the bulk soil samples (pH = 7.8 ± 0.2), This further validates the use of the unplanted soil as the control soil, rather than the pre-planted soil samples as variation in pH

could have had an effect on the microbial community of the soil. Whilst the overall pH of the soil was investigated in this study, it was not possible to determine whether there was a local alteration in pH within the rhizosphere, due to insufficient quantities of soil.

Comparison of the microbial communities of bulk soil

The same trends in CFU counts were observed across TNT concentrations for all bulk soils, whether they were planted with untransformed wild type or NR-expressing tobacco plants or left unplanted. As the concentration of TNT increased, a shift in microbial community occurred from slower growing k-strategists towards faster growing r-strategists. At high concentrations of TNT, the overall culturable bacterial count dropped, presumably due to the toxicity of TNT [72]. The similarity in cell number between unplanted and bulk soils suggested that neither the untransformed wild type nor the NR-expressing tobacco had a significant impact on the abundance of the culturable portion of the microbial community.

Carbon substrate utilization patterns also showed no significant differences between the control unplanted soil and the bulk soil from either of the tobacco plants. For the unplanted and the bulk soils from both tobacco genotypes, the average AAT increased slightly as the concentration of TNT rose from zero to intermediate concentrations (150, 500 mg TNT/kg soil) and then dropped as TNT concentrations increased further. Principal component analysis revealed that PC2 increased with a rise in TNT concentration. Seven of the eight substrates that were loaded strongly on PC2 were also found to load strongly, in an inverse fashion, on the first principal component of soil contaminated with TNT over time. PC2 explained most of the variation observed between the control unplanted soil and bulk soil from both genotypes of tobacco plants. This suggests that the tobacco plants did not have any significant impact on the carbon-utilization pattern of the bulk soil microbial community.

The similarity of the DGGE profiles between unplanted soil and bulk soil (from both tobacco genotypes) also indicated that the tobacco plants did not have a significant impact on the composition of the bulk soil microbial community. The DGGE profiles were comparable to the profiles observed after 30 days of contamination of soil with TNT. Changes observed across the range of TNT, in pre-planted, unplanted and bulk soil, can be attributed solely to the shift in community structure caused by TNT. A shift from slow-growing k-strategists towards faster growing r-strategists has been observed to occur when soil was contaminated with TNT. Pseudomonads were heavily represented in soils with high levels of TNT contamination. There have been numerous reports of pseudomonads transforming TNT in the literature.

The extraction and determination of concentration of nitroaromatics was performed on a mixture of rhizosphere and bulk soil (termed overall soil). The TNT concentration of the overall soil was slightly lower for soil that NR-expressing tobacco had been growing in compared to untransformed wild type tobacco overall soil. The untransformed wild type overall soil was in turn slightly less contaminated with TNT than unplanted soil. A decrease in the local concentration of TNT within the rhizosphere could account for the overall decrease in TNT that was observed, leaving the TNT concentration of the bulk soil unchanged. An earlier study [73] showed that whilst there was no observable change in the 16S rDNA profile of the bulk soil, when plants were added to phytoremediate hydrocarbons, there was a detectable shift in the functional catabolic gene expression. Exploring the catabolic gene expression within both the rhizosphere and the bulk soil would be an interesting extension of this research.

Comparison of the microbial communities of rhizosphere soil

Changes in the microbial ecology of the rhizosphere, compared with the bulk soil, were evident over the range of concentrations of TNT contamination. Enumeration of culturable cells revealed that unamended rhizosphere soil, or rhizosphere soil amended with low concentrations of TNT (up to 50 mg TNT/kg soil), exhibited a much higher CFU count than that observed in bulk soil. This was true for both untransformed wild type and NR-expressing tobacco strains, with no significant difference in CFU observed between the two genotypes. This indicated that the expression of the NR gene within the tobacco had no observable effect on the numbers of the culturable community when there was no, or minimal, TNT contamination.

At intermediate concentrations of TNT amendment (150 and 500 mg TNT/kg soil), a marked difference between the rhizosphere soils of the untransformed wild type and the NR-expressing tobacco was observed. The levels of culturable cells for the untransformed wild type rhizosphere were much lower compared with the cell count of the NR-expressing tobacco rhizosphere. The numbers of culturable bacteria of the untransformed wild type rhizosphere were of the same order of magnitude as that observed in the untransformed wild type bulk soil. The bacterial count of rhizosphere soil from NR-expressing tobacco was considerably higher than that observed in the bulk soil. This marked difference could be due to the conditions of the tobacco plants. At these intermediate concentrations of TNT amendment, the untransformed wild type plants exhibited significantly more severe signs of phytotoxicity compared with the NR-expressing tobacco. The chlorosis exhibited by untransformed wild type plants suggested that photosynthesis was reduced and therefore the amount of exudation by the roots would be reduced. In contrast, one would suppose that the decay of untransformed wild type plant roots, caused by TNT phytotoxicity, would increase the carbon available within the rhizosphere. It must be borne in mind that not only was there a reduction in the CFU per gram of the untransformed wild type rhizosphere soil compared to NR-expressing tobacco rhizosphere soil, there was also a dramatic decrease in the amount of rhizosphere soil. Considerable stunting of root growth and development of the untransformed wild type plant, along with smaller and less dense roots than the NR-expressing tobacco plant, meant that the quantity of rhizosphere soil was low. The higher culturable bacterial count associated with the NR-expressing plant correlated with the reduced phytotoxicity exhibited at these levels of TNT, compared with the untransformed wild type tobacco. The relative health of the NR-expressing tobacco plants at intermediate concentrations of TNT could result in maintenance of root exudation, which would have a positive effect on the rhizosphere microbial community.

Another possible explanation for the relatively high levels of bacterial cells, even at these toxic levels of TNT-amendment, was that the NR-expressing tobacco plants were observed to take up more TNT from soil than the untransformed wild type plants. This could have resulted in a decrease in the local concentration of TNT at the root-soil interface, and therefore a corresponding drop in the toxic effect exerted upon the microbial community. There was an insufficient quantity of rhizosphere soil available for extraction and analysis of TNT to determine whether there was a local reduction in concentration of TNT. Analysis of TNT concentrations in a combination of rhizosphere and bulk soil was undertaken who observed that concentrations of TNT within the soil of NR-expressing tobacco were in general lower than that of untransformed wild type tobacco soil. A microclimate could be envisioned to exist at the root-soil interface. Further investigations would be necessary to support this theory. As well as determining the local concentration of TNT it would also be of interest to determine the

microscopic nature of the microbe-root interactions to see whether a biofilm has been formed around the root.

At the highest levels of soil amendment with TNT, (1500 and 4000 mg TNT/kg soil) only NR-expressing plants had sufficient roots to enable study the rhizosphere, due to the phytotoxicity of the TNT to the untransformed wild type tobacco. Even at these high concentrations of TNT amendment, the culturable bacterial count remained relatively high. One possible explanation could again be the sustained health of the NR-expressing plant resulting in root exudation. At these high, and potentially very toxic, levels of TNT it would seem likely that a local decrease in the concentration of TNT had occurred due to the uptake of TNT by the tobacco plant.

The functional capability of the rhizosphere soils was also explored through investigation of sole carbon substrate utilization patterns (Biolog EcoPlates). The overall metabolic activity (average AAT) was in general higher for the rhizosphere soil compared with the bulk soil. As discussed previously, plant roots impact on the rhizosphere soil in a number of ways from exudation of carbon compounds to alteration of physical and chemical conditions of the soil. It seems likely that the increase in carbon availability, due to root exudation, increased the overall metabolic capabilities of the rhizosphere soil compared with the bulk soil. The metabolic activity of the untransformed wild type rhizosphere soil fluctuated considerably. In general the metabolic activity of untransformed wild type rhizosphere soil did not differ dramatically compared to that of NR-expressing tobacco rhizosphere soil.

Patterns of substrate utilization were discerned using PCA. The first principal component explained a large portion of the variance in carbon substrate utilization observed between rhizosphere and bulk soil. This variance implied that a shift in community structure and functional capability had occurred between the bulk and rhizosphere soil. PC1 was in general positive for the rhizosphere samples, whereas it was negative for all of the unplanted and bulk soil samples. On examination of the substrates that contributed strongly to PC1, it became apparent that the carbohydrates were strongly represented amongst the substrates with a positive loading. Plants are known to exude carbohydrate compounds from their roots [31]; therefore it was perhaps not surprising that the microbial community of the rhizosphere were found to utilize carbohydrate sources more efficiently than the microbial community of the bulk soil. The PC1 of untransformed wild type and NR-expressing rhizosphere soil samples were comparable at concentrations of TNT up to 150 mg/kg. The PC1 of NR at concentrations of 500 mg/kg and above increased compared to that observed at lower concentrations. An equivalent increase in the untransformed wild type rhizosphere soil at 500 mg/kg was not observed. This change in PC1 across the trend of TNT concentrations suggested that a shift in microbial community structure had occurred at the high concentrations of TNT amendment in NR-expressing tobacco rhizosphere soil. This could account for the change in overall metabolic activity observed at these concentrations of TNT with NR-expressing tobacco rhizosphere soil. The trends observed for PC2 for bulk soil samples, across the range of TNT concentrations, were not observed for rhizosphere samples. There appeared to be a shift in functional capability of the rhizosphere soil, perhaps due to a change in community structure. This suggested that the microbial community of the rhizosphere had an altered response to TNT contamination compared to that of the bulk soil.

The genetic diversity of the rhizosphere samples, from a range of concentrations of TNT contamination, was investigated using DGGE. There was a difference in banding profile in the rhizosphere soil compared with the bulk soil. The rhizosphere soil at high concentrations of TNT was not dominated by the same bands as those resulting from the bulk soil. The rhizosphere soils

of NR-expressing and untransformed wild type tobacco tended to cluster together, with differences in TNT concentration distinguishing the clusters, rather than plant type. This indicated that the genetic diversity of the rhizosphere soil of untransformed wild type and NR-expressing tobacco plants did not vary significantly. The concentration of TNT with which soil was contaminated was observed to impact on the genetic diversity of the soil microbial community. A variety of bands emerged in both NR-expressing and untransformed wild type rhizosphere samples as TNT concentrations increased. Although these bands were not in the same place, all of the emergent bands that were sequenced were most closely related to the Bacteriodetes division.

Bacteria of the Bacteriodetes division are ubiquitous [74] and are particularly common in the oceans. There are several reports of Bacteriodetes being identified within the rhizosphere bacterial community [75], however there are no suggestions as to the role that these bacteria play in the rhizosphere habitat. Extensive research has been undertaken into the role that Bacteriodetes play in the aquatic environment, where bacteria of this division are prevalent. Bacteriodetes bacteria appear to have a specialized role in the uptake of dissolved organic material (DOM) and are well known to be proficient in the uptake of biopolymers such as cellulose and chitin, part of the high molecular weight fraction of DOM [74]. The prevalence of Bacteriodetes, could therefore explain the increased utilization of carbohydrates in the rhizosphere samples, compared to the bulk soil. Cellulose, which Bacteriodetes bacteria are proficient at taking up and utilizing, was the substrate that was most strongly loaded on PC1. The prevalence of Bacteriodetes appeared to increase as the concentration of TNT rose. One explanation is that Bacteriodetes bacteria are less susceptible to TNT toxicity then the general bacterial population. Previous studies [72, 76] have shown that different groups of bacteria exhibit varying levels of susceptibility to TNT toxicity. It is possible that the Bacteriodetes bacteria are playing an active role in taking up and transforming TNT. They are renowned for the uptake of organic material. Further studies would be necessary to investigate this possibility. If this physiologically distinct division of bacteria is capable of transforming TNT, then it remains possible that Bacteriodetes have evolved novel mechanisms of TNT-transformation compared to those that have previously been determined.

This study has shown that the presence of either untransformed wild type or NR-expressing tobacco plants has an effect on the microbial community structure of the rhizosphere of TNT-contaminated soil, but not on the bulk soil. It remains unclear whether the presence of the tobacco plant enhanced rhizodegradation. The NR-expressing tobacco plants may have facilitated growth of dense populations of diverse microorganisms with a high catabolic potential, resulting in an increase in TNT transformation. The prevalence of the Bacteriodetes in TNT-contaminated rhizosphere soil may be due to their catabolic abilities. To determine this, further research on isolated and cultured Cytophaga and Flavobacteria from the rhizosphere would need to be carried out. Co-metabolism of TNT when a carbon substrate was present has been shown on multiple occasions [77, 78]. It would be interesting to investigate whether the increase in carbon due to the root exudates leads to more efficient metabolism of TNT by the bacteria present in the rhizosphere. This would be classed as rhizodegradation. The tobacco plants may also play a role in stimulating TNT metabolism by taking up TNT and thereby reducing the concentration of TNT within the rhizosphere, in turn leading to an increase in microbial abundance and activity.

This study has not revealed any significant differences between the impact of the genetically modified NR-expressing tobacco and the untransformed wild type tobacco on the microbial

community of the control, uncontaminated soil. The development and use of genetically modified plants (GMPs) has been a topic of considerable public debate in recent years. Whilst GMPs are of potential benefit for improving agricultural output, as well as for phytoremediation, the impact of GMP use on the environment is still not fully understood. The majority of studies addressing the potential risks of GMP cultivation have considered only aboveground effects. Recently, due to methodological advances in soil microbial ecology, research has begun on the impact of GMPs on the underground environment, in order to gain knowledge of GMP-driven effects on the microbial communities and processes in soil that are essential to key terrestrial ecosystem functions. Two recent reviews of the subject [79, 80] conclude that although such research has advanced our understanding of this topic, a number of knowledge gaps still prevent full interpretation of results, The reviews highlight the failure of most studies to assign a definitively negative, positive or neutral effect to GMP introduction. This study shows that expression of NR by tobacco plants does not have a significant effect on the soil microbial community, when no TNT is present. This is a very encouraging finding, as it would make transgenic NR-expressing plants more acceptable for phytoremediation.

Characterization of XplA expressing Arabidopsis

Liquid culture studies on Arabidopsis plants dosed with 180 μM RDX revealed that *35S-xplA* lines removed all 180 μM RDX from the media within five days. This concentration is over three times that measured in waste water from manufacturing sites[4] and is close to the aqueous solubility limit for RDX (at 25 ºC) [5]. In liquid cultures containing 100 μM RDX, the rates of uptake were similar to those at 180 μM RDX, indicating that even at the solubility limits of RDX, the maximum exposure levels likely to be encountered in contaminated groundwater, the rate of RDX uptake by the *35S-xplA* lines is not reduced. The RDX degradation intermediates, formaldehyde and nitrite, were not detected in liquid media or plant extracts, suggesting that these compounds are metabolized by the endogenous plant enzymes, formaldehyde dehydrogenase and nitrite reductase.

Arabidopsis expressing NR exhibited similar increases in TNT tolerance to those observed in the NR tobacco lines. The line with the highest NR activity, *35S-nfsI-1*, was crossed with *35S-xplA* lines to produce transgenic lines expressing both XplA and NR activities. Liquid culture experiments on these lines showed that the *35S-xplA-nfsI* line removed RDX at rates equivalent to the *35S-xplA* parent lines. However, removal of RDX was inhibited in the presence of TNT. Subsequent analysis revealed that purified XplA is inhibited by TNT.

Arabidopsis lines expressing XplB alongside XplA showed a 40-fold enhancement in the rate of RDX degradation compared to *35S-xplA* lines. We are now investigating the production of a XplA-XplB fusion protein. Such a protein is predicted to have a significantly higher activity than XplA.

Soil studies on Arabidopsis expressing XplA and NR

Soil studied showed that *35S-xplA* plants were significantly larger than the untransformed wild type when grown on RDX-contaminated soil with the shoot tissue contained up to three times more RDX. Additionally, the *35S-xplA* lines achieved higher root and shoot biomasses in the presence of RDX, than when grown on uncontaminated soil. We presume that these lines are using the RDX as a nitrogen source to enhance growth. Further soil studies showed that soil in which *35S-xplA* plants were grown contained between 7.2-fold and 2.4-fold less RDX than soil in which untransformed wild type plants had been grown.

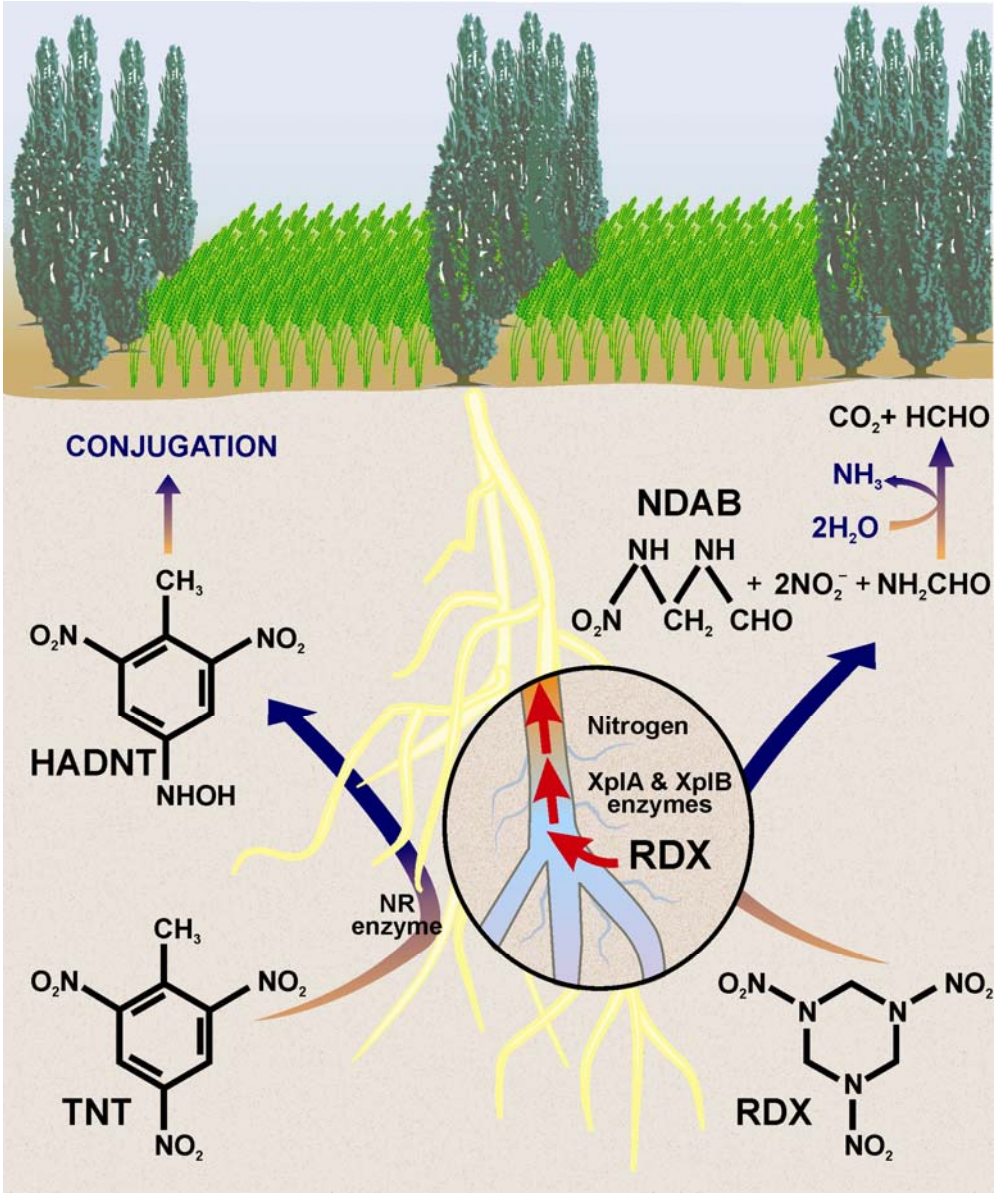


Figure 52. Schematic of proposed method for phytoremediating TNT and RDX from military training ranges using genetically modified plants
NR transforms TNT to less toxic compounds (e.g. HADNTs), which are conjugated to endogenous plant compounds prior to incorporation into the root biomass. RDX is taken up to the aerial parts of the plant where XplA, and reductases partner XplB catabolize RDX to non-toxic compounds.

In soil leachate experiments, the *35S-xplA* lines removed significantly more (53-80 %) RDX from the soil leachate than either untransformed wild type plants (30 %) or *nfsI* only-expressing plants (28 %). The *35S-xplA-nfsI* lines did not perform as well as expected, removing less (20-47 %) than the *35S-xplA* lines. This may be due to gene silencing; the *35S-xplA-nfsI* lines contain

multiple copies of the 35S promoter sequence, known to be a factor inducing gene silencing. We are addressing this in the new SERDP project ER-1498 by employing alternative promoters and additional transgenic lines. In addition, our future studies will include optimization of both XplA by directed evolution, and investigation of the subsequent detoxification steps of NR-produced TNT conjugates by endogenous plant glycosyltransferases.

Overall, the results presented here demonstrate that our proposed method for the phytoremediation of RDX and TNT from contaminated military training ranges (presented in Figure 52) is in principle, viable. RDX is highly mobile in ground water and our soil leachate experiments show that saturating concentrations of RDX can be effectively remediated from groundwater by plants expressing XplA. The NR expressing plants have increased tolerance to, and are able to detoxify, the co-contaminating explosive TNT, and we have produced transgenic plants containing XplA and NR that are able to tackle both explosives together. As Arabidopsis, our model plant system is a relatively small growing, annual, a further magnified effect is predicted in the more robust and larger tree and perennial grass species transformed with these transgenes. These transformed grasses and tree species could be planted along range borders to remove RDX from soil leachate as it leaves the training range.

Our results also demonstrate that transgenic tobacco plants expressing *nfsI* detoxify soil contaminated with TNT, can increase the functional and genetic diversity of the rhizosphere bacterial community in acutely polluted soil compared to wild type plants. This is the first study to demonstrate this and is a step closer to restoring contaminated environments to their natural state.

Transformation of INRA 717 aspen with xplA and nfsI using A. rhizogenes

INRA 717 aspen was transformed with xplA and nfsI using *A. rhizogenes* and gene presence and activities were confirmed by PCR and whole plant and root metabolism assays, confirming that these genes expressed well in a variety of plants. However, the growth characteristics of the aspen transformed with *A. rhizogenes* were poor, and the plants were unsuited to mass propagation.

We are continuing to transform INRA 717 aspens using disarmed *A. tumefaciens.* Several transformed plantlets are developing and will be evaluated for their ability to degrade RDX. Since trees are unlikely to be useful on the training range itself, we envision their main application to be as buffer plantings intercepting groundwater that has become contaminated with RDX either in the past or as a safety factor to prevent migration of contaminated water off base. TNT sorbs strongly to soil surfaces, which prevents it from leaking to the groundwater. Therefore expression of nfsI for the transformation of TNT is not needed to prevent the effects of phytotoxicity on aspens planted over RDX contaminated groundwater. The expression of xplA with or without xplB in such aspen should suffice to produce a treatment effect.

Appendices

List of Technical Publications

Elizabeth L. Rylott, Rosamond G. Jackson, James Edwards, Grant L. Womack, Helena M. B. Seth-Smith, Deborah A. Rathbone, Stuart E. Strand & Neil C. Bruce (2006) An explosive-degrading cytochrome P450 activity and its targeted application for the phytoremediation of RDX. *Nature Biotechnology* 24: 216-9.

Symons ZC, Bruce NC.(2006) Bacterial pathways for degradation of nitroaromatics. *Nat Prod Rep.* 23: 845-50.

Williams RE, Rathbone DA, Scrutton NS, Bruce NC.(2004) Biotransformation of explosives by the old yellow enzyme family of flavoproteins. *Appl Environ Microbiol.* 70: 3566-74.

Travis, E. R., Hannink, N. K. van der Gast, C. J. Thompson, I. P., Rosser, S. J. and Bruce, N. C. (2005) Restoration of the rhizosphere of TNT-contaminated soil planted with transgenic tobacco. (*in preparation*).

Hannink, N. K., Subramanian, Rosser, S. J., Basran, A. Murray, J. A. H., Shanks, J. V. and Bruce, N. C. (2005) Enhanced transformation of TNT by tobacco plants expressing a bacterial nitroreductase. (*submitted for publication* to Plant Biotechnology).

Two posters were presented at the Nov. 2006 SERDP/ESTCP Symposium
This work was presented at:

- The International Poplar Conference (a platform talk), in China
- The American Plant Biology Conference, Boston
- The SETAC (Society of Environmental Toxicology and Chemistry) World Congress 2004. Portland Oregon.

Page left blank intentionally

References

1. Hannink, N. K., Rosser, S. J., French, C. E., Basran, A., Murray, J. A., Nicklin, S. & Bruce, N. C. (2001)Phytodetoxification of TNT by transgenic plants expressing a bacterial nitroreductase *Nat. Biotechnol.* **19**, 1168-1172.

2. Peterson, M. M., Horst, G. L., Shea, P. J., Comfort, S. D. & Peterson, R. K. (1996)TNT and 4-amino-2,6-dinitrotoluene influence on germination and early seedling development of tall fescue *Environ. Pollut.* **93**, 57-62.

3. Scheidemann, P., Klunk, A., Sens, C. & Werner, D. (1998)Species dependent uptake and tolerance of nitroaromatic compounds by higher plants *J. Plant Physiol.* **152**, 242-247.

4. Jackson, M., Green, J. M., Hash, R. L., Lindsten, D. C. & Tatyrek, A. F. (1978)Nitramine (RDX-HMX) Wastewater Treatment at the Holston Army Ammunition Plant *U. S. Army Armament Research and Development Command: Dover, NJ* **Report ARLCD-77013**.

5. Lynch, J. C., Myers, F. M., Brannon, J. M. & Delfino, J. J. (2001)Effects of pH and temperature on the aqueous solubility and dissolution rate of 2,4,6-trinitrotoluene (TNT), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-1,3,5,7-tetracine (HMX) *J. Chem. Eng. Data* **46**, 1549-1555.

6. Anlezark.G.M., Melton, R. G., Sherwood, R. F., Coles, B., Friedlos, F. & Knox, R. J. (1992)Purification and Properties of a Nitroreductase Enzyme from *Escherichia coli* - a Potential Enzyme for Antibody-Directed Enzyme Prodrug Therapy *Biochemical Pharmacology* **44**, 2295.

7. watanabe, M., Ishidate, M. & Nohmi, T. (1990)Nucleotide-sequence of *Salmonella typhimurium* nitroreductase gene *Nucleic Acids Research* **18**, 1059.

8. Bryant, C., Hubbard, L. & McElroy, W. D. (1991)Cloning, nucleotide sequence, and expression of the nitroreductase gene from *Enterobacter cloacae J Biol. Chem.* **266**, 4126-4130.

9. Koder, R. L. & Miller, A. F. (1998)Steady-state kinetic mechanism, stereospecificity, substrate and inhibitor specificity of *Enterobacter cloacae* nitroreductase *Biochimica Et Biophysica Acta-Protein Structure and Molecular Enzymology* **1387**, 395-405.

10. McCormick, N. G., Feeherry, F. E. & Levinson, H. S. (1976)Microbial transformation of 2,4,6-trinitrotoluene and other nitroaromatic compounds *Appl. Environ. Microbiol.* **31**, 949-958.

11. Leskovac, V., Svircevic, J., Trivic, S., Popovic, M. & Radulovic, M. (1989)Reduction of aryl-nitroso compounds by pyridine and flavin coenzymes *Int. J. Biochem.* **21**, 825-834.

12. Liu, D., Thomson, K. & Anderson, A. C. (1984)Identification of nitroso-compounds from biotransformation of 2,4-dinitrotoluene *Applied and Environmental Microbiology* **47**, 1295-1298.

13. Thompson, P. L., Ramer, L. A. & Schnoor, J. L. (1998)Uptake and transformation of TNt by hybrid popular trees *Environ. Sci. Technol.* **32**, 975-980.

14. Larson, S. L., Jones, R. P., Escalon, L. & Parker, D. (1999)Classification of explosives transformation products in plant tissue *Environmental Toxicology and Chemistry* **18**, 1270-1276.

15. Wayment, D. G., Bhadra, R., Lauritzen, J., Hughes, J. B. & Shanks, J. V. (1999)A transient study of the formation of conjugates during TNT metabolism by plant tissues. *International J. Phytoremed.* **1**, 227-239.

16. Best, E. P., Zappi, M. E., Fredrickson, H. L., Sprecher, S. L., Larson, S. L. & Ochman, M. (1997)Screening of aquatic and wetland plant species for phytoremediation of explosives-contaminated groundwater from the Iowa Army Ammunition Plant *Ann. N. Y. Acad. Sci.* **829**, 179-194.

17. Trombly, J. (2009)Engineering enzymes for better bioremediation *Environ. Sci. Technol.* **29**, 560A-564A.

18. Gorge, E., Brandt, S. & Werner, D. (1994)Uptake and metabolism of 2,4,6-trinitrotoluene in higher plants *Environmental Science and Pollution Research* **4**, 229-233.

19. Gong, P., Wilke, B. & Fleischmann, S. (1999)Soil-based phytotoxicity of 2,4,6-trinitrotoluene (TNT) to terrestrial higher plants *Arch. Environ. Contam Toxicol.* **36**, 152-157.

20. Bekkedal, M. Y. V., Ritchie, G. D., Stil, K. R. & Rossi, J. (2002)A proposed working definition for the novel concept of neurobehavioral hormesis *Human and Ecological Risk Assessment* **8**, 1815-1823.

21. Allender, W. J., Cresswell, G. C., Kaldor, J. & Kennedy, I. R. (1997)Effect of lithium and lanthanum on herbicide induced hormesis in hydroponically-grown cotton and corn *Journal of Plant Nutrition* **20**, 81-95.

22. Peterson, M. M., Horst, G. L., Shea, P. J. & Comfort, S. D. (1998)Germination and seedling development of switchgrass and smooth bromegrass exposed to 2,4,6-trinitrotoluene *Environ. Pollut.* **99**, 53-59.

23. Schneider, K., Oltmanns, J., Radenberg, T., Schneider, T. & PaulyMundegar, D. (1996)Uptake of nitroaromatic compounds in plants - Implications for risk assessment of ammunition sites *Environ. Sci. Pollution Res.* **3**, 135-138.

24. Hughes, J. B., Shanks, J. V., Vanderford, M., Lauritzen, J. & Bhadra, R. (1997)Transformation of TNT by aquatic plants and plant tissue cultures *Environ. Sci. Technol.* **31**, 266-271.

25. Harvey, S. D., Fellows, R. J., Cataldo, D. A. & Bean, R. M. (1990)Analysis of 2,4,6-trinitrotoluene and its transformation products in soils and plant tissues by high-performance liquid chromatography *International J. Chromatography* **518**, 361-374.

26. Palazzo, A. J. & Leggett, D. C. (1986)Effect and disposition of TNT in a terrestrial plant *Journal of Environmental Quality* **15**, 49-52.

27. Jaeger, C. H., Lindow, S. E., Miller, S., Clark, E. & Firestone, M. K. (1999)Mapping of sugar and amino acid availability in soil around roots with bacterial sensors of sucrose and Tryptophan *Appl. Environ. Microbiol.* **65**, 2685-2690.

28. Lemanceau, P., Corberand, T., Gardan, L., Latour, X., Laguerre, G., Boeufgras, J. M. & Alabouvette, C. (1995) Effect of 2 plant species, Flax (*Linum Usitatissinum* L.) and Tomato (*Lycopersicon Esculentum* Mill), on the diversity of soilborne populations of fluorescent Pseudomonads *Appl. Environ. Microbiol.* **61**, 1004-1012.

29. Yang, C. H. & Crowley, D. E. (2000)Rhizosphere microbial community structure in relation to root location and plant iron nutritional status *Appl. Environ. Microbiol.* **66**, 345-351.

30. Cunningham, S. D., Shann, J. R., Crowley, D. E. & Anderson, T. A. (1997)Phytoremediation of Contaminated Water and Soil in *Phytoremediation of soil and water contaminants*, edited by E. L. Kruger, T. A. Anderson and J. R. Coats. *American Chemical Society, Washington D. C.*

31. Bertin, C., Yang, X. & Weston.L.A. (2003)The role of root exudates and allelochemicals in the rhizosphere *Plant and Soil* **256**, 67-83.

32. Fan, T. W. M., Lane, A. N., Pedler, J., Crowley, D. E. & Higashi, R. M. (1997)Comprehensive analysis of organic ligands in whole root exudates using nuclear magnetic resonance and gas chromatography mass spectrometry *Analytical Biohemistry* **251**, 57-68.

33. Delhaize, E., Hebb, D. M. & Ryan, P. R. (2001)Expression of a Pseudomonas aeruginosa citrate synthase gene in tobacco is not associated with either enhanced citrate accumulation or efflux *Plant Physiol* **125**, 2059-2067.

34. Morris, C. E. & Monier, J. M. (2003)The ecological significance of biofilm formation by plant- associated bacteria *Annual Review of Phytopathology* **41**, 429-453.

35. George, T. S., Gregory, P. J., Robinson, J. S. & Buresh, R. J. (2002)Changes in phosphorus concentrations and pH in the rhizosphere of some agroforestry and crop species *Plant and Soil* **246**, 65-73.

36. Clausen, J., Robb, J., Curry, D. & Korte, N. (2004)A case study of contaminants on military ranges: Camp Edwards, Massachusetts, USA *Environ. Pollut.* **129**, 13-21.

37. Fournier, D., Halasz, A., Spain, J., Fiurasek, P. & Hawari, J. (2002)Determination of key metabolites during biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine with *Rhodococcus* sp. strain DN22 *Appl. Environ. Microbiol.* **68**, 166-172.

38. Fournier, D., Halasz, A., Spain, J., Spanggord, R. J., Bottaro, J. C. & Hawari, J. (2004)Biodegradation of the hexahydro-1,3,5-trinitro-1,3,5-triazine ring cleavage product 4-nitro-2,4-diazabutanal by *Phanerochaete chrysosporium Appl. Environ. Microbiol.* **70**, 1123-1128.

39. Seth-Smith, H. M., Rosser, S. J., Basran, A., Travis, E. R., Dabbs, E. R., Nicklin, S. & Bruce, N. C. (2002)Cloning, sequencing, and characterization of the hexahydro-1,3,5-trinitro-1,3,5-triazine degradation gene cluster from *Rhodococcus rhodochrous Appl. Environ. Microbiol.* **68**, 4764-4771.

40. Hannink, N. K., Subramanian, M., Rosser, S. J., Basran, A., Murray, J. A., Shanks, J. V. & Bruce, N. C. (2007)Enhanced transformation of tnt by tobacco plants expressing a bacterial nitroreductase *Int. J. Phytoremediation.* **9**, 385-401.

41. Gleave, A. P. (1992)A versatile binary vector system with a T-DNA organisational structure conducive to efficient integration of cloned DNA into the plant genome *Plant Mol. Biol.* **20**, 1203-1207.

42. Murashige, T. & Skoog, F. (1962)A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiol. Plant.* **15**, 473-496.

43. Haderlein, S. B., Weissmahr, K. W. & Schwarzenbach, R. P. (1996)Specific adsorption of nitroaromatic: Explosives and pesticides to clay minerals *Environ. Sci. Technol.* **30**, 612-622.

44. Comfort, S. D., Shea, P. J., Hundal, L. S., li, Z., Woodbury, B. L., Martin, J. L. & Powers, W. L. (1995)TNT transport and fate in contaminated soil *Journal of Environmental Quality* **24**, 1174-1182.

45. Guckert, J. B., Carr, G. J., Johnson, T. D., Hamm, B. G., Davidson, D. H. & Kumagai, Y. (1996)Community analysis by Biolog: Curve integration for statistical analysis of activated sludge microbial habitats *Journal of Microbiological Methods* **27**, 183-197.

46. Garland, J. L. & Mills, A. L. (2009)Classification and characterization of heterotrophic microbial communities on the basis of patterns of community level sole carbon source utilization *Applied and Environmental Microbiology* **57**, 2359.

47. Whiteley, A. S. & Bailey, M. J. (2000)Bacterial community structure and physiological state within an industrial phenol bioremediation system *Appl. Environ. Microbiol.* **66**, 2400-2407.

48. Guerineau, F., Lucy, A. & Mullineaux, P. (1992)Effect of two consensus sequences preceding the translation initiator codon on gene expression in plant protoplasts *Plant Mol. Biol.* **18**, 815-818.

49. Gleave, A. P. (1992)A versatile binary vector system with a T-DNA organisational structure conducive to efficient integration of cloned DNA into the plant genome *Plant Mol. Biol.* **20**, 1203-1207.

50. Gleave, A. P. (1992)A versatile binary vector system with a T-DNA organisational structure conducive to efficient integration of cloned DNA into the plant genome *Plant Mol. Biol.* **20**, 1203-1207.

51. Rylott, E. L., Jackson, R. G., Edwards, J., Womack, G. L., Seth-Smith, H. M., Rathbone, D. A., Strand, S. E. & Bruce, N. C. (2006)An explosive-degrading cytochrome P450 activity and its targeted application for the phytoremediation of RDX *Nat. Biotechnol.* **24**, 216-219.

52. Hughes, J. B., Wang, C., Bhadra, R., Richardson, A., Bennett, G. N. & Rudolph, F. B. (1998)Reduction of 2,4,6-trinitrotoluene by *Clostridium acetobutylicum* through hydroxylamino-nitrotoluene intermediates *Environ. Toxicol. Chem.* **17**, 343-348.

53. Garland, J. L. & Mills, A. L. (1991)Classification and characterization of heterotrophic microbial communities on the basis of patterns of community level sole carbon source utilization *Applied and Environmental Microbiology* **57**, 2359.

54. Reed, D. L. & Hafner, M. S. (2002)Phylogenetic analysis of bacterial communities associated with ectoparasitic chewing lice of pocket gophers: A culture- independent approach *Microbial Ecology* **44**, 78-93.

55. Hurek, T., Wagner, B. & Reinhold Hurek, B. (1997) Identification of N_2 fixing plant- and fungus-associated *Azoarcus* species by PCR-based genomic fingerprints *Applied and Environmental Microbiology* **63**, 4331-4339.

56. Quatrini, P., Scaglione, G., Cardinale, M., Caradonna, F. & Puglia, A. M. (2002)*Bradyrhizobium* sp nodulating the Mediterranean shrub Spanish broom (*Spartiumn junceum* L.) *Journal of Applied Microbiology* **92**, 13-21.

57. Cleary, J. M., Lauro, F. M. & Bartlett, D. H. (2009)A 16S based phylogeny of polysaccharide producing *Sphingomonas* strains. (Unpublished) *Submitted (17-APR-2002) to the EMBL/GenBank/DDBJ databases*.

58. Glockner, F. O., Zaichikov, E., Belkova, N., Denissova, L., Pernthaler, J., Pernthaler, A. & Amann, R. (2000)Comparative 16S rRNA analysis of lake bacterioplankton reveals globally distributed phylogenetic clusters including an abundant group of actinobacteria *Applied and Environmental Microbiology* **66**, 5053-5065.

59. Friedrich, M. M. (2006)Characterisation of bacteria from hexane degrading biofilters. (Unpublished) *Unpublished*.

60. Dedysh, S. N., Khmelenina, V. N., Suzina, N. E., Trotsenko, Y. A., Semrau, J. D., Liesack, W. & Tiedje, J. M. (2002)*Methylocapsa acidiphila* gen. nov., sp nov., a novel methane- oxidizing and dinitrogen-fixing acidophilic bacterium from *Sphagnum* bog *International Journal of Systematic and Evolutionary Microbiology* **52**, 251-261.

61. Coleman, N. V., Spain, J. C. & Duxbury, T. (2002)Evidence that RDX biodegradation by Rhodococcus strain DN22 is plasmid-borne and involves a cytochrome p-450 *J Appl. Microbiol.* **93**, 463-472.

62. Yamamoto, S. & Harayama, S. (1998)Phylogenetic relationships of *Pseudomonas putida* strains deduced from the nucleotide sequences of gyrB, ropD and 16S rRNA genes *International Journal of Systematic Bacteriology* **48**, 813-819.

63. Mohn, W. W., Wilson, A. E., Bicho, P. & Moore, E. R. (1999)Physiological and phylogenetic diversity of bacteria growing on resin acids *Systematic and Applied Microbiology* **22**, 68-78.

64. Pacheco, C. C., Passos, J. F., Moradas-Ferreira, P. & DeMarco, P. (2003)Strain PM2, a novel methylotrophic fluorescent *Pseudomonas* sp *FEMS Microbiol. Lett.* **277**, 279-285.

65. Brinkmeyer, R., Knittel, K., Jurgens, J., Weyland, H., Amann, R. & Helmke, E. (2003)Diversity and structure of bacterial communities in arctic versus antarctic pack ice *Appl. Environ. Microbiol.* **69**, 6610-6619.

66. Lucas, F. S., Broennimann, O., Febbraro, I. & Heeb, P. (2003)High diversity among feather-degrading bacteria from a dry meadow soil *Microbial Ecology* **45**, 282-290.

67. Kisand, V. & Wikner, J. (2003)Combining culture-dependent and -independent methodologies for estimation of richness of estuarine bacterioplankton consuming riverine dissolved organic matter *Appl. Environ. Microbiol.* **69**, 3607-3616.

68. Tamaki, H., Hanada, S., Kamagata, Y., Nakamura, K., Nomura, N., Nakano, K. & Matsumura, M. (2003)*Flavobacterium limicola* sp nov., a psychrophilic, organic-polymer-degrading bacterium isolated from freshwater sediments *International Journal of Systematic and Evolutionary Microbiology* **53**, 519-526.

69. O'Sullivan, L. A., Weightman, A. J. & Fry, J. C. (2002)New degenerate Cytophaga-Flexibacter-Bacteroides-specific 16S ribosomal DNA-targeted oligonucleotide probes reveal high bacterial diversity in River Taff epilithon *Applied and Environmental Microbiology* **68**, 201-210.

70. Futamata, H., Harayama, S., Hiraishi, A. & Watanabe, K. (2009)Functional and structural analyses of trichloroethylene- degrading bacterial communities under different phenol-feeding conditions: laboratory experiments *Appl. Microbiol. Biotechnol.* **60**, 594-600.

71. Hannink, N. K., Rosser, S. J. & Bruce, N. C. (2002)Phytoremediation of Explosives *Critcal Reviews in Plant Sciences* **21**, 511-538.

72. Fuller, M. E. & Manning.J.F. (1998)Evidence for differential effects of 2,4,6-trinitrotoluene and other munitions compounds on specific subpopulations of soil microbial communities *Environmental Toxicology and Chemistry* **17**, 2185-2195.

73. Sicilianon, S. D., Germida, J. J., Banks, K. & Greer, C. W. (2003)Changes in microbial community composition and function during a polyaromatic hydrocarbon phytoremediation field trial *Appl. Environ. Microbiol.* **69**, 483-489.

74. Kirchman, D. L. (2002)The ecology of *Cytophaga-Flavobacteria* in aquatic environments *FEMS Microbiol. Ecology* **39**, 91-100.

75. Gardener, B. B. M. & Weller, D. M. (2001)Changes in populations of rhizosphere bacteria associated with take-all disease of wheat *Applied and Environmental Microbiology* **67**, 4414-4425.

76. Fuller, M. E. & Manning, J. F., Jr. (1997)Aerobic gram-positive and gram-negative bacteria exhibit differential sensitivity to and transformation of 2,4,6-trinitrotoluene (TNT) *Curr. Microbiol.* **35**, 77-83.

77. Boopathy, R., Kulpa, C. F., Manning.J. & Montemagno, C. D. (1994)Biotransformation of 2,4,6-trinitrotoluene (TNT) by co-metabolism with various co-substrates - a laboratory-scale study *Bioresource Technology* **47**, 205-208.

78. Boopathy, R. & Manning.J. (1999)Surfactant-enhanced bioremediation of soil contaminated with 2,4,6-trinitrotoluene in soil slurry reactors *Water Environment Research* 119-124.

79. Kowalchuk, G. A., Bruinsma, M. & van Veen, J. A. (2003)Assessing responses of soil microorganisms to GM plants *Trends in Ecology and Evolution* **18**, 403-410.

80. Bruinsma, M., Kowalchuk, G. A. & van Veen, J. A. (2003)Effects of genetically modified plants on microbial communities and processes in soil *Biology and Fertility of Soils* **37**, 329-337.