FINAL REPORT

Allelochemical Control of Non-Indigenous Invasive Plant Species Affecting Military Testing and Training Activities

SERDP Project RC-1388

OCTOBER 2010

Jorge M. Vivanco
Mark W. Paschke
Colorado State University

Ragan Callaway
University of Montana

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LIST OF ACRONYMNS

AC - Activated carbon
ARS - Agricultural Research Service
DMACA - dimethylaminocinnamaldehyde
DNA - Deoxyribonucleic acid
DOD - Department of Defense
EC50 - Effective concentration (Concentration of a compound that reduces growth by 50% relative to control).
EMS - ethyl methanesulfonate
GO - gene ontology
HPLC - High performance liquid chromatography
HPLC - High-pressure Liquid Chromatography
MB - Methyl benzoate
MB - methyl benzoate
MT - Montana
PCR - Polymerase chain reaction
SERDP - Strategic Environmental Research and Development Program
USDA - United States Department of Agriculture
USFS - United States Forest Service
VOC - Volatile organic compound
WA - Washington
WI - Wisconsin
YTC - Yakima Training Center

ACKNOWLEDGEMENTS

The project participants wish to express their gratitude to the staff of SERDP for providing funds to attain these studies as well for their constant guidance and support through the length of this project. We also thank the numerous undergraduate, graduate students, post-docs and collaborators that participated in this project.

KEYWORDS

Allelochemicals
Allelopathy
Competition
Genomics
Invasive Plants
Knapweed
Metabolomics
Microbes
Phytotoxins
Restoration
Revegetation
Roots
Soil

ABSTRACT

Allelopathy is a relatively controversial concept in the ecological literature as it is hard to quantify in the field. Interest in allelopathy has increased in recent years with reports of invasive plants using allelopathy as an invasion mechanism. The present project aimed to understand this process under a variety of conditions including natural habitats and to utilize this basic knowledge in strategies to control invasive plant species affecting military testing and training. We reasoned that greater knowledge of allelopathy in the context of invasion biology could lead to more sustainable measures of invasive plant control and management. In a series of studies we characterized new allelochemicals produced by a variety of invasive plants. We screened large numbers of native plants for resistance to allelochemicals, and in the process highly competitive species were identified. Additionally, we tested new ideas about using native allelopathic smother crops against invaders. The selection of native plant species capable of establishing within invasive plant infestations was a modest success as shown in greenhouse and field studies.

The project also generated basic knowledge to contribute to the ecological literature by demonstrating that allelopathy is a conditional biological occurrence and that certain biological or environmental triggers must exist to make allelopathy apparent in the field. Similarly this project has contributed basic knowledge on the plasticity of invasive species and how their intrinsic biochemistry related to defense and aggression (invasion) is control by the presence of other neighboring individuals. These basic and applied studies and others described in this report are likely to hold promise for more fully understanding and managing plant invasions.

OBJECTIVES

Our main objective was to develop an understanding of the allelopathic properties of several invasive plants in order to improve methods for controlling these and other invasives. Accordingly, we sought to learn more about the nature of allelopathy and invasiveness, and weak links in the allelopathic chain that might be exploited in order to control the spread of invasive plants. In pursuit of this goal, we investigated the impact of various management strategies on allelopathic invasive species, as well as the duration and long-term effect of allelopathic chemicals in the soil after the invasive's removal. We also worked to determine and describe the mechanisms used by allelopathic plants to neutralize the effects of their own toxins.

Our ultimate purpose was to develop useful products and practical information for direct transmission to participating installations for in-site use. Specific objectives associated with our multidisciplinary project are summarized below.

Isolation and characterization of allelochemicals from invasive plants found on military bases.

Using allelopathy for the control of invasive plants.

Biological degradation of allelochemicals.

Identify native plants that are resistant to allelochemicals.

Explore how various control strategies for spotted knapweed impact the amount of allelochemicals released into the soil and revegetation efforts.

Understand the mechanisms of allelochemical detoxification.

Integrate allelochemical control of invasive species with other proven control strategies.

BACKGROUND

Invasive plant species are a persistent problem for land managers in the western United States. Department of Defense (DOD) installations have special difficulty due to military training activities, which frequently disturb large areas of ground and lead to infestations of invasive plants that degrade environmental quality. The DOD strives to manage its lands responsibly and in line with federal environmental regulations while continuing to test and train troops. In order to continue to perform large-scale training operations, it is in need of an effective, economical, and ecological method for combating various invasive plants. The knapweeds (*Centaurea maculosa* L., *Acroptilon repens* (L.) DC., *C. diffusa* Lam.) are among the worst invasive offenders in the U.S., infesting over 4.3 million hectares in 14 western states and two Canadian provinces (DiTomaso 2000, Sheley et al. 1998). Along with leafy spurge (*Euphorbia esula*) and Canada thistle (*Cirsium arvense*), these invasive rangeland plants are notorious for their ability to negatively affect soil quality through the release of natural plant toxins known as allelochemicals among other qualities that make these plants good invaders (Hierro and Callaway 2003, Steenhagen and Zimdahl 1979, Kazinczi et al. 2001).

Although the economic and biological costs associated with exotic plant invasion are large, the mechanisms of plant invasion remain poorly understood (Levine et al. 2003; Pimentel et al. 2000). The introduction of exotic plant species can be devastating to native ecosystem biodiversity, leading to extinction of native plant species (Pimentel et al. 2000) or major alterations in the structure of higher trophic levels (Levine et al. 2003). In addition, exotic plant invasion can change general ecosystem properties such as nutrient cycling, hydrology and fire regimes (Levine et al. 2003). For the most part, studies related to the impacts of exotic plant invasion have focused on above ground flora and fauna (Levine et al. 2003). However, there has been recent interest in determining how invaders impact below ground soil microbial community structure and function (Klironomos 2002; Reinhart and Callaway 2004; Wardle 2006; Wardle et al. 2004; Wolfe and Klironomos 2005).

Allelochemistry (plant secretion of phytotoxins) has recently re-emerged as a possible mechanism for the success of some invasive weeds (for example see: Callaway and Aschehoug 2000). As proposed here, we believe that integrated research strategies that recognize the importance of allelochemicals in invasions can suggest novel approaches to fighting exotic invaders. For example, just as weeds are able to evolve resistance to man-made herbicides, native species can possess or evolve resistance to allelochemicals from invasive species (Callaway and Ridenour 2004). Constitutive and evolved resistance in native species to the allelopathic effects of invaders might be used to reduce our reliance on synthetic treatments, and to add to the arsenal of insect biocontrol, cultural practices, and mechanical methods we currently have available for integrated weed management systems.

The rationale for initiating this project was based on studies published by the Vivanco and Callaway laboratories reporting high concentrations of catechin (an allelochemical purported produced and released by *C. maculosa* - Spotted knapweed) in soils surrounding spotted knapweed. Upon the start of the project, we embarked on an expedition to determine the kinetics of catechin presence in the soil throughout the year (Perry et al. 2007). Through these studies,

we found that catechin is present in the soil at very low concentrations during the year but that at specific time points the concentrations of catechin in the soil could be rather high - similar to the results reported prior to start of the project. In addition, several laboratories including the Vivanco lab had difficulty re-isolating catechin from the root exudates of spotted knapweed plants grown *in vitro*. Recently, the riddle has been partially solved; a third party laboratory discovered that spotted knapweed roots release catechin during the day and that this compound decreases its concentration in the exudates through the night (Tharayil and Triebwasser, 2010). The reason for this cyclic secretion has not been unraveled but it resembles the situation that we uncovered in the field.

Other studies performed during the project focused on the isolation of new phytotoxins produced by other invasive species and testing the effect of those toxins on suite of native plants. Through this approach, we sought to identify native plants could be better competitors against invasives in the field.

Our project investigated the mechanisms of plant invasions with the ultimate goal of developing economic, ecologically benign methods of control and advancing scientific understanding. The project made progress on numerous fronts one of those being the development of novel ways to restore native plant communities after invasive plant suppression. The desired condition of natural plant communities is for them to be sustainable and capable of resisting future invasions, which has the added benefits of maintaining native grassland diversity and providing habitat for wildlife. We conducted experiments in which we seeded areas with collections of native species that were developed to maximize their invasive plant resistance. This approach is potentially powerful, yet underutilized, in restoration contexts. Additionally, we sought to improve our understanding of the nature of invasiveness by creating the first cDNA library of invasive spotted knapweed, which would allow us to investigate the genomic basis of invasiveness to an extent previously impossible. .

The present project was initiated due to a belief that allelopathy may be an important reason for the success of some invasive species such as spotted knapweed. However, research performed during the course of the project has revealed crucial aspects of allelopathy that are worth studying further. For, instance, allelopathy is conditional, allelopathy is not due to a single compound, the stability of an allelochemical in the soil is of crucial importance, allelopathy depends on the presence of given neighbors and on plant density, allelopathy might be indirect and through the negative effect on soil microbial populations. All these aspects are explained in detail in this project,

METHODS AND RESULTS

ISOLATION AND CHARACTERIZATION OF ALLELOCHEMICALS FROM INVASIVE PLANTS

Besides the knapweeds, there are other invasive species of importance to western U.S. military bases that may possess allelochemical properties. There is evidence in the literature that leafy spurge, yellow star thistle and Canada thistle have allelopathic properties. We worked to characterize these allelochemicals, and screen additional invasive plants present in military sites for useful allelochemicals. Studies were related to the isolation and characterization of phytotoxins (allelochemicals) produced by a variety of invasive plants. The isolation of allelochemicals was conducted from the root exudates of plants cultured under laboratory conditions or using the roots of field collected plants. Our general approach was to examine the phytotoxicity of crude and solvent-extracted root exudates against the model plant *Arabidopsis thaliana* under *in vitro* conditions in liquid media and then purify and identify the phytotoxic compounds.

To obtain root exudates, seeds of the invasives leafy spurge and yellow starthistle and the N. American species mule's ears, Canada goldenrod, common milkweed, annual ragweed, and Canadian horseweed were surface-sterilized and germinated on solid MS media. Fifty seedlings were transferred into 400 ml of liquid MS media in 1 L flasks. After 6 wks, the plants were treated with chitosan, a root exudate elicitor. The MS media and root exudates were collected 3 d later. To obtain chloroform and ethyl acetate extracts of the root exudates, a 250 ml portion of the crude root exudates was mixed with 250 ml chloroform. The chloroform layer was then concentrated under a vacuum. The remaining 250 ml of exudates were mixed with 250 ml ethyl acetate, and the ethyl acetate layer was concentrated under a vacuum. The remaining water phase was then concentrated under a vacuum. To assess the phytotoxicity of crude root exudates and their extracted fractions, seeds of bioassay plants were surface-sterilized and germinated on solid MS media. Seven day-old plants were transferred into 1 ml of liquid MS media in 12 or 24-well plates. After 24 h, the plants were treated with varying concentrations of crude or extracted root exudates, with 4 replicates per treatment. The crude root exudates and water phase were applied directly to the liquid media in which the plants were growing. The chloroform and ethyl acetate extracts were resuspended in methanol, filtered, and applied to fresh 12-well plates. The methanol was then allowed to evaporate to avoid effects on the plants, after which 1 ml of liquid MS media was added to each well and the plants were transferred to the wells. Plants were blotted dry and weighed 7 d after treatment.

<u>Root exudates of the invasive yellow starthistle have only weak activity</u>

Phytotoxicity bioassays suggested that *Centaurea solstitialis* L. (yellow star-thistle), an invasive plant in western North America, does not rely on phytotoxic root exudates for invasion of California grasslands. Treatment with crude root exudates and chloroform-extracted root exudates from *C. solstitialis* reduced growth of the model plant *Arabidopsis thaliana*. However, high concentrations of the exudates (50% v/v or 500 $\mu g\ ml^{-1}$) were required to inhibit *A. thaliana* growth and did not result in *A. thaliana* mortality, suggesting the presence of only a weak growth inhibitor (Figure 1). Moreover, high concentrations of *C. solstitialis* crude root exudates did not affect the growth of six native grass species often displaced by *C. solstitialis* invasions in California grasslands (Qin et al.2007).

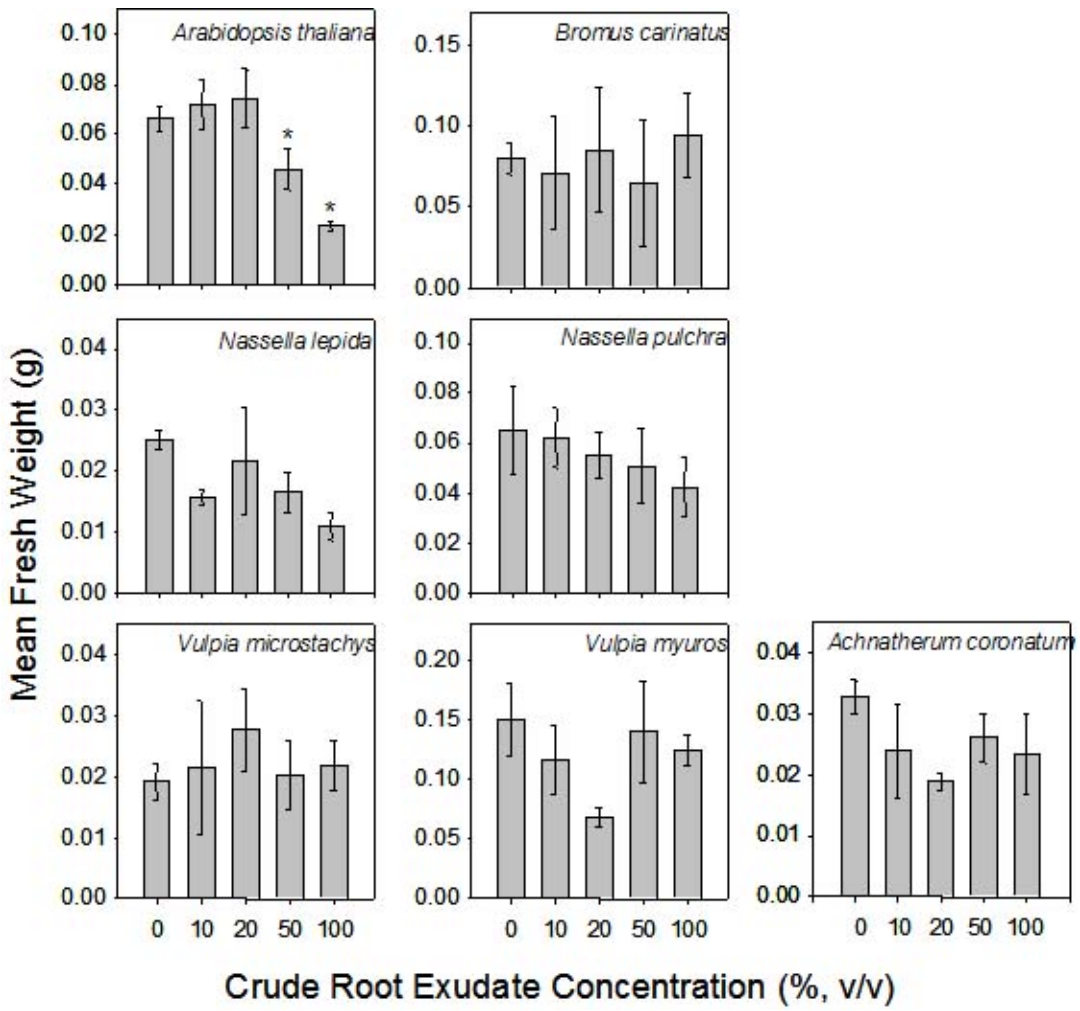

Figure 1. Yellow starthistle crude root exudates have little effect on growth of the model plant *Arabidopsis thaliana* and six native California grasses commonly displaced by yellow starthistle. (*) Indicates a mean significantly lower than the control. Error bars are one s.e.

<u>Root extracts and exudates of the invasive plant leafy spurge contain phytotoxins</u>

We conducted a bioactivity-driven fractionation of root extracts and exudates from the invasive plant leafy spurge (*Euphorbia esula* L.), and structurally characterized jatrophane diterpenes and ellagic acid derivatives. Ellagic acid derivatives and one of the jatrophane diterpenes, esulone A, have been previously reported from leafy spurge, but another of the jatrophane diterpenes, kasuinine B, has not. We found that these compounds are phytotoxic but affect plants in different ways, either inducing overall plant necrosis or reducing root branching and elongation. Several of the compounds from leafy spurge roots were also present in leafy spurge root exudates, suggesting that these compounds could accumulate in the leafy spurge rhizosphere as well as leaching into the soil from decaying roots. Moreover, leafy spurge root exudates exhibited phytotoxic activity, suggesting that leafy spurge roots may exude sufficient quantities of phytotoxic compounds to influence other plants. Further studies are needed to determine

whether any of these compounds are present in the leafy spurge rhizosphere or bulk soil. Thus, research is also needed to examine the effects of these compounds on native North American plants displaced by leafy spurge at concentrations produced by leafy spurge under natural conditions. Of particular interest is the observation that kansuinine B and trimethylellagic acid were both present in the chloroform-extracted leafy spurge root exudates, since these compounds seem to have complementary activities. Under natural circumstances these two types of compounds might act in coordination in the rhizosphere resulting in an overall phytotoxic effect on neighboring plants. However, further ecological studies are needed to justify this claim (Qin et al. 2006).

Allelopathic potential of *Solidago canadensis*

Solidago canadensis L. (Canada goldenrod) is an exceptionally successful worldwide invader of North American origin that has to date conquered Europe, large parts of Asia, Australia and New Zealand. However, the traits enabling the species to successfully establish in natural ecosystems around the world and dominate the new ecosystems remain unclear. Studying this plant is of outmost importance for the revegetation process in North America because it could be a very strong competitor of invasive plants from Eurasia.

One mechanism explaining the success of invasives may be the production and release of allelopathic compounds by the invader that, due to a lack of co-evolutionary history, have harmful effects on plant neighbors in the introduced range. We partially tested this hypothesis by growing seven competing native European plant species either with the introduced *Solidago canadensis*, one of the most successful invasive plants in Europe or on soil precultivated with *S. canadensis*. We added activated carbon to the soil to neutralize organic chemical compounds with putative allelopathic effects. Furthermore, we added unsterilized soil inocula from the introduced (Switzerland) or native (USA) range to the soil to test potential confounding effects of soil microbes on invasion success. Untreated sterilized soil served as control. Five out of the seven native species were more competitive against the invasive species in soils with activated carbon than without, supporting the allelopathy hypothesis. However, competitive outcomes were also influenced by the two sources of soil inoculum and by interactive effects of soil inoculum and *Solidago* origin suggesting that soil microbes alter allelopathic effects. *Achillea millefolium*, the species least affected by the presence of *Solidago canadensis* and with no response to the activated carbon treatment is the only species used in this experiment reported to grow within *Solidago* stands in Europe, whereas the other European species tested tend to grow at the periphery of invasive *Solidago* stands.

The crude extract of the root exudates of *Solidago* showed a significant inhibitory effect on growth of *Arabidopsis*. The magnitude of inhibition was directly proportional to the concentration of the extract added. The growth of *Arabidopsis* seedlings decreased when the *Solidago* exudates were applied in doses between 25 and 250 µg/ml, whereas complete mortality occurred at 500 µg/ml. A double blind chemical analysis by LC-MS of *Solidago* root extracts revealed four main chemical compounds that had different molecular masses and were consistently present in the 40 different *Solidago* samples analyzed. The four chemical substances were named after their molecular mass (MS). Polyacetylenes and diterpenes have already been isolated from roots of *S. canadensis*. However, the molecular masses of the compounds isolated

in this study are higher than any of the previously detected compounds present in the roots. The UV spectrum suggests that the compound with mass 517 is a polyacetylene derivative maybe with sugar residues incorporated which could increase the water solubility of the compounds and thus increase the molecular mass while other compounds isolated in this study may be diterpene lactone derivatives. The secondary chemical compound that occurred at lowest concentrations (MS 517) did not significantly differ between native and invasive (diploid) populations or among different ploidy levels in the native range; but the three other compounds showed different concentrations among ploidy levels and in the case of MS 349 and MS 363 between diploid native vs. invasive populations. However, none of the four compounds had a higher concentration in invasive populations compared with plants from the native range. MS 349 and 363 were present in the highest concentrations in the native American hexaploid and tetraploid populations, whereas in MS 335 and MS 517 the concentration was highest in native American diploid plants. In invasive, diploid populations, the concentration of all four compounds was on average about half the concentration (albeit with variation among populations) of that observed in diploid native American plants. This suggests lower investment by invasive plants into secondary compounds and raises the question of a higher susceptibility of plant competitors in the invasive than in the native range to these putatively allelopathic substances (Abhilasha et al. 2008).

Isolation of allelochemicals from Russian Knapweed

Acroptilon repens (L.) DC (Russian knapweed; formerly *Centaurea repens*) is a perennial herbaceous plant belonging to the family Asteraceae. Its highly competitive nature and broad ecological adaptability make it a persistent problem in North America. This plant is native to Mongolia, western Turkestan, Iran, Turkish Armenia and Asia Minor, and was introduced into North America in the early 1900's, primarily as a contaminant of Turkestan alfalfa (*Medicago sativa*) seeds. This plant is now widely distributed in the United States, and has been reported in 22 western and mid-western states where it competes successfully with agricultural/range crops and native plants. Like most invasive species, *A. repens* primarily invades disturbed ecosystems, and it has invaded about 1.5 million acres in North America and each year its territory expands by roughly 8%.

A bioassay-guided fractionation of the root extracts of this plant led to the isolation of five polyacetylenic compounds, of which one [5′-methoxy-1′-(5-prop-1-yn-1-yl-2-thienyl)-hexa-2′,4′-diyin-6′-yl acetate] was previously unreported. The structures of these compounds were elucidated on the basis of spectroscopic analysis (IR, ESIMS, ^{1}H, ^{13}C NMR and 2D NMR). All of the compounds obtained, except 1-chloro-4-(5-penta-1,3-diyn-1-yl-2-thienyl)but-3-yn-2-ol, showed phytotoxic activity against *Arabidopsis thaliana* seedlings. The presence of 4′-chloro-1′-(5-penta-1,3-diyn-1-yl-2-thienyl)-but-2′-yn-3′-ol was detected in the root exudates of aeroponically grown *A. repens* plants. None of the polyacetylenes isolated in this study were found in Colorado soils collected between September 2006-July2007 in an *A. repens* colonized site. However, one specific type of polyacetylene [1 -(5-penta-1,3-diyn-1-yl-2-thienyl)-but-2′-yne-3′,4′-diol] was found in *A. repens* infested soil from Yakima, Washington in June 2007. In this soil we also detected two other polyacetylenes (M_r: 354 and 386), whose structures could not be further investigated due to a lack of material, but whose UV spectra were consistent with those of typical polyacetylenes. These compounds may represent conjugates. We have not

analyzed roots or root exudates from *A. repens* found growing at Yakima, WA soils, so there could be qualitative or quantitative differences in polyacetylene content or processing between the Washington and Colorado *A. repens* populations. Root exudation may be influenced by several factors in natural ecosystems, and different soil conditions or microorganism content could affect the stability of exuded polyacetylenes at different sites. Additionally, it is quite possible that different ecotypes of *A. repens* could produce and secrete slightly different quantities of phytotoxins. Furthermore, UV light causes the rapid degradation of polyacetylenes, thus suggesting that the amount of sunlight at different sights could affect the stability and lifespan of these compounds in the soil. An in-depth analysis of different soil and plant types or environmental conditions between the two sites of collection (Colorado and Washington) and independent study of root exudations of the Washington population are needed to clarify these results. Contrary to previous reports the compound 7,8-benzoflavone was not detected in root exudates, nor was it encountered in extracts of roots, aerial parts or infested soil (Quintana et al. 2008).

A selective, sensitive, and rapid in-field assay for soil catechin

*S*potted knapweed root exudates have been found to possess phytotoxic properties, these exudates have been found by chemical analysis to contain catechin, and catechin supplied exogenously was found to inhibit growth and germination at concentrations reported to be exuded from spotted knapweed under lab and field conditions. However, there is a great deal of variation in the levels of catechin recovered as root exudate from both lab and field studies, with some groups reporting only trace levels while others report milligram quantities per gram of soil. Further, a recent study found that soil catechin concentrations can vary from very high to absent from one month to the next. The interpretation of these results are complicated by the fact that catechin is a relatively unstable compound, is often found at relatively low concentrations, interacts with soil cations to form insoluble complexes and/or degradation products, and exhibits extremely variable accumulation patterns. This variation in soil catechin concentrations suggests that frequent measurements at multiple sites, monitoring at daily or even hourly intervals, may be required to understand catechin dynamics in spotted knapweed soils, and thus the role of catechin in spotted knapweed invasion. However, such a large study would be difficult given the time and cost of soil extraction and analysis by current methods. A faster, less expensive method for soil catechin detection is needed. We developed a novel method that utilizes the colorimetric reagent dimethylaminocinnamaldehyde (DMACA) in an acidic ethanol solution for detection of soil catechin. This method is selective and extremely sensitive and can be used in the field for qualitative, but not quantitative, analysis. This assay will allow for a greater understanding of the role of catechin as an allelochemical (Broeckling et al. 2008).

Isolate and characterize allelochemicals from other invasive plants

Previously it has been shown that the floral scent of snapdragon flowers consists of a relatively simple mixture of volatile organic compounds (VOCs). These compounds are thought to be involved in the attraction of pollinators; however, little is known about their effect on other organisms, such as neighboring plants. We found that VOCs from snapdragon flowers inhibit Arabidopsis root growth. Out of the three major snapdragon floral volatiles, myrcene, E-B-ocimene, and methyl benzoate (MB), MB was found to be primarily responsible for the

inhibition of root growth. Ten umol MB reduced root length by 72.6%. We employed a microarray approach to identify the MB target genes in Arabidopsis that were responsible for the root growth inhibition phenotype in response to MB. These analyses showed that MB treatment affected 1.33% of global gene expression, including cytokinin, auxin and other plant-hormone-related genes, and genes related to seed germination processes in Arabidopsis. Accordingly, the root growth of cytokinin (*cre1*) and auxin (*axr1*) response mutants were less affected than that of the wild type by the volatile compound: roots of the treated mutants were reduced by 45.1% and 56.2%, respectively, relative to untreated control mutants. Interestingly, two other members of the Scrophulariaceae family, yellow toadflax (*Linaria vulgaris*) and Dalmatian toadflax (*Linaria genistifolia* spp. *dalmatica*), are known to be aggressive invasive plants in North America, and may present valid models for testing the biological effects reported in this study under realistic field conditions, if they emit MB or other allelopathic VOCs (Horiuchi et al. 2007).

Three compounds were isolated from Canada thistle roots collected in the field. These compounds were isolated based on abundance and phytotoxicity. These were: Siringin, β-sitosterol and aplotaxene. Siringin and β-sitosterol were isolated from Canada thistle for the first time. Of these, only siringin showed moderate phytotoxic activity.

ALLELOPATHY FOR THE CONTOL OF INVASIVE PLANTS

We investigated the phytoxicity and synergism of the combination of several allelochemicals and their potential for practical applications in invasive plant management. When this approach proved ineffective we switched gears to explore the use of native allelopathic species for the control of invasives. We followed this line of investigation based on reports of these allelopathic North American species invading European plant communities.

We tested various knapweed allelochemicals as foliar herbicides. However, these initial experiments were not successful, due to the low stability and solubility of the allelochemicals when used as foliar herbicides. Accordingly, we changed our focus to explore allelochemicals as soil-applied herbicides and to use native allelopathic plants as a control measure for invasives. To test the efficacy of (±)-catechin and 7,8-benzoflavone as soil drenches for invasive plant control, we conducted a greenhouse experiment examining effects of the phytotoxins alone and in combination on five important invasive plants: *Bromus tectorum* L. (cheatgrass), *Centaurea solstitialis* L. (yellow star thistle), *Cirsium arvense* L. (Canada thistle), *Phragmites australis* (Cav.) Trin. ex Steud. (common reed), and *Salsola tragus* L. (Russian thistle). We applied (±)-catechin (allelochemical of spotted knapweed), 7, 8-benzoflavone (allelochemical of Russian knapweed), and a mixture of the two compounds to germinated seedlings at four concentrations (0, 0.25, 0.50, and 1.00 mg/ml) biweekly to determine the most effective rates of application for invasive plant control. In conjunction with this experiment, we collected and analyzed soil samples to create a time series of phytotoxin concentrations following application in order to predict the persistence of (±)-catechin and 7,8-benzoflavone activity in soil drenches and to understand potential effects of different invasive plants on phytotoxin degradation. Aboveground and belowground biomass was harvested eight weeks after the first dose. Results indicated that there were no significant effects of the phytotoxins on the invasive plants. As indicated below in the report, it appears that allelochemicals degraded rapidly in this

experimental system, suggesting that duration of exposure may have limited any potential phyotoxic effects.

We subsequently explored the implications of these results with additional studies (see below). We have learned that *in situ*, these allelochemicals may be short-lived in soil and may be released in seasonal and spatially localized pulses. Therefore, soil drenches with these "average" soil concentrations may be ecologically unrealistic. Additionally, we suspect that the greatest hope for finding potent allelochemicals for combating invasive species may rest not with invasive species, but rather with our native allelopathic species. These native species should produce allelochemicals that are novel for combating invasive species.

Native allelopathic plants as smother crops for control of invasives

Planting cover crops to compete with invasive plants has been explored in agriculture and ecological restoration (Blackshaw et al., 2006; Landhausser et al., 1996; Ledgard & Davis, 2004; Morgan, 1997; Perry & Galatowitsch, 2003; Sheley et al., 2006; Shirley, 1994; Singh et al., 2003). However, cover crops often fail to improve desired species success, mainly because they do not act selectively (De Haan et al., 1994; Hoffman et al., 1993; Lanini et al., 1991; Ledgard & Davis, 2004; Perry & Galatowitsch, 2003). As our understanding of allelopathy as a mechanism of invasion matured during the early phase of our project, we realized that allelopathic cover crops, might be more likely to act selectively against non-native plants, while allowing native, desired species to establish. In native undisturbed plant communities, allelopathy may be relatively ineffective, since plant species that frequently interact with allelopathic plants would be expected to develop resistance to the allelochemicals over time (Fitter, 2003). In contrast, in novel interactions between native and introduced species, allelopathy may be more intense, since neither species has had time to develop resistance to the novel allelochemicals (Rabotnov, 1982). This logic forms the basis of the novel weapons hypothesis, which posits that some invasive species are so successful because they are allelopathic and produce phytotoxins that are novel to the native species in their invaded range (Callaway & Ridenour, 2004). By the same logic, however, native species that are allelopathic may be particularly effective against invasive species, which have not had time to develop resistance to native allelochemicals. Those same native allelochemicals would be expected to have relatively little effect on other native species, which have had the time to develop resistance.

Many North American rangeland species are thought to be allelopathic, including annual ragweed (*Ambrosia artemisiifolia*), common sunflower (*Helianthus annuus*), Canada goldenrod (*Solidago canadensis*), and littleleaf pussytoes (*Antennaria microphylla*). Annual ragweed, which is invasive in Europe (Beres et al., 2002), produces phytotoxic phenoloids and terpenoids that inhibit germination and seedling growth of several cultivated species (Bruckner, 1998). Annual ragweed root exudates and shoot extracts also inhibit germination and growth of early-seral species, but not later-seral species, in native North American habitats (Jackson & Willemsen, 1976). Common sunflower, which is naturalized in Europe (Faure et al., 2002), also produces a number of phytotoxic compounds that inhibit a variety of plant species (Azania et al., 2003; Beres & Kazinczi, 2000; Irons & Burnside, 1982; Leather, 1983; Macias et al., 1998; Macias et al., 1998; Maruthi & Sankaran, 2001; Morris & Parrish, 1992; Ohno et al., 2001), including early-seral species, but not later-seral species, in native North American habitats (Wilson & Rice, 1968). Canada goldenrod, which is invasive in Europe, produces a phytotoxic,

long-chain polyacetylene, cis-Dehydromatricaria ester (Tsao & Eto, 1996), and our previous studies further elucidated the role of allelopathy in the invasiveness of this species (Abhilasha et al. 2008). Finally, littleleaf pussytoes produces two phytotoxic phenolic acids, hydroquinone and caffeic acid, that inhibit germination and seedling growth of the invasive species leafy spurge (*Euphorbia esula*) and may allow littleleaf pussytoes populations to resist leafy spurge invasion (Barkosky et al., 1999; Barkosky et al., 2000; Hogan & Manners, 1990; Manners & Galitz, 1986).

In a greenhouse experiment, we examined the effectiveness of four native and putatively allelopathic smother crops for controlling four invasive species and increasing success of four western North American grassland species (desired species). The four invasive species were grown in pair-wise mixtures with the four desired species to observe effects of the invasive species on the desired species. In addition, each invasive species x desired species pair was grown with and without each of the four potential cover crops to determine whether the cover crops improved desired species success in competition with the invasive species (Table 1). Each of the invasive species also was grown without a desired species, with and without each cover crop, to examine effects of the cover crops on the invasive species specifically. Activated carbon, which adsorbs organic compounds in soil, was used to test for a role of allelochemicals in the plant interactions. Finally, each of the 12 species also was grown alone with and without activated carbon to test for direct effects of the carbon. Each of the 172 treatment combinations (four cover crops x four invasive species x four desired species x two activated carbon treatments, four cover crops x four invasive species x two activated carbon treatments, 12 species x two activated carbon treatments) was replicated ten times. Six months after transplanting, aboveground biomass in the cones was harvested, separated among individuals, dried at 60°C to constant mass, and weighed. Plant mortality also was recorded.

Table 1. Cover crops, invasive species, and desired species included in the greenhouse cover crop experiment.

Group	Common Name[1]	Scientific Name	Family	Life History
Cover crops	annual ragweed	*Ambrosia artemisiifolia* L.	Asteraceae	annual
	common sunflower	*Helianthus annuus* L.	Asteraceae	annual
	Canada goldenrod	*Solidago canadensis* L.	Asteraceae	perennial
	littleleaf pussytoes	*Antennaria microphylla* Rydb.	Asteraceae	perennial
Invasive species	cheatgrass	*Bromus tectorum* L.	Poaceae	annual
	Japanese brome	*Bromus japonicus* L.	Poaceae	annual
	Canada thistle	*Cirsium arvense* (L.) Scop.	Asteraceae	perennial
	whitetop	*Cardaria draba* (L.) Desv.	Brassicaceae	perennial
Desired species	hairy false goldenaster	*Heterotheca villosa* (Pursh) Shinners	Asteraceae	perennial
	green needlegrass	*Nassella viridula* (Trin.) Barkworth	Poaceae	perennial
	western wheatgrass	*Pascopyrum smithii* (Rydb.) A. Löve	Poaceae	perennial
	upright prairie coneflower	*Ratibida columnifera* (Nutt.) Woot. & Standl.	Asteraceae	perennial

[1]Nomenclature follows the USDA plants database (http://plants.usda.gov/).

Results suggested that the annual smother crops might be effective for controlling invasive annuals and facilitating native perennials. Planting annual ragweed (*Ambrosia artemisifolia*) and common sunflower (*Helianthus annuus*) reduced biomass of the invasive species cheatgrass (*Bromus tectorum*), Japanese brome (*Bromus japonicus*), Canada thistle (*Cirsium arvense*), and whitetop (*Cardaria draba*). The annual smother crops also reduced desired species biomass in competition with the perennial invasives, but either increased or did not affect desired species biomass in competition with the annual invasives. Planting the perennial smother crops, Canada goldenrod (*Solidago canadensis*) and littleleaf pussytoes (*Antennaria microphylla*), rarely inhibited invasive species, but did increase desired species biomass. Our hypothesis that the smother crops would inhibit invasive species via allelopathy was only partially supported in this study. In most cases, activated carbon did not influence effects of the smother crops on the invasive species or the desired species, suggesting that organic compounds did not have a role in those interactions. In only two cases, the activated carbon treatment suggested that organic compounds may have been responsible for negative effects of smother crops on invasive species: effects of annual ragweed on Canada thistle and effects of common sunflower on cheatgrass in the presence of western wheatgrass. Conversely, the activated carbon treatment also suggested that organic compounds may have moderated the negative effect of common sunflower on cheatgrass in the presence of green needlegrass. Activated carbon did not influence biomass in the species monocultures, indicating that effects of activated carbon on species interactions were not caused or masked by direct effects of activated carbon on other environmental conditions besides allelochemicals. Thus, our results suggest that allelopathic effects of the smother crops were rare in our experiment and strongly dependent on target species identity and community composition. Results from this greenhouse study were reported in Perry et al. (2009). Our subsequent field experiments (see below) tested the efficacy of these smother crops under more ecologically relevant field conditions.

BIOLOGICAL DEGRADATION OF ALLELOCHEMICALS

In order to effectively restore sites invaded by invasive allelopathic plants, or to use allelochemicals as herbicides, it was important for us to understand the persistence of these compounds in soils. Therefore the relative degree of persistence of the various allelochemicals in a variety of soils was determined. This was a long-standing question in allelopathy research and to the best of our knowledge the presence of any given allelochemical in a natural soil has never been followed through a long time course.

In situ persistence and degradation of allelochemicals from spotted knapweed

We monitored the concentrations of this allelochemical (catechin) at the same site in Missoula by sampling rhizosphere soil on a regular basis throughout parts of 2005 and 2006. This work was reported in Perry et al. (2007). In the summer of 2005 we didn't find any catechin in several field sites in Montana with similar results in 2006 through April. However, we were able to detect catechin in May 2006. The highest concentration we found in May 2006 was quite high: 2.18 mg/g. However, most were lower than that, with a mean of ~0.5 mg/g. From these results we concluded in the 2007 publication (Perry et al. 2007) that secretion of this compound may be seasonal and may even vary by year. At this point, it does not appear that catechin is a long-

lived compound in soils. Subsequent attempts by us to find catechin in soils beneath spotted knapweed at Fort McCoy, WI were unsuccessful.

Because (±)-catechin is a chelator, another approach we took was to compare the effects of pure (±)-catechin to (±)-catechin-metal complexes. We found that the presence of metals causes a rapid decline in the concentration of pure (±)-catechin in solution. But very importantly, some (±)-catechin-metal complexes were more phytotoxic than (±)-catechin alone (Figure 2) (Pollock et al. 2009). In contrast, other (±)-catechin-metal complexes were less toxic than pure (±)-catechin. Our results, which were published in 2009 (Pollock et al. 2009) demonstrate substantial conditionality in the effects of a root-exuded chemical due to just one aspect of soil chemistry, and the toxicity of chelation-derived complexes of (±)-catechin indicates that the allelopathic effect of the exudate does not depend on high concentrations of the pure form in the soil.

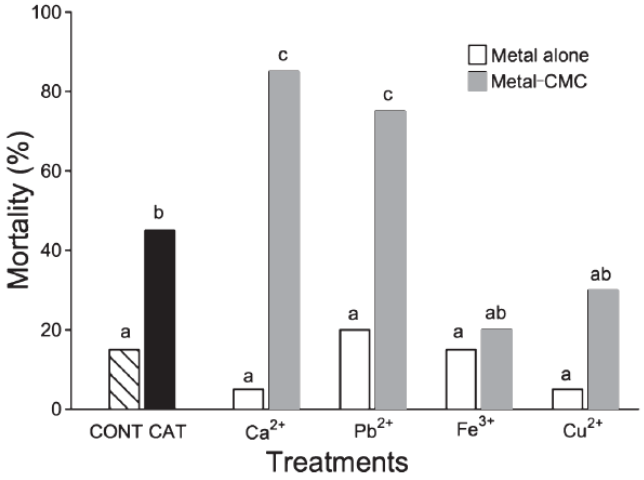

Figure 2. Percent mortality of *Festuca idahoensis* and *Koeleria macrantha* (combined) under drought-like conditions for controls with no catechin added, CONT; catechin added, CAT; metals-alone, Metalalone; and CMCs, Metal-CMC. Shared letters above designate no significant differences among means ($P < 0.05$) as determined from paired logistic regression comparisons (SPSS 15.0, 2006).

Stability and phytotoxicity of catechin in different soils

We conducted experiments in India and the USA to understand the dynamics of soil concentrations and phytotoxicity of catechin to other plants *in vitro* and in soil (Inderjit et al. 2008). Experiments with single and pulsed applications into soil were conducted in the field. Experimental application of catechin to soils always resulted in concentrations that were far lower than the amounts added but within the range of reported natural concentrations. Pulses replenished catechin levels in soils, but consistently at concentrations much lower than were applied, and even pulsed concentrations declined rapidly. Different natural soils varied substantially in the retention of catechin after application but consistent rapid decreases in concentrations over time suggested that applied experimental concentrations might overestimate concentrations necessary for phytotoxicity by over an order of magnitude. Catechin was not phytotoxic to *Bambusa arundinacea* (bamboo) in natural Indian soil in a single pulse, but soil concentrations at the time of planting seeds were either undetectable or very low. However, a single dose of catechin suppressed the growth of bamboo in sand and in soil mixed with organic matter. Catechin also suppressed the growth of *Koeleria macrantha* in soils from Montana and Romania, and in field applications at 49 ug/l. Multiple pulses of catechin were inhibitory at very low concentrations in Indian soil.

In situ persistence and degradation of the allelochemical from Russian knapweed

Soils were collected from *Acroptilon repens* (L.) DC. (Russian knapweed) infestations from Colorado and Wyoming in 2005 and 2006 for analysis of the allelochemical of *A. repens*, 7, 8-benzoflavone. A high performance liquid chromatography (HPLC) method was developed to detect this compound in soil. The maximum observed concentration of 7, 8-benzoflavone, 82 μg g^{-1} soil, occurred in early spring of 2005 but by summer no 7, 8-benzoflavone was detected. 7, 8-Benzoflavone was not detected at any site during the relatively dry spring of 2006. This pattern may indicate that 7, 8-benzoflavone production varies during growing season, and that it

might be used by Russian knapweed to inhibit the early developmental stages of other species and function as an interspecific inhibitor. Additional soil samples were collected in 2009 from beneath Russian knapweed infestations at Yakima Training Center, WA. We did not find any 7, 8-benzoflavone in these samples.

Degradation of catechin and 7,8-benzoflavone in artificial soil

Allelochemicals produced by invasive plants may have persistent effects on native plant communities; even after invasive plant populations are controlled. Information on degradation rates of allelochemicals in soil is necessary for predicting whether allelochemicals from invasive plants will have such persistent effects and for developing management strategies to address those effects. We measured loss rates of two allelochemicals, catechin and 7,8-benzoflavone, produced by spotted knapweed and Russian knapweed (*Acroptilon repens*), respectively, in an artificial soil in a greenhouse.

The experiment was conducted using 3.8-cm-diameter-by-21-cm-depth pots filled with a 1:1:1 mixture of silica sand, fine vermiculite, and calcine clay. The allelochemicals, catechin and 7,8-benzoflavone, were applied in powder form to the soil surface of separate pots at dose rates of 10 and 5 mg pot^{-1}, respectively. Each of the two chemical treatments (catechin, 7,8-benzoflavone) was replicated 25 times. The experiment was arranged in a blocked, split-plot design, with five blocks; the two chemical treatments were grouped in two "whole plots" within each block. The pots were watered as needed throughout the experiment to maintain moist soil conditions while minimizing leaching from the bottoms of the pots. Also, they were fertilized weekly with a complete nutrient solution.

The day after chemical application, and again two, four, six, and eight weeks after application, five replicates of each treatment (one replicate per block) were harvested and extracted to quantify allelochemical concentrations. To collect samples for extraction, the soil in the top 10 cm of each pot was collected and homogenized. A 3-cc subsample of this soil was placed in 10 ml methanol. Then, the remaining soil in each pot was homogenized and a 3-cc subsample of the lower soil was placed in 10 ml methanol. The samples were stored in methanol at 4°C until processed.

To prepare the samples for analysis, the samples were shaken for 12 hours on a rotary shaker and then centrifuged for five minutes at 7,000 rpm. The supernatants were transferred to fresh tubes and another 5 ml methanol was added to the remaining soil. The samples in 5 ml methanol were shaken for another 12 hours on a rotary shaker and centrifuged for five minutes at 7,000 rpm. The resulting supernatants were added to the earlier supernatants, concentrated under blown N_2, rinsed twice with 0.75 ml methanol, transferred to fresh tubes, centrifuged for five minutes at 14,000 rpm, transferred again to fresh tubes, concentrated under blown N_2, resuspended in 0.4 ml methanol, and stored at 0°C until analysis. The extracted soil samples were dried at 65°C to a constant weight and weighed to determine concentrations on a per g dry soil basis.

Catechin and 7,8-benzoflavone concentrations were quantified by HPLC and compared to 1 mg ml^{-1} standards. HPLC separations used mobile phase solutions of (A) 1% acetic acid in distilled water and (B) absolute methanol, with a multistep gradient of 0-5 min, 5% B; 5-15 min, increased to 20% B; 15-20 min, 20% B; 20-40 min, increased to 100% B; 40-50 min, 100% B;

50-55 min, 5% B. The column was a reverse phase, 5 µm C_{18} (4.6 x 150 mm) (Dionex Corp., Sunnyvale, CA), the flow rate was 1 ml min^{-1}, the sample injection volume was 20 µl, and absorbance was measured at 280 nm.

No catechin was found in the samples even just one day after chemical application, suggesting very rapid degradation, binding, or leaching of catechin from the artificial soil. In contrast, 7-8-benzoflavone persisted in the artificial soil throughout the eight-week experiment (Figure 3). 7,8-Benzoflavone concentrations were much greater in the upper portions of the pots, where the 7,8-benzoflavone was added, than in the lower portions of the pots, suggesting that 7,8-benzoflavone had limited mobility in the soil. In the upper portions of the pots, 7,8-benzoflavone concentrations declined slowly over the course of the experiment, indicating that 7,8-benzoflavone slowly degraded in or leached from the artificial soil. A linear regression analysis estimated the rate of decline as 3.00 ± 1.43 ug g^{-1} wk^{-1}. In field studies of 7,8-benzoflavone concentrations, the highest concentration observed was ~80 ug g^{-1} (Alford et al. 2007). Our greenhouse results suggest that 7,8-benzoflavone would persist for ~27 weeks, or half of a year, if it was present at 80 ug g^{-1} when Russian knapweed was removed from a site. Thus, persistent 7,8-benzoflavone in the soil might be expected to influence native plant establishment in the first growing season after Russian knapweed eradication. However, the lack of 7,8-benzoflavone in most field soils studied calls into question the significance of this compound as an allelochemical. In fact, our subsequent studies (Quintana et al. 2008) suggest that compounds other than 7,8-benzoflavone may be responsible for allelopathy in Russian knapweed.

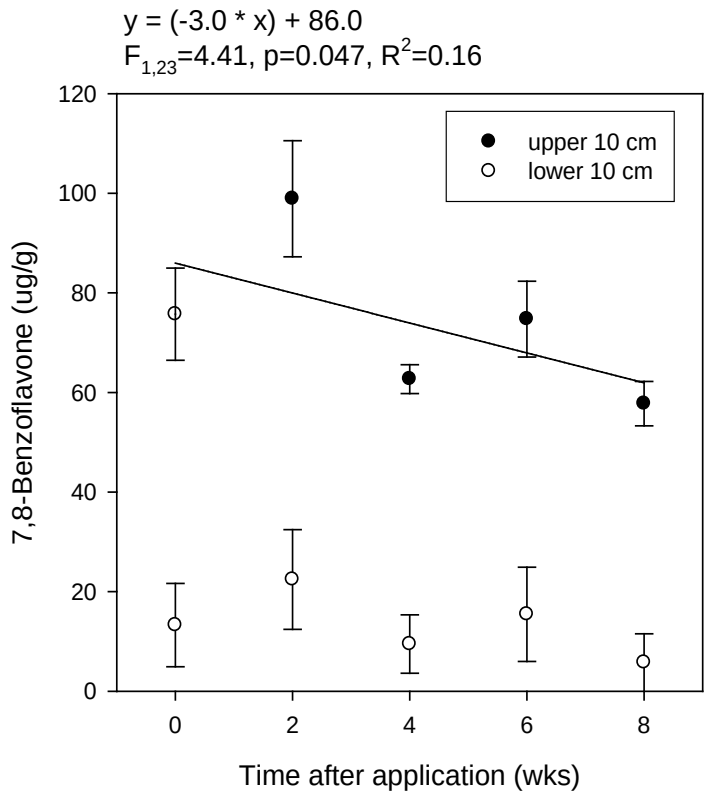

Figure 3. 7,8-Benzoflavone concentrations over time in artificial soil treated with 5 mg 7,8-benzoflavone. Samples were collected from both the upper and the lower portions of experimental pots. Linear regression analysis indicated that only the concentrations in the upper portions changed significantly with time. The fitted line and statistical results for the concentrations in the upper portions are shown. Error bars are 1 SEM. N=5.

IDENTIFICATION OF NATIVE PLANTS THAT ARE RESISTANT TO ALLELOCHEMICALS

Military lands are undergoing profound community and ecosystem changes due to plant invasions. Land managers have employed a number of tools in the fight against invasive plants including herbicide, biological control insects, and seeding with mixes of grass species, which are often invasives themselves. Surprisingly, judicious but aggressive seeding with native species as invasive control has only recently been implemented and to a relatively small degree. This is because the superior colonizing and competitive abilities of invasives often limits the restoration and rehabilitation of infested sites using expensive native seed. Even when invasive plant treatments using herbicides, biocontrol agents, or other control techniques effectively suppress target species, often invaders are the first to return or are replaced by other invaders. There is a crucial need for determining native seed mixes and application techniques to better compete with invaders and prevent re-invasion. Several knapweed species, introduced to North America from Eurasia, are a major concern for land managers because of their ability to rapidly establish and dominate in disturbed areas and in relatively undisturbed grasslands. Whatever the mechanisms of invasion the bottom line is that spotted knapweed is able to establish and flourish in many grasslands and exclude large numbers of natives in many areas where it establishes.

Identifying competitive native species; and perhaps more importantly competitive suites of native species, for seeding into restored grasslands may prove a valuable and overlooked tool. Therefore, we sought to identify native species and particular ecotypes of native species that could be good competitors with invasive allelopathic plants, which could be used to reclaim infestations of allelopathic invaders. We then tested these species in field trials at Fort McCoy, WI, Yakima Training Center, WA and additional sites in Montana.

Screening of western US grassland plants for restoration after spotted knapweed invasion

A variety of native plants of the intermountain west were screened under laboratory conditions for their possible resistance to catechin. Based on these studies, we found eight species with EC50s greater than 3.0 mg/ml that were identified as resistant to catechin, the purported allelochemical produced by spotted knapweed, and that were likely suitable for revegetation of spotted knapweed-infested areas. Catechin resistance was positively correlated with mean seed mass, suggesting that seed carbohydrate reserves may allow seedlings to detoxify catechin, develop barriers to catechin exposure, or sustain a positive growth rate despite (±)-catechin-induced cell death. Future efforts to identify allelochemical-resistant grassland species would most profitably focus on large-seeded species. These studies were published in the journal *Restoration Ecology* (Perry et al. 2005). The plant species examined in this publication among others were also grown in greenhouse and field experiments in direct competition with spotted knapweed to assess the correspondence between (±)-catechin tolerance and general competitive ability with spotted knapweed (see below). There was little correlation between catechin resistance in the laboratory experiment (Perry et al. 2005) and competitive ability with spotted knapweed in the greenhouse experiment. However, the greenhouse experiment yielded important insights into abilities of grassland species of the intermountain west to compete with spotted knapweed (Table 2).

Table 2. Index of competitive ability (RRII) with spotted knapweed for 12 species commonly used in grassland restoration in the intermountain west. RRII is calculated as the difference between biomass of the target species in monoculture and in mixture with the competitor species, divided by the sum of the biomasses in monoculture of the two species. Catechin sensitivity is taken from Perry *et al*. 2005.

Species	RRII	Competitive Ability	Catechin sensitivity
Sphaeralcea coccinea	0.12	Weakest competitor	Sensitive
Hedysarum boreale	0.18		Resistant
Festuca idahoensis	0.25		Very sensitive
Artemisia ludoviciana	0.28		Sensitive
Gallardia aristata	0.38		Resistant
Poa secunda	0.39		Very sensitive
Pseudoroegneria spicata	0.54		Sensitive
Achillea millefolia	0.70		Very sensitive/sensitive
Helianthus annuus	0.71		Sensitive
Elymus trachycaulus	0.80		Sensitive
Bouteloua gracilis	0.83		Sensitive
Thinopyrum intermedium	0.83	Strongest competitor	Very sensitive

Screening of tallgrass prairie plants for restoration after spotted knapweed invasion

Although spotted knapweed initially invaded western North America, it is now spreading east and invading tallgrass prairie in the Midwest and Northeast U.S. To identify native plants suitable for revegetation of spotted knapweed infestations in tallgrass prairie and to prepare for field-testing at Fort McCoy, WI, we conducted additional screening experiments for catechin resistance among tallgrass prairie species.

(±)-Catechin resistance was evaluated for 19 native tallgrass prairie species, including eight grasses and 11 forbs (Table 3). *Festuca idahoensis* (Idaho fescue) was also included as a (±)-catechin-sensitive control. Seeds of the tallgrass prairie species were obtained from Prairie Nursery (Westfield, WI). *Festuca idahoensis* seeds were obtained from Granite Seed (Lehi, UT).

Table 3. Native tallgrass prairie species screened for (±)-catechin resistance.

Species	Common Name	Family	Growth Form
Andropogon gerardi	big bluestem	Poaceae	Grass
Bouteloua curtipendula	sideoats grama	Poaceae	Grass
Elymus canadensis	Canada wildrye	Poaceae	Grass
Heliopsis helianthoides	smooth oxeye	Asteraceae	Forb
Hesperostipa spartea	porcupinegrass	Poaceae	Grass
Liatris aspera	tall blazingstar	Asteraceae	Forb
Lupinus perennis	sundial lupine	Fabaceae	Forb
Monarda fistulosa	wild bergamot	Lamiaceae	Forb
Monarda punctata	spotted beebalm	Lamiaceae	Forb
Panicum virgatum	switchgrass	Poaceae	Grass
Penstemon grandiflorus	large beardtongue	Scrophulariaceae	Forb
Polygonatum canaliculatum	Solomon's seal	Liliaceae	Forb
Rudbeckia hirta	blackeyed Susan	Asteraceae	Forb
Schizachyrium scoparium	little bluestem	Poaceae	Grass
Sorghastrum nutans	Indiangrass	Poaceae	Grass
Sporobolus heterolepis	prairie dropseed	Poaceae	Grass
Symphyotrichum laeve	smooth blue aster	Asteraceae	Forb
Symphyotrichum oolentangiense	skyblue aster	Asteraceae	Forb
Verbena stricta	hoary verbena	Verbenaceae	Forb

The 20 species were treated with four catechin concentrations (0, 0.5, 1.0, and 4.0 mg ml^{-1}), with four replicates per species x treatment combination. The experiment was set-up using sterile techniques in a transfer hood. (±)-Catechin treatments were applied to groups of 25 seeds of each species on sterile Whatman #41 ashless filter paper in sterile 60 mm Petri dishes. Prior to treatment, the seeds were surface sterilized in 10% bleach fortified with a drop of tween for 10 minutes, rinsed three times with sterile distilled water and submerged for one hour in sterile distilled water. (±)-Catechin (Shivambu International, New Delhi, India) was dissolved in methanol, diluted with distilled water to create a 4 mg ml^{-1} (±)-catechin, 10% methanol solution, and filter-sterilized with a 2 µm syringe filter. The (±)-catechin solution and a sterile 10% methanol solution were added to the dishes so that all treatments including the controls received the same volume of methanol with the appropriate (±)-catechin concentration in 2 ml of liquid. After applying the (±)-catechin, the dishes were left open in the transfer hood for two hours to allow the methanol to evaporate. The evaporated liquid was then replaced with sterile water. The dishes were closed, sealed with parafilm, and arranged at random in a 16 h 25ºC day, 8 h 15ºC night incubator.

The experiment was maintained for 21 d. Dish locations in the incubator were rearranged at random weekly. Dishes were watered with sterile water in a transfer hood as needed. After 21 d, the number of germinated seeds in each dish was counted, and the root and shoot lengths of each germinated seedling were measured.

Effects of (±)-catechin on percent germination and mean root and shoot elongation were examined using linear regression analysis. When necessary, the dependent variables (germination, root length, shoot length) were transformed to correct significantly unequal variances, and the independent variable (catechin concentration) was transformed to correct significant non-linearity. Standard statistical methods involving linear regression of the log

means and log standard deviations for the treatments for each species was used to identify the appropriate transformations for the dependent variables. The Box-Tidwell approach was used to identify the appropriate transformations for the independent variable. Percent germination was arcsin, square root transformed for all analyses. EC50s, or the estimated concentrations required to reduce germination or growth by 50%, are a standard measure of toxin resistance. To allow for comparisons among species, EC50s for (±)-catechin were calculated from the fitted lines for all cases of significant negative effects of (±)-catechin. All statistical analyses were conducted using PROC GLM in SAS statistical software (version 9.1, SAS Institute, Inc., Cary, NC).

Germination was insufficient to evaluate effects of (±)-catechin on germination or growth of two of the species, *Hesperostipa spartea* (porcupinegrass) and *Polygonatum canaliculatum* (Solomon's seal). Seedling root growth was insufficient to evaluate effects of (±)-catechin on root growth for an additional two species, *Liatrus aspera* (tall blazingstar) and *Sporobolus heterolepis* (prairie dropseed).

(±)-Catechin treatment reduced seedling root elongation for eight species, including four grasses (Figure 4) and four forbs (Figure 5). Of the grasses, *Bouteloua curtipendula* (sideoats grama) was the most sensitive, strongly reduced by even the lowest (±)-catechin treatment (Dunnett's one-tailed t-test, α=0.05). All four forbs that were affected by (±)-catechin treatment were, like *B. curtipendula*, inhibited even by the lowest (±)-catechin treatment (Dunnett's one-tailed t-test, α=0.05). *Verbena stricta* (hoary verbena) root elongation also appeared to be reduced by (±)-catechin treatment but this trend was not significant, perhaps because of low replication due to poor germination in some treatments. EC50s for root length varied from 0.86 to 4.61 mg ml^{-1} among the significantly inhibited species (Table 3).

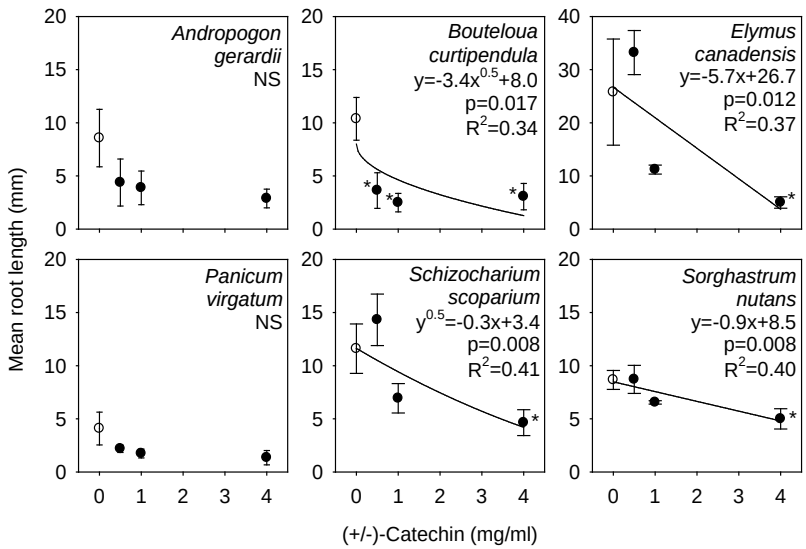

Figure 4. Significant effects of catechin on seedling root elongation of six of the grass species. Fitted lines and P and R^2 values were determined using linear regression analysis. (*) indicates means that were significantly lower than the controls (Dunnett's one-tailed t-test, α=0.05). Error bars are one standard error of the mean.

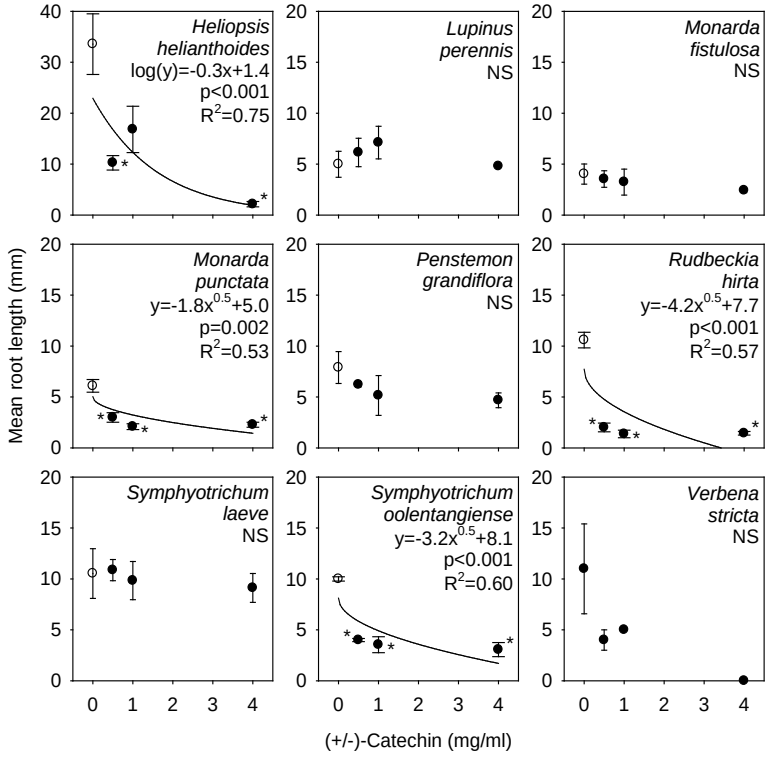

Figure 5. Significant effects of catechin on seedling root elongation of nine of the forb species. Fitted lines and P and R2 values were determined using linear regression analysis. (*) indicates means that were significantly lower than the controls (Dunnett's one-tailed t-test, α=0.05). Error bars are one standard error of the mean.

(±)-Catechin treatment reduced germination of two species, *Lupinus perennis* (silky lupine) and *Verbena stricta* (hoary verbena) (Figure 6), but did not affect germination of the other species examined.. Effects of (±)-catechin were considerably stronger for *V. stricta* germination than for *L. perennis* germination (Table 4). (±)-Catechin treatment did not significantly reduce seedling shoot elongation for any species.

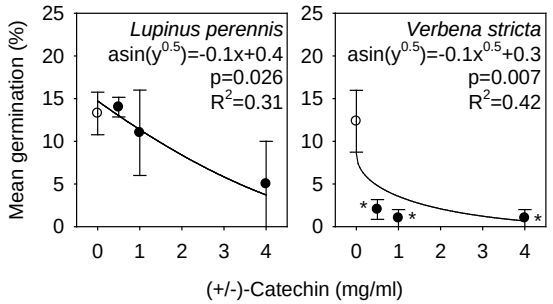

Figure 6. Significant effects of catechin on germination of two tallgrass prairie species. Fitted lines and P and R^2 values were determined using linear regression analysis. (*) indicates means that were significantly lower than the controls (Dunnett's one-tailed t-test, α=0.05). Error bars are one standard error of the mean.

Table 4. Estimated concentrations of (±)-catechin that reduce root length and germination by 50% (EC50) for species significantly inhibited by (±)-catechin treatment. EC50s were determined from the fitted lines shown in Figures 5-7. Species with roots that were not affected by (±)-catechin treatment were considered (±)-catechin-resistant.

Trait	Species	EC50 (mg ml^{-1})	Resistance
Root elongation	*Rudbeckia hirta*	0.86	Most sensitive
	Heliopsis helianthoides	1.11	
	Bouteloua curtipendula	1.41	
	Symphyotrichum oolentangiense	1.61	
	Monarda punctata	1.97	
	Elymus canadensis	2.32	
	Schizachyrium scoparium	2.94	
	Sorghastrum nutans	4.61	
	Andropogon gerardi	---	Resistant
	Lupinus perennis	---	Resistant
	Monarda fistulosa	---	Resistant
	Panicum virgatum	---	Resistant
	Penstemon grandiflorus	---	Resistant
	Symphyotrichum laeve	---	Resistant
	Verbena stricta	---	Resistant
Germination	*Verbena stricta*	0.68	Most sensitive
	Lupinus perennis	2.38	

Natural selection for resistance to the allelopathic effects of invasive plants

We previously reported (Callaway et al. 2005) that selection for resistance to the allelochemicals produced by spotted knapweed is already occurring in the natural populations of North American native plants. Efforts were focused on multiplying these plants to obtain seed. We subsequently conducted a series of experiments with seeds of *Pseudoroegneria spicata* collected from populations throughout the Northwest United States and found substantial population variation within this species (Figure 7).

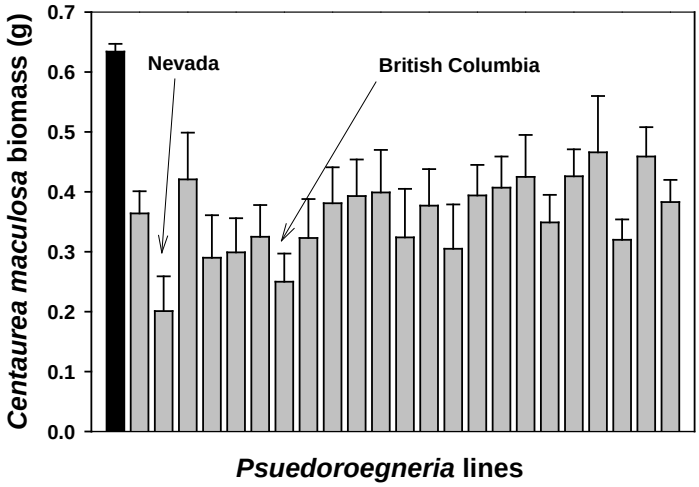

Figure 7. Total biomass of knapweed grown alone (black bar) and grown with bluebunch wheat grass from different populations. The highly suppressive effects of bluebunch wheat grass from Nevada and British Columbia are emphasized.

Allelochemical resistance of grass cultivars bred for use on military lands

Grass cultivars that have been bred for use on military lands by Tony Palazzo and coworkers at the U.S. Army Corps of Engineers, Engineering Research and Development Center in Hanover, NH (ESTCP project #RC-200401) might be particularly appropriate for revegetating infested areas if those cultivars are also resistant to allelochemicals from invasives. We examined the resistance of four grass cultivars, SERDP *Agropyron fragile*, Vavilov *A. fragile*, SERDP *A. trachycaulum*, and Pryor *A. trachycaulum* to catechin, a previously reported phytotoxin from spotted knapweed .

(±)-Catechin resistance was evaluated for the four cultivars and for two additional grass species, one known to be catechin-sensitive (*Festuca idahoensis*) and one known to be catechin resistant (*Bromus marginatus*). Seeds of the four cultivars were obtained from Tony Palazzo. *Festuca idahoensis* and *Bromus marginatus* seeds were obtained from Granite Seed (Lehi, UT).

The 6 cultivars were treated with six catechin concentrations (0, 0.125, 0.25, 0.5, 1.0, and 4.0 mg ml^{-1}), with five replicates per species x treatment combination. The experiment was set up using sterile techniques in a transfer hood. (±)-Catechin treatments were applied to groups of 25 seeds of each cultivar on sterile Whatman #41 ashless filter paper in sterile 60 mm Petri dishes. Prior to treatment, the seeds were surface sterilized in 10% bleach fortified with a drop of tween for 10 minutes, rinsed three times with sterile distilled water and submerged for one hour in sterile distilled water. (±)-Catechin (Shivambu International, New Delhi, India) was dissolved in methanol, diluted with distilled water to create a 4 mg ml^{-1} (±)-catechin, 10% methanol solution, and filter-sterilized with a 2 µm syringe filter. The (±)-catechin solution and a sterile 10% methanol solution were added to the dishes so that all treatments including the controls received the same volume of methanol with the appropriate (±)-catechin concentration in 2 ml of liquid. After applying the (±)-catechin, the dishes were left open in the transfer hood for two hours to allow the methanol to evaporate. The evaporated liquid was then replaced with sterile water.

The dishes were closed, sealed with parafilm, and arranged at random in a 16 h 25ºC day, 8 h 15ºC night incubator.

The experiment was maintained for 35 d. Dish locations in the incubator were rearranged at random weekly. Dishes were watered with sterile water in a transfer hood as needed. After 35 d, the number of germinated seeds in each dish was counted, and the root and shoot lengths of each germinated seedling were measured.

Effects of (±)-catechin on percent germination and mean root and shoot elongation were examined using linear regression analysis. When necessary, the dependent variables (germination, root length, shoot length) were transformed to correct significantly unequal variances, and the independent variable (catechin concentration) was transformed to correct significant non-linearity. Standard statistical methods involving linear regression of the log means and log standard deviations for the treatments for each species was used to identify the appropriate transformations for the dependent variables. The Box-Tidwell approach was used to identify the appropriate transformations for the independent variable. Percent germination was arcsin, square root transformed for all analyses. EC50s, or the estimated concentrations required to reduce germination or growth by 50%, are a standard measure of toxin resistance. To allow for comparisons among species, EC50s for (±)-catechin were calculated from the fitted lines for all cases of significant negative effects of (±)-catechin. All statistical analyses were conducted using PROC GLM in SAS statistical software (version 9.1, SAS Institute, Inc., Cary, NC).

All six cultivars exhibited significant reductions in mean root length with catechin treatment (Figure 8). However, the four cultivars bred for use on military lands were considerably less inhibited by catechin treatment than the catechin-resistant control, *Bromus marginatus*. Pryor *A. trachycaulum* was the most catechin resistant in terms of root elongation (EC50=3.6 mg/ml), SERDP *A. trachycaulum* and SERDP *A. fragile* were intermediate (EC50=2.4 mg/ml), and Vavilov *A. fragile* was the least resistant (EC50=1.7 mg/ml). Shoot length was significantly reduced by catechin treatment only for Vavilov *A. fragile* and *Bromus marginatus.* Germination was significantly reduced by catechin treatment for several of the cultivars, but not for Pryor *A. trachycaulum* or Vavilov *A. fragile*.

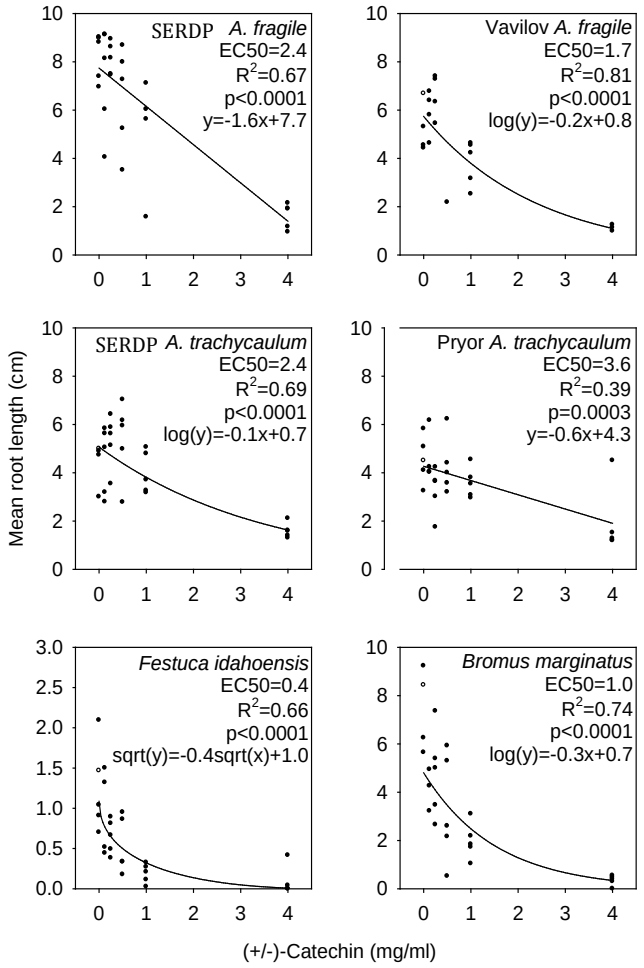

Figure 8. Significant effects of catechin on seedling root elongation of four cultivars bred for use on military lands, plus a previously identified catechin-sensitive species (*F. idahoensis*) and a previously identified catechin-resistant species (*B. marginatus*). Fitted lines and P and R^2 values were determined using linear regression analysis. EC50 values (estimated catechin concentrations required to reduce root length by 50%) were calculated from the fitted lines.

Effects of allelochemicals on legumes and rhizobium

Our initial findings indicated that *Lupinus sericeus* (silky lupine), among other legumes, was relatively resistant to spotted knapweed invasion and allelochemistry. We hypothesized that legume species may exhibit resistance to flavonoids in knapweed root exudates and may serve as candidate species for management efforts. Because legumes form symbiotic relationships with Rhizobia, these bacteria were also evaluated for allelochemical resistance. In this study we examined four legume species for effects of 7,8-benzoflavone and (±)-on rhizosphere interactions involving legume roots and associated Rhizobia. Pure cultures of four Rhizobia strains exhibited varied responses when grown with 7,8-benzoflavone or (±)-catechin. *Medicago sativa* (alfalfa) and its bacterial symbiont, *Sinorhizobium meliloti*, exhibited allelochemical resistance that varied with (±)-catechin concentration when grown *in vitro*. Four legume species were grown under greenhouse conditions. Plants that were inoculated and nodulated generally exhibited no response to 7,8-benzoflavone or (±)-catechin treatments. Plants that were not inoculated exhibited stronger responses. Therefore inoculation and nodulation may confer resistance to allelochemicals. These results, when coupled with previous research and field

observations, suggest that legumes may not be susceptible to knapweed allelopathy and may be good choices in restoration of knapweed infestations when inoculated, particularly on sites with low soil nitrogen (Alford et al. 2009).

Greenhouse competition experiments

To identify additional species for use in restoration of spotted knapweed infestations, we examined competitive ability against spotted knapweed for 39 species: 21 species commonly planted in grassland restorations in the western U.S., 12 tallgrass prairie species, and six invasive species including spotted knapweed (Table 5).

Each of the 39 species was grown either alone or in combination with spotted knapweed in a greenhouse in 2.4 L pots filled with 1 L of potting soil over 1.4 L of 20/30 grit silica sand. Spotted knapweed was also grown alone. The spotted knapweed seeds were sown four weeks after the competitor species, to allow the competitor species to establish without spotted knapweed competition. Interactions between the western grassland species and spotted knapweed were examined in one experiment in 2005, while interactions between the tallgrass prairie species and spotted knapweed were examined in a separate experiment in 2006. Twenty weeks after planting spotted knapweed, aboveground and belowground biomass of all plants was harvested.

Results of this experiment indicated that many species native to the Palouse and intermountain prairie of northwestern America elicit strong competitive effects on knapweed and demonstrate strong competitive responses to knapweed (Figure 9). There was a strong relationship between competitive effect and response, indicating that some native species may possess strong overall potential in restoration efforts (Figure 10).

Species that grew larger in monoculture tended to reduce spotted knapweed biomass more than the species that grew smaller in monoculture, suggesting that rapid growth rate was an important predictor of competitive ability with spotted knapweed under our experimental conditions. Averaged across species, competition with spotted knapweed increased root:shoot ratios of prairie species, suggesting that competition may have been for belowground resources.

Table 5. Species examined for competitive ability with spotted knapweed (*Centaurea maculosa*).

Species	Common Name	Family	Origin	Form
Western grassland:				
Achillea millefolium	Common yarrow	Asteraceae	N, I	F
Artemisia frigida	Prairie sagewort	Asteraceae	N	S
Artemisia ludoviciana	White sagebrush	Asteraceae	N	S
Bouteloua gracilis	Blue grama	Poaceae	N	G
Bromus marginatus	Mountain brome	Poaceae	N	G
Elymus trachycaulus	Slender wheatgrass	Poaceae	N	G
Festuca idahoensis	Idaho fescue	Poaceae	N	G
Gaillardia aristata	Common blanketflower	Asteraceae	N	F
Geranium viscosissimum	Sticky purple geranium	Geraniaceae	N	F
Hedysarum boreale	Boreal sweetvetch	Fabaceae	N	L
Helianthus annuus	Common sunflower	Asteraceae	N	F
Hesperostipa comata	Needle and thread	Poaceae	N	G
Koeleria macrantha	Prairie junegrass	Poaceae	N	G
Leymus cinereus	Basin wildrye	Poaceae	N	G
Linum perenne	Blue flax	Linaceae	I	F
Lupinus sericeus	Silky lupine	Fabaceae	N	F,S
Poa sandbergii	Sandberg bluegrass	Poaceae	N	G
Poa secunda	Sandberg bluegrass	Poaceae	N	G
Pseudoroegneria spicata	Bluebunch wheatgrass	Poaceae	N	G
Sphaeralcea coccinea	Scarlet globemallow	Malvaceae	N	S
Thinopyrum intermedium	Intermediate wheatgrass	Poaceae	I	G
Tallgrass prairie:				
Andropogon gerardi	Big bluestem	Poaceae	N	G
Bouteloua curtipendula	Sideoats grama	Poaceae	N	G
Elymus Canadensis	Canada wildrye	Poaceae	N	G
Heliopsis helianthoides	Smooth oxeye	Asteraceae	N	F
Monarda fistulosa	Wild bergamot	Lamiaceae	N	F,S
Monarda punctata	Spotted beebalm	Lamiaceae	N	F,S
Panicum virgatum	Switchgrass	Poaceae	N	G
Rudbeckia hirta	Blackeyed susan	Asteraceae	N	F
Schizachyrium scoparium	Little bluestem	Poaceae	N	G
Sorghastrum nutans	Indiangrass	Poaceae	N	G
Symphyotrichum leave	Smooth blue aster	Asteraceae	N	F
Symphyotrichum oolentangiense	Skyblue aster	Asteraceae	N	F,S
Invasives				
Bromus tectorum	Cheatgrass	Poaceae	I	G
Euphobia esula	Leafy spurge	Euphorbiaceae	I	F
Linaria dalmatica	Dalmation toadflax	Scrophulariaceae	I	F
Potentilla recta	Sulphur cinquefoil	Rosaceae	I	F
Sisymbrium altissimum	Tall tumblemustard	Brassicaceae	I	F

Nomenclature follows the U.S. Department of Agriculture National Resources Conservation Service plants database (http://plants.usda.gov).

N = native (N); I = introduced. G = grass, F = forb, S = subshrub/shrub. A = annual, B = biennial, P = perennial.

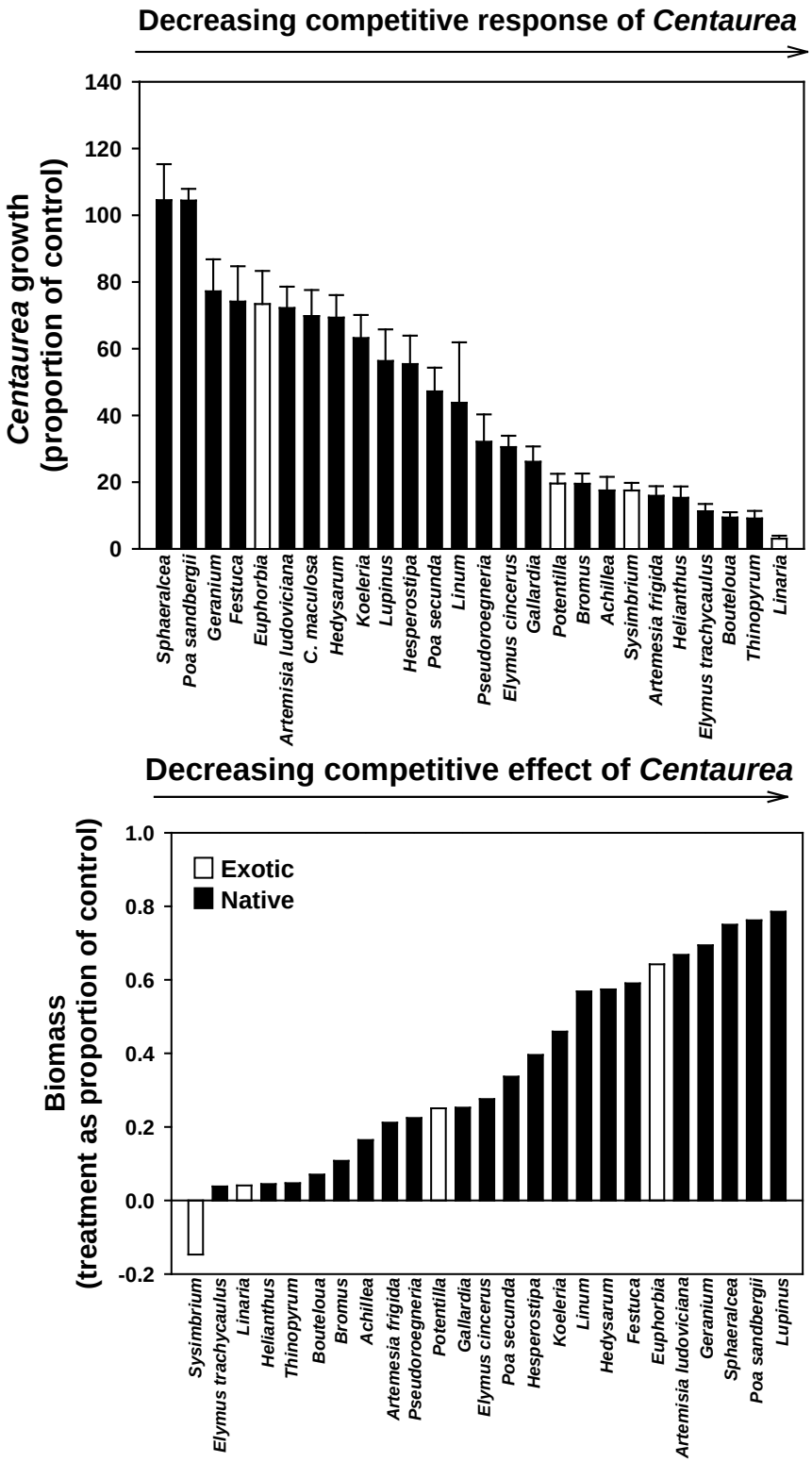

Figure 9. Mean competitive effects of knapweed on native species, and competitive responses of knapweed to native species.

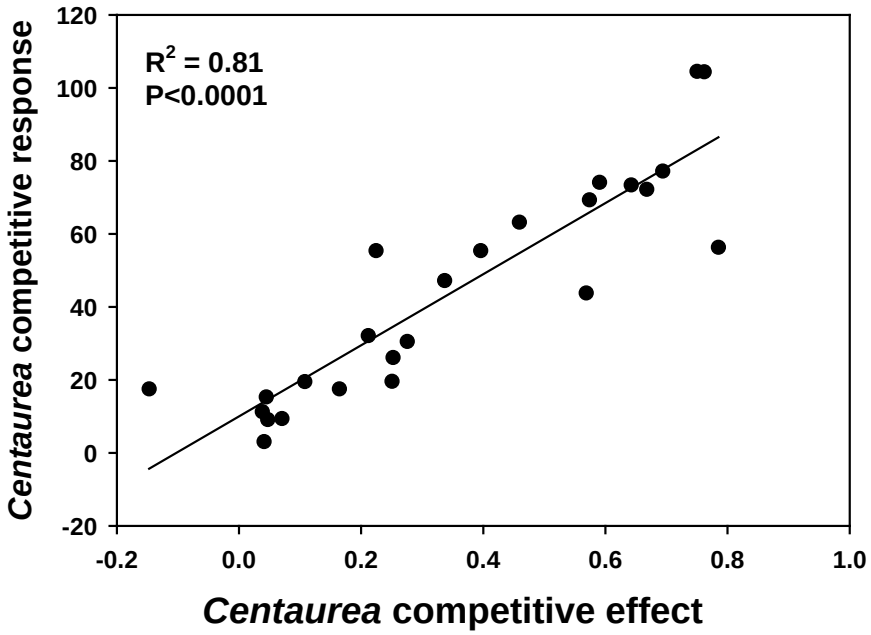

Figure 10. Relationship between competitive effect and response interactions for native North American species and spotted knapweed.

Facilitative effects of *Lupinus* and *Gallardia* on Spotted Knapweed

On The University of Montana's research grounds located at Fort Missoula (46.839919°N, 114.054154°W), we set up 1m^2 plots to determine the effects of two different native forbs on native grasses in the presence of spotted knapweed. We choose the forbs, *Gaillardia aristata* (blanketflower) and *Lupinus sericeus*, because of our previous work demonstrating these plants may exude their own chemicals with the potential to nullify some of the toxic effects of spotted knapweed. In the spring of 2006, we established 300 plots, each plot randomly assigned either a *Lupinus* or *Gallardia*, or without either species. Once these plants established in 2008, we planted a native grass, Idaho fescue (*Festuca idahoensis)*, in each of the plots. Several weeks later we planted small rosettes of spotted knapweed into some of the plots to determine if the native forbs 'shield' their grass neighbor from the potentially deleterious effects of spotted knapweed. We found that *Lupinus* and *Gallardia* strongly suppressed spotted knapweed but we found no significant positive effect of this suppression on *Festuca* (data not shown); perhaps due to increased competitive effects of the other native species.

Field experiments for identifying strong native competitors against Spotted Knapweed

At Fort Missoula we set up field plots where we established from seed over 25 different native grasses and forbs in plots. In the fall of 2007 we planted a small knapweed as close to the target native plant as possible. The plots were harvested in the fall of 2008. Native species showed tremendous variation in their effect on spotted knapweed, from very weak competitors such as *Linum lewisii* and *Vulpia octoflora* to very good competitors, such as *Agropyron smithii* and

Achillea millefolia. The crucial results from this experiment demonstrate that large numbers of native species may be used as good competitors in restoration treatments (Figure 11).

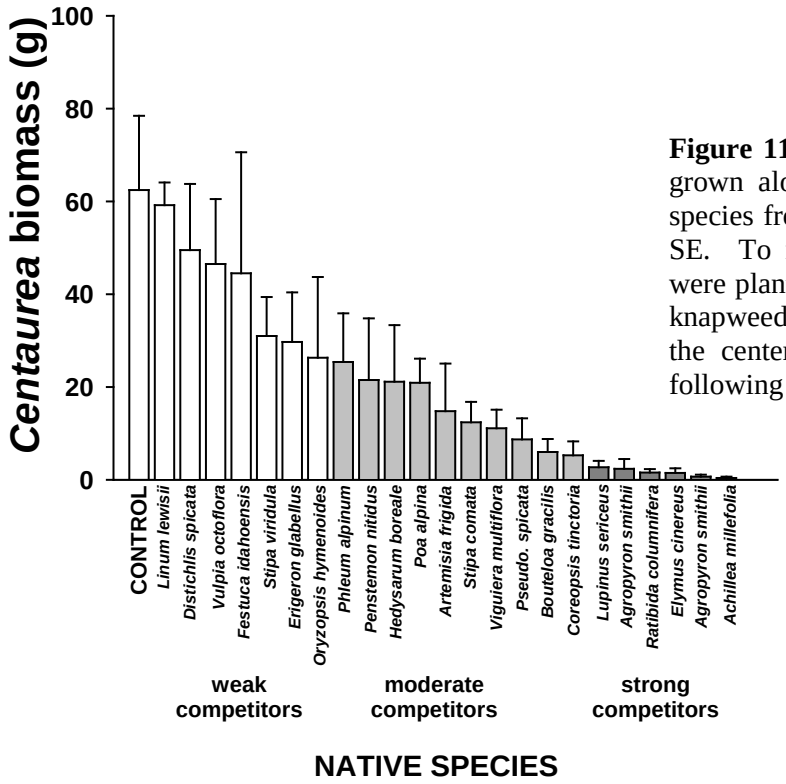

Figure 11. Biomass of spotted knapweed when grown alone (control) or with different native species from Montana. Bars show means and 1 SE. To mimic invasive situations the natives were planted on season ahead of knapweed, and knapweed seedling were transplanted 5 cm from the center of the native in the spring of the following year.

<u>Field seeding of diverse assemblages of native species against Spotted Knapweed</u>

In a knapweed-invaded grassland we set up an experiment to compare native plants (grasses + forbs) that demonstrate strong growth against knapweed (= strong natives) versus native plants that did not demonstrate strong growth when grown with knapweed (= weak natives). The study site was "Frenchtown" township, located ≈10 km west of Missoula (4 6.592311^{o}N, 114.090040^{o}W) and has a knapweed cover about 30%. Also, to explore the role of diversity for resistance to invasion, we divided our 'strong' and 'weak' treatments into low diversity (three species) and high diversity (eleven species), for a total of five treatments (Strong low; strong high; weak low; weak high; control) (Table 6). The experiment was seeded in the fall 2007 and responses have been monitored for reinvasion since 2009.

Table 6. Seed addition experiments, began in Fall 2007, at Frenchtown and Mansion Heights locations, Montana. 100 grams of seed per 2-x 2-m plot were added.

Taxa	Common	Category	diversity mix	seeds/g	Seed (g) /plot (low/high)	root habit	Family
Festuca idahoensis	Idaho fescue	weak	low/high	680	33.33/9.09	bunch	Poaceae
Monarda fistulosa	wild bergemont	weak	low/high	1190	33.33/9.09	rhizo	Lamiaceae
Poa sandbergii	Sandbergii's bluegrass	weak	low/high	2,311	33.33/9.09	bunch	Poaceae
Koeleria macrantha	prairie junegrass	weak	high	3,360	9.09	bunch	Poaceae
Artemesia ludoviciana	Prairie sage	weak	high	3,488	9.09	rhizo	Asteraceae
Psuedoroegneria spicata	bluebunch wheatgrass	weak	high	305	9.09	bunch	Poaceae
Geranium viscosissimum	sticky geranium	weak	high	110	9.09	tap	Geraniaceae
Penstemon nitidus	smooth blue beard tongue	weak	high	484	9.09	tap	Scrophulariaceae
Chrysopsis villosa	hairy golden aster	weak	high	2,000	9.09	tap	Asteraceae
Hedysarum boreale	northern sweetvetch	weak	high	105	9.09	tap	Fabaceae
Potentilla arguta	tall cinquefoil	weak	high	8,112	9.09	tap	Rosaceae
	total seeds per gram			22145			
Stipa comata	needle and thread	strong	low/high	136	33.33/9.09	bunch	Poaceae
Artemesia frigida	fringed sage	strong	low/high	13000	33.33/9.09	tap	Asteraceae
Elymus cinerus	basin wild rye	strong	low/high	360	33.33/9.09	bunch	Poaceae
Lupinus sericeus	silky lupine	strong	high	125	9.09	tap	Fabaceae
Gaillardia aristata	blanketflower	strong	high	310	9.09	tap	Asteraceae
Coreopsis tinctoria	plains coreopsis	strong	high	3,440	9.09	biennial/tap	Poaceae
Festuca idahoensis	Idaho fescue	weak	high	680	9.09	bunch	Poaceae
Monarda fistulosa	wild bergemont	weak	high	1,190	9.09	rhizo	Lamiaceae
Poa sandbergii	Sandbergii's bluegrass	weak	high	2,311	9.09	bunch	Poaceae
Solidago canadensis	Canada goldenrod	strong	high	1,829	9.09	rhizo	Asteraceae
Achillea millefolium	yarrow	strong	high	2,700	9.09	rhizo	Asteraceae
	Total seeds per gram			26081			

We also conducted an experiment similar to that at the Frenchtown site in an old growth bunchgrass prairie near Missoula, Montana that is moderately invaded by knapweed (30% cover) ('Mansion Heights'; 46.482610^{o}N, 114.010161^{o}). We established plots to measure the effects of native plant diversity and differences in competitive 'strength' on knapweed invasion. To establish a 'zero point' we sprayed a composite-specific herbicide (Transline) with a short residual time (30-60 days) on the plots. After 50 days in the fall of 2007, we seeded in our different treatments (strong low; strong high; weak low; weak high; strong high + spray control; and control) (Table 6). These plots have been monitored since 2009.

On May 7th 2007 we applied three treatments of seeding to 75 burn piles in the Ninemile Valley, Montana (47.119210^{o}N, 114.487827^{o}W; elevation 1,021m). The three treatments are "USFS

mix" (prairie junegrass *Koeleria macrantha*; bluebunch wheatgrass *Psuedoroegneria spicata*; Idaho fescue *Festuca idahoensis*; , and basin wild rye *Elymus cinereus*; n=30), "competitive mix" (prairie junegrass; bluebunch wheatgrass; Idaho fescue; basin wild rye; blanketflower *Gaillardia aristata*; yarrow *Achillea millefolium*; fringed sage *Artemesia frigida*; and silky lupine *Lupinus sericeus*; n=30), and "no seed" controls (n=15). According to the Bitterroot National Forest botanist, the target-seeding amount is approximately 80 seeds per square foot (= 860 seeds m^{-2}). The burn pile plots in the Ninemile are approximately 1 meter square, therefore we applied 860 seeds per plot. The USFS mix received four species of grass comprised of 215 seeds each (~1.8 grams total). The competitive mix included 8 species of grasses and broadleaf plants comprised of 108 seeds each (~2.2 grams total). For each of the seeding treatments the seeds was scattered by hand onto the piles and lightly raked in. The no-seed controls were raked to control for that effect. On August 10, 2007 we recorded the cover of all species that established during this first growing season. The cover and diversity of invasives did not differ among treatments (5-6 species in each treatment) and the species of primary interest, spotted knapweed, had only initiated establishment (<1% average cover). The cover of natives in the no-seed control was 5.9% of the ground surface, 11.6% in the USFS mix treatment, and 20.2% in the competitive mix treatment. Native diversity was 11 species in the control, 14 species in the USFS treatment, and 19 species in the competitive treatment. Because seeding treatments can take several years to reveal their efficacy, we will continue to monitor these plots in future years

In an experiment to test the importance of reseeding knapweed resistant species in grassland-forest mixtures, we used post-logging "burn piles" as experimental sites near Lake Como, MT (46.040679^{o}N, 114.265482^{o}W). The sites were burned in the spring and seeded just two weeks post burn. These burn piles are point sources of invasion in these habitats. For one treatment we planted the standard Forest Service mix of native grass species and in another treatment we planted a mix of native forbs and grasses that have demonstrated good growth when with knapweed. We also applied a fall seed application treatment to another set of the spring-treated burn piles. This experiment was started in the spring of 2007. Spotted knapweed colonized all treatments similarly, and in 2010 we found no differences among any of the treatments (ANOVA, $F_{treatment}$=0.934, P=0.459).

In the field trials above we have investigated seeding in native species in areas highly disturbed (burn piles) and relatively natural areas (bunchgrass prairie). At the field site reported here we targeted the highest density spotted knapweed site we could find in the vicinity, where knapweed formed a virtual monoculture. At this site (Beavertail Hill) we planted combinations of knapweed resistant and non-resistant species (Table 7) in the field in a site very highly invaded by spotted knapweed (46.726332°N, 113.598471°W). The other plants on site were either grasses or forbs of European origin. However, the site is surrounded by hillsides of intact remnant prairie with a variety of native grasses, forbs, shrubs, and trees. We set up five treatments in 2m x 2m plots, each replicated 10 times (Table 8).

Table 7. Native plant species seeded into replicated test plots in Montana containing spotted knapweed to evaluate competitive ability.

Species	Competitor Category	Amount per plot
Festuca idahoensis	'weak'	12 grams
Poa sandbergii	'weak'	7 grams
Psuedoroegneria spicata	'weak'	25 grams
Koeleria macrantha	'weak'	5 grams
Sphaeralcea coccinea	'weak'	3 grams
Geranium viscosissimum	'weak'	4 grams
Lupinus sericeus	'tough'	4 grams
Gaillardia aristata	'tough'	5 grams
Stipa comata	'tough'	30 grams
Artemesia frigida	'tough'	2 grams

Note: 'weak' species added to all seed addition treatments

Table 8 The Beavertail Hill, MT plots and treatments used to investigate spotted knapweed control measures on soil catechin production.

Plot # & Name	Treatments/additions	Notes
1 - Control	Mowed + herbicide; no seeds added	
2 – Weak	Mowed + herbicide; 'weak seed mix added	
3 – Tough	No Mow, No herbicide; 'tough' seed mix added	
4 – Tough	Mowed + herbicide; 'tough' seed mix added	
5 – Tough exclosure	Mowed + herbicide; 'tough' seed mix added	To control for massive rodent population on site

The plots were seeded initially May 12, 2006 and again on October 13th, 2006.

We found that the seeding process was ineffective in this very highly invaded site; we established very low abundances of a few native species, including bluebunch wheatgrass and yarrow, but intense reinvasion by knapweed was the overwhelming result in all plots. However, the rodent exclusion treatment did present some success with seeded natives. This implies that the rodent pressure on the seeds and new seedlings was intense. Finally, most of this site was severely burned in the summer of 2007, but not all plots burned at equal intensity, and thus this experiment has been terminated.

SPOTTED KNAPWEED CONTROL STRATEGIES IMPACT ON SOIL CATECHIN AND REVEGETATION EFFORTS

Common forms of weed control have the potential to influence allelochemical production. We conducted field experiments to investigate the effects of control measures (grazing, mowing, herbicides, biocontrols) on the exudation of knapweed allelochemicals and the effects of increased exudation on native plant communities and successful revegetation.

These studies examined if a series of control strategies widely used to control invasives (i.e. fire, mowing, etc) had an effect on the secretion of catechin by spotted knapweed. It seems that from our current understanding of the conditionality of catechin's allelopathy that these are not appropriate triggers.

Spotted knapweed seems to compensate, and even overcompensate, when the plants are exposed to mechanical (i.e. mowing; herbicide) and natural (i.e. herbivory by ungulates and insects) stressors. In one field study, spotted knapweed produced more of the allelochemical catechin when infected with a root boring larvae of the biocontrol moth, *Agapeta zoogana* (Thelen et al. 2005). Although we were able to obtain soil samples from natural sites where knapweed was grazed on by sheep (Mount Jumbo, MT); where a concurrent spotted knapweed mowing study was being conducted (also Mount Jumbo); and where herbicide was applied (most public lands where knapweed exists in close proximity to urban areas), our results were inconclusive. Additional annual soil samples from the military bases did not find any soil catechin at the time of sampling.

We may not have evidence of chemical response of spotted knapweed to the above stressors, but we know this plant can still be virulent when it is injured (as with the response to biocontrol insects noted above). For example, it is apparent that spotted knapweed's seeds are the first to germinate and colonize after the residual chemical effects of herbicide dissipate (typically three years), but this could be due to the atypical fall germination of spotted knapweed (most Rocky Mountain natives germinate in the spring). We also know that knapweed can adapt its habit to mowing, i.e. growing more laterally close to the ground but still able to flower and produce viable seeds. And, to our knowledge, no study has shown that grazing injures or causes any long-term harm to spotted knapweed.

BIOCHEMICAL MECHANISM OF RESISTANCE TO ALLEOCHEMICALS USED BY NATIVE PLANTS

The phytoxicity of catechin was investigated with the objective of eventually turning the invasive weapon used by spotted knapweed against itself as well as harnessing it for broad-spectrum invasive plant control strategies. When this proved impractical, we turned to developing broad-based genomics and metabolomics understanding of the invasion capabilities of spotted knapweed. We then sought to develop a solid understanding of the negative effects of spotted knapweed root exudates on the surrounding soil micorbiome and how this effect might have negative consequences on native plants.

We have identified one biochemical mechanism that allows resistant species to counteract the effect of an allelochemical. We have found that *Lupinus sericeus* (silky lupine) and *Gaillardia grandiflora* (blanketflower) increase exudation of organic acids into the rhizosphere in response

to exposure to the phytotoxic compound catechin, which is reported to be secreted by the invader spotted knapweed. Spotted knapweed also appears to secrete less catechin *in vitro* when exposed to oxalic acid, and *in vivo* when growing near *L. sericeus* plants, suggesting an active two-way chemical exchange between these plant species. Mechanistically, oxalic acid works exogenously to block generation of O_2 radicals in susceptible plants, reducing oxidative damage generated by catechin. Furthermore, field experiments show that *L. sericeus* indirectly facilitates native grasses in grasslands invaded by spotted knapweed, and this facilitation is correlated with the presence of oxalic acid in the soil. Addition of exogenous oxalic acid to native grasses and *Arabidopsis thaliana* grown *in vitro* alleviated the phytotoxic effects of catechin, supporting the field experiments and indicating that root secreted oxalic acid may also act as a chemical facilitator for plant species that do not produce the compound (Weir et al. 2006).

Screening for mutants resistant to the catechin

A powerful approach for determining the biological functions of genes in an organism is to produce mutants with altered phenotypes and physiological responses or that show varied responses to specific treatments. Various approaches for mutagenesis are available and we selected mutants of Arabidopsis produced by treating the seeds with ethyl methanesulfonate (EMS) that produces random point mutations.

We conducted this study by screening a large set of Arabidopsis EMS mutants. Mutant Arabidopsis are Colombia-0 ecotype and are provided by Lehle Seed Company. Mutants are organized into parental lines, harvested from approximately 1,000 M1 parents per parental group. Approximately 2,000 mutants are screened per parental line, reaching a goal of 40,000 total mutants screened. Over 20,000 mutants have been screened. Of the 20,000 mutants screened so far, eight resistant mutants have been found in the first round of screening. Some of these mutants show no cell death on the roots. When homozygous seeds from those mutants were obtained the catechin resistance phenotype originally observed in the plants disappeared suggesting that the original observation might have been a pleotropic effect not necessarily related to the mutation.

Molecular characterization of genes involved in catechin detoxification

In order to identify potential genes involved in the ability of spotted knapweed to become a successful invader, we have generated a cDNA library by pooling several spotted knapweed individuals grown from seed collected from four different sites in Montana. The library represents 4423 unique transcripts, with an average trimmed sequence length of 784 bp. Seventy-seven percent of sequences showed significant homology ($E<10^{-4}$) to existing proteins in the NCBI database and could be grouped based on gene ontology (GO) assignments (Figure 12). Many of the sequences are homologous to proteins involved in plant secondary metabolism, defense, and evolution, and are good candidates for further study of the genetic basis of the detoxification of allelochemicals by spotted knapweed; most importantly, these marker genes will allow us to examine the invasiveness of this species at the molecular level. Understanding the genetic basis of evolution for increased invasiveness in some plants is critical to understanding the mechanisms through which invasions occur and thus identifying species with a higher likelihood of becoming invasive, as well as devising innovative control measures (Broz et al. 2007a).

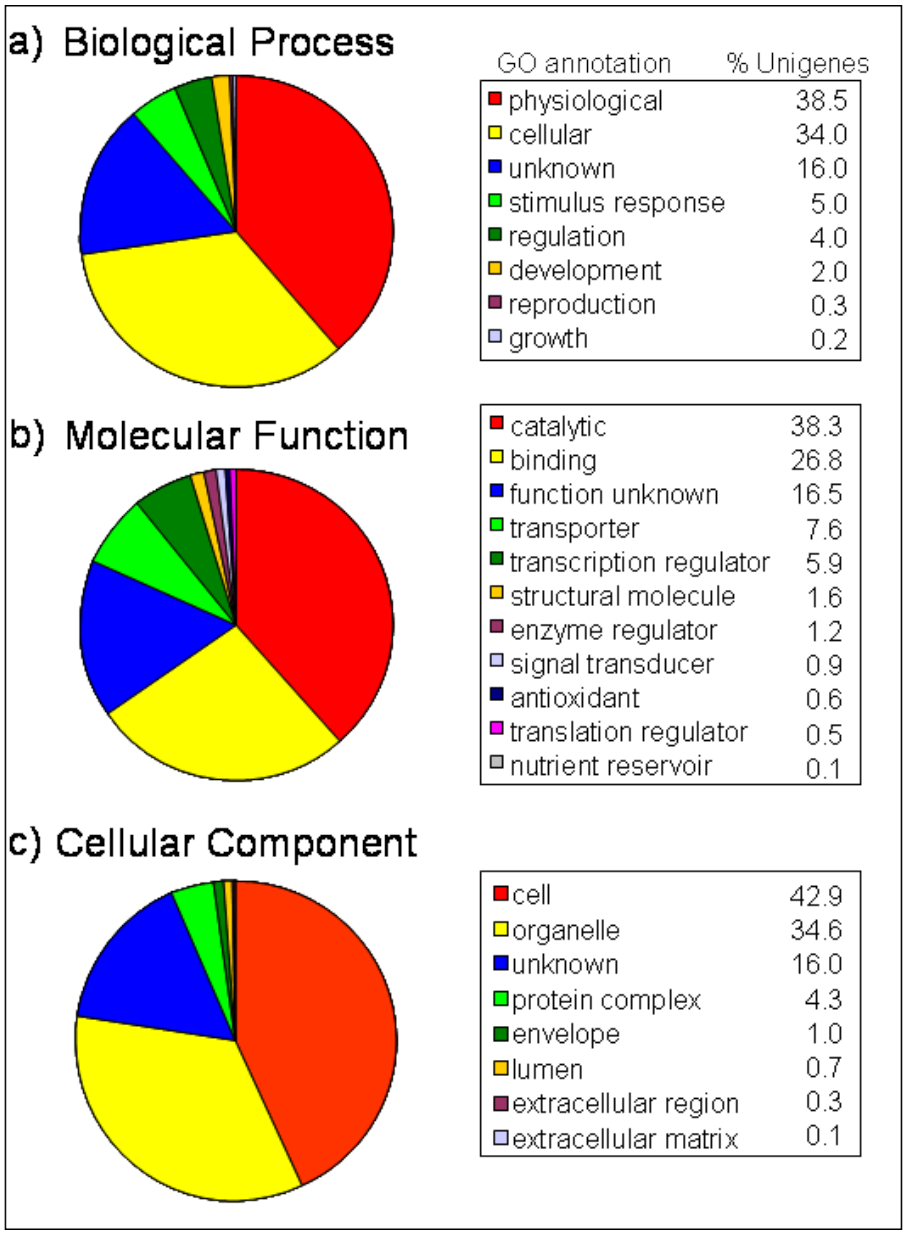

Figure 12. Gene Ontology annotation of *Centaurea* Unigenes. A normalized cDNA library was created from whole plants representing seven invasive populations of spotted knapweed (*Centaurea maculosa).* Five thousand ESTs were sequenced from the 5' end (Agencourt biosciences), and assembled into 4,423 contigs, or "Centaurea Unigenes". Unigenes were translated in all frames and the resulting amino acid sequences were used as BLAST queries. Top BLAST hits provided annotation and functional categorization (gene ontology assignment) for each Centaurea Unigene. Not all unigenes could be annotated by GO programs. Computational analysis was done using the PLAN database.

This was the first report of a cDNA library from an invasive weed. The *Centaurea* cDNA library, consisting of 4423 unique transcripts (unigenes), represents an initial step towards looking at gene-specific expression in this species, and will pave the way for creation of other resources such as microarray chips that can help provide a view of global gene expression in invasive spotted knapweed and its native counterparts. These technologies can likely be extrapolated to look at other invasive knapweeds (*C. diffusa, C. solstitialis, C. virgata* and *Acroptilon repens*) also problematic in North America. By comparing native and invasive spotted knapweed plants under different stresses, including herbivory and pathogen infection, it will be possible to test hypotheses such as EICA using molecular resources coupled with classical (physiological/ecological) techniques.

This technology will also be useful to help understand differences in gene expression between diploid and tetraploid spotted knapweed populations, and give insight into the effects of chromosome doubling and polyploidization events in the plant world. Additionally, by looking at secondary metabolite accumulation and the genes responsible for their production in spotted knapweed , it may be possible to knock out those genes, create mutants defective in the production of allelochemicals, and to finally determine unequivocally whether allelopathy (novel weapons) is involved in the invasive success of some weeds.

Understanding the genetic basis of evolution for increased invasiveness in exotic plants is critical to understanding the mechanisms through which exotic invasions occur. The *Centaurea* cDNA library provides a unique resource that will be valuable to geneticists, molecular biologists, and ecologists alike.

Identification of biochemical/ molecular mechanisms used by spotted knapweed to detoxify catechin

The flavonol (±)-catechin is an allelochemical produced by the invasive plant spotted knapweed. The full effects of (±)-catechin on plant communities in both the native and the introduced ranges of spotted knapweed remain uncertain due to the lack of persistence of this compound in the soil. While high soil (±)-catechin concentrations are known to inhibit the growth and survival of susceptible plants, the effects of low (±)-catechin concentrations have not been explored. Recent studies indicate that (±)-catechin concentrations in spotted knapweed soils can be high but are often very low, and that relatively low (±)-catechin concentrations also occur in microsites within areas of high soil (±)-catechin. In this study, by supplementing plant growth media with (±)-catechin, we show that low (±)-catechin concentrations may induce growth and defense responses in neighboring plants. Lower-than-MIC (minimum inhibitory concentration) doses of the allelochemical induced growth in *Arabidopsis thaliana*; plants treated with 25 µg ml^{-1} (±)-catechin accumulated more than twice the biomass of untreated control plants. Further, pre-treatment of *A. thaliana* roots with low concentrations of (±)-catechin induced resistance to the bacterial pathogen *Pseudomonas syringae* pv *tomato* DC3000 in *A. thaliana* leaves. Low doses of (±)-catechin resulted in moderate increases in ROS in the meristems of treated plants, which may have loosened the cell walls and thus increased growth. Experiments with *A. thaliana* mutants indicated that (±)-catechin induces pathogen resistance by up-regulating defense genes via the SA / NPR1 dependent pathway. Our results suggest that the growth and defense-inducing

effects of (±)-catechin are concentration-dependent, as (±)-catechin at higher concentrations is phytotoxic, thus suggesting the potential for hormesis to occur in nature (Prithiviraj et al. 2007).

Additionally, we have pursued studies to determine if spotted knapweed allelopathy is also manifested against soil microbes, which may have an overall effect on plant community fitness. We collected soils from sites populated by the spotted knapweed. Microbial species diversity in high-density stands of spotted knapweed was compared to those found in adjacent low-density stands at two sites. High density spotted knapweed stands were near monocultures, consisting almost entirely of spotted knapweed plants, whereas low density stands contained isolated spotted knapweed ($\geq$ 1 m spacing) along with a wide variety of native grasses and other plant species. DNA isolated from soil samples was analyzed by real-time PCR and length heterogeneity analysis.

Total amplified microbial DNA (an index of biomass) and microbial phylotype richness varied between the two sites; however, at both sites the high-density spotted knapweed stand was associated with significant declines in fungal abundance and diversity. Bulk soil from high-density stands of spotted knapweed contained over 80% less fungal DNA, associated with the decline in abundance of six phylotypes, compared to low density stands (Figure 13A & 13B). In soils obtained from spotted knapweed rhizospheres, fungal biomass was reduced nearly seven times in high-density stands compared to low density stands (Figure 13A).

Total fungal biomass in the rhizosphere of *Pseudoroegneria spicata*, a native grass present in the low density stand, was significantly lower than that of the spotted knapweed rhizosphere, but was greater than that of the spotted knapweed rhizosphere of the high density stand. A comparison of the individual abundance of each phylotype present in the various rhizospheres showed that seven phylotypes were significantly reduced in the spotted knapweed low density and sixteen in the spotted knapweed high density (Figure 13C). Conversely, various phylotypes increased in abundance in the spotted knapweed rhizosphere as compared to the rhizosphere of *Poa secunda*. For example, six phylotypes were significantly increased in the spotted knapweed rhizosphere of both the low and high density stands. Though previous work revealed that spotted knapweed disrupts the arbuscular mycorrhizal fungal community of native and naturalized grasses, our results present the first analysis of the effects of spotted knapweed on the broader soil microbial community.

To examine if and at what distance spotted knapweed could affect the microbial community present in the soil rhizosphere of native grasses, we collected soils from another low-density stand of spotted knapweed (> 5 m spacing), but focused on a native grass species (*Poa secunda*) growing at various distances from spotted knapweed adults. Rhizosphere soils collected from *P. secunda* growing directly adjacent to spotted knapweed had significantly higher fungal biomass than those collected from distances further away (Figure 14A). This could be an additive effect, as the rhizospheric zone of both plant species overlapped at this distance. Interestingly, *P. secunda* growing 15 cm from spotted knapweed had the lowest amount of microbial biomass out of all distances tested. A more detailed analysis of the individual phylotype abundances suggests that this pattern of biomass is the net effect of two different effects of spotted knapweed on fungi in the rhizosphere. For example, total abundance (Σ peak heights) of some phylotypes decreases within 15 cm of a spotted knapweed plant; whereas, other phylotypes show a dramatic increase

within the spotted knapweed rhizosphere. When the resultant total abundance of all phylotpyes is determined (Figure 14B), this pattern is consistent with our observed estimates of total fungal biomass. It should also be noted that spotted knapweed roots were not apparent in grass rhizosphere samples at a distance of 15 cm or more from the spotted knapweed plant, suggesting that diffusible root exudates may be partially responsible for the observed decrease in microbial biomass at this distance (Broz et al. 2007b).

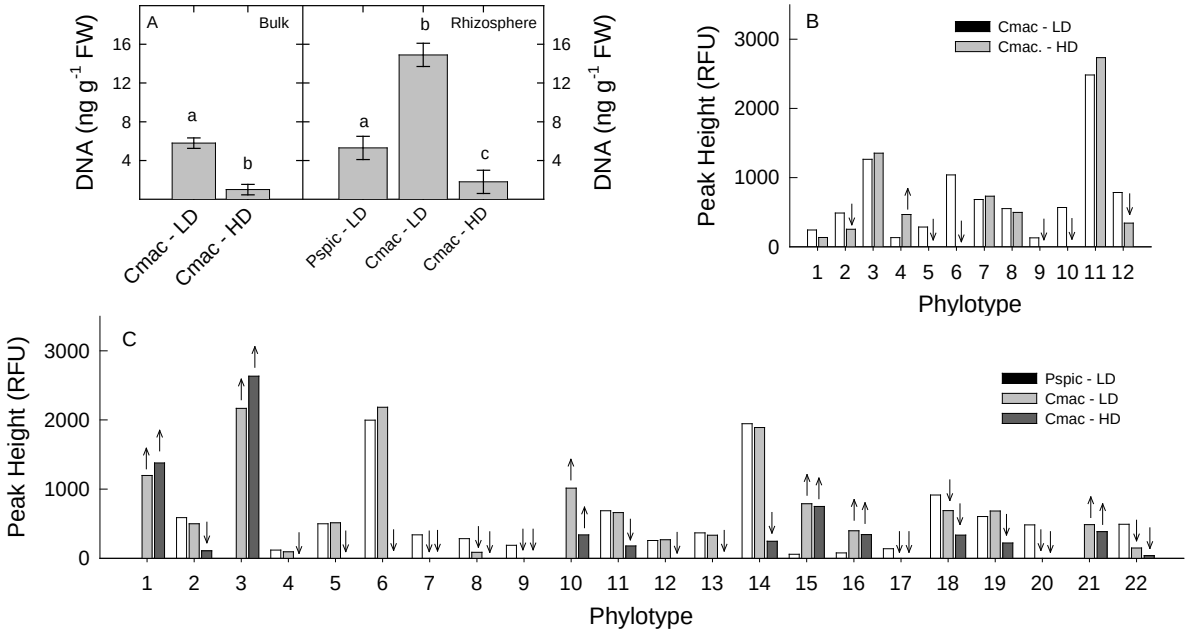

Figure 13. Total fungal DNA (panel A) and individual phylotype abundance (peak height) from bulk (panel B) and rhizosphere soil (panel C) from two sites in Montana that contained adjacent high and low density stands of spotted knapweed. Pspic – LD = *Pseudoroegneria spicata* in the low density stands, Cmac – LD = *Centaurea maculosa* in the low density stands, Cmac – HD = spotted knapweed in the high-density stands. Bars are LSmeans and standard errors; means with different letters are significantly different ($P < 0.05$, panel A). Bars are LSmeans and standard errors, arrows indicate significantly increasing or decreasing phylotype abundance between high and low density stands ($P < 0.05$, panel B). Bars are LSmeans and standard errors, arrows indicate significantly increasing or decreasing phylotype abundance relative to the *P. spicata* rhizosphere ($P < 0.05$, panel C).

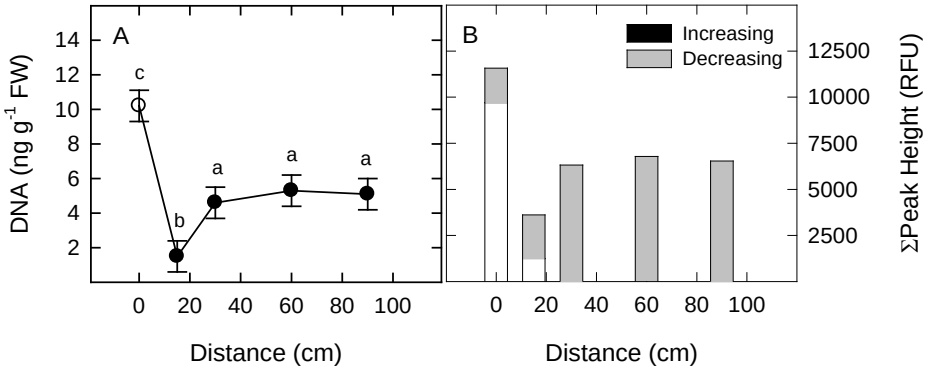

Figure 14. Total fungal DNA (panel A) and phylotype abundance (panel B) from the rhizosphere of *Poa secunda* plants at various distances from a *Centaurea maculosa* plant. Points are LS means and standard errors, means with different letters are significantly different from the control, *P. secunda* at 90 cm ($P < 0.05$, panel A). Bars are the total abundance (Σ of peak heights) for all phylotypes in each of two categories, significantly increasing or decreasing, based on the pair wise comparison between the *P. secunda* rhizophere at 0 and 90 cm ($P < 0.05$, panel B).

Can spotted knapweed culture pathogens of native plants?

This study developed knowledge of how the allelopathy of spotted knapweed manifested against soil microbes. For instance, in 2007 we published that spotted knapweed can alter the native microbial community composition within its own rhizosphere and that of neighboring native plants. At higher densities, the effect of spotted knapweed on native soil microbial communities was even more deleterious. We thus surmised that this invasive can have extreme effects not only on visible aboveground biodiversity but also on the native soil microbial community that extends beyond its rhizosphere. We have now determined the composition of the microbial community in the soils of spotted knapweed at high and low densities.

We used conserved primers to amplify portions of ribosomal regions from DNA extracted from knapweed-infested soils. These PCR fragments were then subject to 454 sequencing in order to get a general idea of microbial genera present in knapweed-infested soils. Members of the phylum ascomycota were the most predominant in an analysis of fungal sequences, followed by members of basidiomycota, zygomycota and a single oomycota. Common soil fungi genera including *Peniciullum* and *Aspergillus* were identified in the analysis. A variety of genera that contain known species of potential plant pathogens were identified as well. Sequences sharing strong homology to *Rhizoctonia*, *Sclerotina*, *Alternaria*, *Pythium*, *Phaeosphaeria*, *Plicaturopis* and *Phoma* species were found in knapweed-infested soils. Interestingly, many of these fungal genera, including *Alternaria* and *Phoma* were identified in a study of spotted knapweed seed endophytes.

We used the same spotted knapweed infested soils in greenhouse studies to determine if the microbial communities present would impact biomass accumulation in spotted knapweed or the native bunch grass Idaho fescue. Half of the soil used in this experiment was sterilized in order

to remove microbial community members. Both plant types tended to have reduced biomass accumulation in non-sterile soil versus sterile soil, which may suggest that plant pathogens are present in the original soil communities. These results are currently being prepared for publication.

Use of molecular resources developed for spotted knapweed to understand its invasiveness

Based on the realization during the course of the project that the allelochemical potential of catechin might be conditional, we embarked on a series of studies to comprehend the invasion potential of spotted knapweed at the genomics level. Therefore, we developed a molecular understanding of gene expression in spotted knapweed by co-hybridization studies using an *Arabidopsis* microarray. In these studies, we found that spotted knapweed gene expression changes depending on the presence of susceptible (to invasion) or resistant neighbors (Table 9). These studies have the potential to be transformative because it indicates that the individual physiology of spotted knapweed could be triggered by the presence of particular plant neighbors (Broz et al. 2008), which is accordance with other published information showing that plants can sense somehow the presence of neighbors (Gruntman and Novoplansky, 2004; Mahall and Callaway, 1991; Duddle and File, 2007). However, the results of our studies provide for a mechanism that might allow for invasive success at the community level.

These studies provide new information about gene regulation in plant competition; however, they are limited in scope. The data describe interactions between *Centaurea* and specific neighbors at only one moment in time, but competition is a process and competitive interactions may be better understood by testing plants from a series of time points. Because plants were grown in natural soils, microorganisms may also have had an influence on both the competitive results and the trends in gene expression that were observed. Thus, it could be beneficial to analyze transcript profiles of *Centaurea* grown in various environmental conditions, including a sterile environment. It would also be beneficial to analyze the response of the *Centaurea* leaf transcriptome in order to better understand competitor impacts on the shade avoidance response. In the field, each member of a plant community is likely to have multiple plant neighbors and will be subject to environmental characteristics that may influence competitive ability. This study explored the interaction of an invasive plant with only two potential neighbors in a greenhouse setting. This experimental design reflects, to some extent, the lack of plant diversity found in communities invaded by *Centaurea*. However, extrapolating these results to plant communities in the field is beyond the scope of this experiment.

In addition, it is difficult to know how much of the *Centaurea* genome is being sampled using our experimental platform. Many of the spots on the *Arabidopsis* microarray failed to hybridize reliably with *Centaurea* cDNAs, greatly reducing the amount of genes that could be analyzed. Q-PCR techniques revealed neighbor dependant differences in *Centaurea* gene expression, but these were not consistently in agreement with the results of the microarray. However, using either analysis, it appears that competition and neighbor identity are having an effect on *Centaurea* gene expression.

Table 9. Differentially expressed genes identified in micro-array analysis of competition experiments.

DOWN in BOTH					
ID	Function	FunCat	Ave C–G (*P* value)	Ave C–F (*P* value)	Two sample *t*-test *P* value
At5g60790	ABC Transporter, GCN subfamily	CIP, EIP	0.020 (0.031)	0.020 (0.015)	0.941
At1g71810	ABC1 family, possible chaperone	GIP	0.221 (0.015)	0.290 (0.013)	0.255
At1g05120	RAD16 homologue, DNA repair	GIP	0.219 (0.002)	0.369 (0.026)	0.016
At5g47920	Unknown	U	0.013 (0.039)	0.019 (0.016)	0.313
At2g03360	Unknown	U	0.017 (0.044)	0.017 (0.014)	0.836
BACT22F8	Unknown	U	0.014 (0.013)	0.014 (0.038)	0.928

UP in BOTH					
ID	Function	FunCat	Ave C–G (*P* value)	Ave C–F (*P* value)	Two sample *t*-test *P* value
At1g70490	ADP ribosylation, GTPase family	GIP	150.606 (0.0377)	84.816 (0.0348)	0.279

DOWN in GAILLARDIA, NO CHANGE in FESTUCA					
ID	Function	FunCat	Ave C–G (*P* value)	Ave C–F (*P* value)	Two sample *t*-test *P* value
At3g08580	Adenine nucleotide translocator	CPS	0.431 (0.025)	4.172 (0.200)	0.038
At2g43640	SRP14, RNA binding	GIP	0.331 (0.011)	0.644 (0.102)	0.012
At5g56670	S30, 40S ribosome	GIP	0.390 (0)	1.140 (0.627)	0.026
At3g44010	S29, 40S ribosome	GIP	0.025 (0.044)	1.478 (0.593)	0.047
At2g20690	Riboflavin synthase	M	0.465 (0.046)	1.130 (0.178)	0.005
At4g23600	COR13, Cysteine/ethylene synthesis	M	0.228 (0.008)	1.780 (0.249)	0.012
At5g06440	Unknown	U	0.495 (0.049)	0.728 (0.057)	0.024
At1g28400	Unknown	U	0.189 (0.011)	0.558 (0.029)	0.001
At4g18420	Unknown	U	0.445 (0.012)	1.138 (0.664)	0.042
AL161514	Unknown	U	0.377 (0.039)	1.280 (0.470)	0.013

NO CHANGE in GAILLARDIA, DOWN in FESTUCA					
ID	Function	FunCat	Ave C–G (*P* value)	Ave C–F (*P* value)	Two sample *t*-test *P* value
At1g22750	Unknown	U	0.549 (0.019)	0.149 (0.019)	0.002

Table 9. Continued.

NO CHANGE in GAILLARDIA, UP in FESTUCA					
ID	Function	FunCat	Ave C–G (*P* value)	Ave C–F (*P* value)	Two sample *t*-test *P* value
At1g76540	Cyclin dependant kinase	CPS, M	0.567 (0.357)	3.091 (0.002)	0.049
At5g50120	Transducin, Gprotein complex	CPS	0.449 (0.187)	2.148 (0.039)	0.025
At4g05320	SEN3, UBQ10	GIP, EIP	0.545 (0.106)	6.270 (0.016)	0.002
At5g44190	GLK2, myb family	GIP	0.591 (0.272)	3.556 (0.028)	0.026
At5g60120	TOE2, AP2 domain	GIP	0.480 (0.319)	3.606 (0.010)	0.044
At4g27330	SPL, MADS-box	GIP	0.552 (0.393)	5.676 (0.019)	0.041
At5g35770	SAP, development	GIP	0.463 (0.177)	2.884 (0.032)	0.016
At2g24060	IF3, translation initiation	GIP	0.467 (0.349)	4.811 (0.037)	0.048
At4g13850	GRP2, RNA binding	GIP	0.473 (0.307)	4.159 (0.045)	0.038
At5g02570	Histone 2B	GIP	0.381 (0.266)	3.687 (0.015)	0.038
At5g53120	Spermidine synthase (polyamines)	M	0.469 (0.292)	4.646 (0.009)	0.029
At2g27760	IPT2, Cytokinin synthase	M	0.470 (0.231)	3.757 (0.030)	0.021
At5g47980	Acyltransferase	M	0.420 (0.288)	3.697 (0.002)	0.041
At2g27730	Photorespiration	M	0.695 (0.302)	3.698 (0.021)	0.014
AB016892	Unknown	U	0.409 (0.278)	4.629 (0.007)	0.032
At2g13320	Unknown	U	0.503 (0.314)	4.106 (0.019)	0.034
AC007293	Unknown	U	0.331 (0.225)	3.640 (0.022)	0.031
At3g07030	Unknown	U	0.578 (0.349)	3.780 (0.019)	0.034
AC023912	Unknown	U	0.705 (0.030)	4.277 (0.010)	0.0002
AF367321	Unknown	U	0.431 (0.127)	3.326 (0.032)	0.008
AL163527	Unknown	U	0.398 (0.249)	3.050 (0.003)	0.037
AP000368	Unknown	U	0.454 (0.226)	3.911 (0.048)	0.021
AV441101	Unknown	U	0.567 (0.323)	2.587 (0.008)	0.046
AA394491	Unknown	U	0.531 (0.316)	3.503 (0.002)	0.036
AA728481	Unknown	U	0.502 (0.317)	3.839 (0.046)	0.039

This is the first report of using cross-species hybridization to microarray and Q-PCR in order to identify genes involved in competition between invasive and native plants. Future studies should be aimed at identifying transcriptional changes in both *Centaurea* and a variety of native plant competitors, in order to gain greater insight into the mechanisms of plant competition. By further characterizing competitive systems, it may be possible to identify molecular factors that increase plant competitive ability and facilitate invasion by exotics.

Based on these genetic studies, we took the knowledge that plant neighbors influence gene expression of spotted knapweed to analyze the metabolome of the invasive when it interacts with different neighbors in the greenhouse and in the field. Coincidentally, we found that spotted knapweed individuals accumulate increased levels of defense related secondary metabolites and reduced levels of primary metabolites when growing in conspecific versus heterospecific field stands (Table 10). In a greenhouse experiment designed to further investigate these results, we found that spotted knapweed plants accumulated less biomass and had higher amounts of total phenolics when grown with a conspecific versus a heterospecific plant neighbor, but only when the plants were elicited with jasmonic acid to mimic herbivory. These results indicate that an individual spotted knapweed plant can differentially modify its defense response strategy based on the composition of the plant community in which it grows: conspecific plant neighbors result in increased accumulation of defense related secondary metabolites, whereas heterospecific neighbors lead to increases in primary metabolism and biomass production. Our results suggest that plant neighbor identity, although generally unaccounted for in biological studies, is an important factor in individual plant biochemistry and physiology that necessitates further study (Broz et al. 2010).

Table 10. Compounds identified by GC-MS demonstrating significant ANOVA effects from stand type (conspecific or heterospecific).

Metabolites higher in heterospecific stands		
Compound	*Fold change (Conspecific/Heterospecific)*	*ANOVA p-value (stand type)*
Glycine	0.702	0.0023
Cytosine	0.370	0.0019
L-Alanine	0.483	<0.0001
L-Aspartic acid	0.562	0.001
L-Threonine	0.578	0.001
L-Proline	0.367	0.0035
Ethanol amine	0.812	<0.0001
Pyroglutamic acid	0.621	<0.0001
4-aminobutyric acid	0.599	0.0029
3-hydroxybenzoate	0.923	0.0077
Glycerol	0.856	0.0096
Catechol	0.818	0.0096
Ribose	0.763	0.0092
Fructose	0.716	0.0013
Fructose	0.733	0.0015
Maleic acid	0.575	0.0005
Succinic Acid	0.781	0.001
Fumaric Acid	0.651	<0.0001
Phosphoric acid (polar)	0.789	0.0064
Phosphoric acid (non-polar)	0.807	0.0094
Glycerophosphate	0.706	0.0032
Phytol	0.743	0.0053
Linoleic acid	0.794	0.0119
Hexacosanol	0.805	0.0057
Hexacosanoic acid	0.738	0.0035
Octacosanol	0.829	0.0064

Metabolites higher in conspecific stands		
Compound	*Fold change (Conspecific/Heterospecific)*	*ANOVA p-value*
Quinic Acid	1.199	0.0012
Inositol-like	1.586	0.0025
Inositol-like	1.316	0.0001
Galactose	1.245	0.0009
Galactonic acid	1.218	0.0002
Chlorogenic acid	2.080	0.0111

Although the perception of and response to neighbors is widely recognized in other taxa ranging from microorganisms to mammals (Diggle et al. 2007; Lyon 2007; Greene and Gordon, 2007; Martin et al., 2008; Dulac and Wagner, 2006; Sapolsky 2005), it remains understudied in the field of plant biology. Our results indicate that greenhouse-grown spotted knapweed individuals modify their defensive chemistry based on the identity of their plant neighbor. In addition, spotted knapweed individuals were found to exhibit different metabolic profiles in the field based on stand type (heterospecific or conspecific), which is likely due to a combination of factors including plant neighbor identity and rates of specialist herbivory. Whether or not a majority of plant species are able to differentially sense and respond to different plant neighbors

remains to be determined. If plants are indeed capable of these processes it will have large implications for both the study and human management of ecological systems.

Plant ploidy and invasiveness

To further these studies we used the EST library for spotted knapweed (Broz et al. 2007) to understand the genes in spotted knapweed related to invasion. We found that introduced populations of spotted knapweed exhibit reduced expression of transcripts related to constitutive defense relative to their native tetraploid counterparts, as might be expected based on ideas of enemy release and rapid evolution (Broz et al. 2009). Measurements of several vegetative traits were similar for all geo-cytotypes; however, fecundity of tetraploids was significantly greater than diploids, due in part to their polycarpic nature.

We selected three distinct PAL unigenes for analysis of secondary metabolite-related transcript, as this enzyme represents the first enzymatic step in the flavonoid synthesis pathway which contributes isoflavones, anthocyanins, condensed tannins and other secondary metabolic compounds in plants (La Camera et al. 2004; Treutter 2005; Winkel-Shirley 2001). Flavonoids are often stored in plant tissues as 'pre-formed' defense compounds and may act as pathogen and herbivore deterrents. The expression of PAL gene transcripts in addition to the secondary metabolites resulting from the flavonoid pathway are known to be important in plant defense against pathogens, herbivores and environmental stresses. A chitinase and a beta-1,3-glucanase were selected to analyze defense-related transcription, as these transcripts represent members of the PR family of proteins, which have been widely implicated in plant resistance to pathogens (Doxey et al. 2007; Jwa et al. 2006; Kasprzewska 2003). Different forms of chitinase are involved in both active and passive defense responses in plants. Glucanases have also been implicated in plant resistance to pathogens, and beta-1, 3-glucanases comprise part of the PR-2 group of pathogenesis-related genes. The fact that PAL, chitinase and glucanase transcripts were all reduced in introduced tetraploids compared to native tetraploids (Table 11) might suggest that populations of plants from the introduced range will be less defended against herbivores than natives, as is generally predicted by the EICA hypothesis. Recent reports indicate that introduced spotted knapweed plants are better defended against both generalist and specialist enemies than natives (Ridenour et al. 2008). This observation, in combination with the current study, may suggest that introduced populations have a higher potential degree of defense induction. However, the current study only measured levels of genes that may be involved in constitutive defense. Thus, our results must be interpreted with caution with regard to ecological hypotheses of plant defense in biological invasions.

We have demonstrated that the quantitative analyses of gene expression in native and introduced plant populations reveal trends that may provide additional insight into ecological hypotheses. However, the mechanisms underlying the observed changes in gene expression remain unclear, and further work is needed in this area. A better understanding of the genetic and molecular basis of invasiveness in exotic plants is not only an interesting case study in evolution, but is important to further our understanding how these invasions occur, and to choose appropriate management interventions. The techniques used in our study can provide an important complement to classical ecological measurements of plant fitness and competitive success.

Table 11. Relative gene expression values of *C. maculosa* geo-cytotypes.

	EU 2x vs EU 4x		Relative Expression			EU 4x vs US 4x	
Gene	**t**	**p-value**	**EU 2x**	**EU 4x**	**US 4x**	**t**	**p-value**
Actin	0.84	0.411	0.80 [a]	1.00 [a]	0.69 [a]	1.41	0.174
COX	0.96	0.348	1.25 [a]	1.00 [a]	0.86 [a]	0.63	0.538
UBQ	0.84	0.413	1.24 [a]	1.00 [a]	1.07 [a]	0.26	0.795
PAL 1	1.20	0.245	0.71 [ab]	1.00 [b]	**0.42 [a]**	**3.06**	**0.006**
PAL 2a	**4.91**	**<0.001**	**0.37 [a]**	1.00 [b]	**0.39 [a]**	**4.00**	**<0.001**
PAL 2b	**8.19**	**<0.001**	**0.21 [b]**	1.00 [c]	**0.06 [a]**	**8.19**	**<0.001**
Chitinase	0.47	0.644	0.89 [ab]	1.00 [b]	**0.59 [a]**	**2.14**	**0.045**
Glucanase	0.90	0.373	0.72 [ab]	1.00 [b]	**0.41 [a]**	**2.42**	**0.025**
TE	**2.41**	**0.025**	**0.50 [a]**	1.00 [b]	**0.42 [a]**	**3.06**	**0.006**
RAD	1.55	0.136	0.61 [a]	1.00 [a]	0.57 [a]	1.78	0.090

INTEGRATIVE FIELD STUDIES

Like other control strategies, we recognized that allelochemical control of invasives would not be a panacea, but has the potential to provide additional and better invasive plant control and revegetation strategies. Therefore, it was important for us to determine how allelochemical control strategies interact with other proven technologies such as biocontrol, mechanical and cultural control practices. The information and products from all the objectives were used to design factorial field studies addressing the control and ecology of invasive allelopathic plants. Allelochemical smother crops, the use of allelochemical-resistant native revegetation species, and biocontrols were evaluated at Yakima Training center, WA and Fort McCoy, WI. Results from greenhouse and lab experiments played a role in determining the treatment combinations used at each site. As these studies were underway, insights led to other investigations at alternate field sites.

In November 2005, we established field plots at Fort McCoy, WI in locations dominated by the two invasive plants of interest, spotted knapweed and leafy spurge (*Euphorbia esula*). The Fort McCoy field experiment was designed to test:

- whether allelochemical-resistant native species are more effective than standard restoration species for revegetating spotted knapweed and leafy spurge infested sites,
- whether allelopathic native species can be used to reduce reestablishment of spotted knapweed and leafy spurge (allelopathic smother crops),
- whether tillage and/or activated carbon addition can facilitate native species establishment, and
- whether herbivory of spotted knapweed and leafy spurge from biocontrol organisms affects interactions between invasive plants and native species (invasive plants were treated with systemic insecticide to selectively remove biocontrols).

- Further, we used the experimental plots to study the effects of biocontrol organisms and different native seed mixes on allelochemical production by the invasive plants.

In November 2005, we established field plots at Yakima Training Center (YTC) in locations dominated by Russian knapweed. The field experiment at YTC examined:

- whether allelochemical-resistant native species are more effective than standard restoration species for revegetating Russian knapweed infested sites,
- whether allelopathic native species can be used to reduce reestablishment of Russian knapweed, and
- whether tillage and/or activated carbon addition can facilitate native species establishment.
- Further, we used the experimental plots to study the effects of the different native seed mixes on Russian knapweed allelochemical production.

Specific hypotheses that focused on the invasive plants of interest in these two studies were:

1. The cover, biomass, and density of invasive plants of interest will be lowest on plots seeded with the allelopathic seed mix compared to all other seed mixes.
2. The cover, biomass, and density of invasive plants of interest will be lowest on plots seeded with the resistant seed mix compared to those seeded with the standard seed mix.
3. The cover, biomass, and density of invasive plants of interest will be lowest on plots that were tilled and received activated carbon compared to those that were tilled but did not receive activated carbon.
4. The cover, biomass, and density of invasive plants of interest will be lowest on plots that were tilled compared to those that were not tilled.

Hypotheses that focused on the plant community as a whole in these two studies were:

1. The cover, biomass, and species richness of native plant species will be greatest on plots that were seeded compared to the control plots that did not receive seed.
2. The cover, biomass, and species richness of native plant species will be greatest on plots that were seeded with the standard or resistant seed mix compared to those that were not seeded (control plots) or those that received the allelopathic seed mix.

At each site, we evaluated the ability of four types of native seed mixes to establish and compete effectively with invasive allelopathic plants: (1) a standard revegetation mix, (2) an allelochemical-resistant mix, (3) an allelopathic mix, and (4) a control (no seeds) (Tables 12 and 13). The four seed mixes were examined in factorial experiments with three activated carbon treatments: (1) 1 kg m^{-2} of activated carbon tilled into the soil, (2) a tilled control, and (3) an untilled control. Activated carbon sorbs organic compounds such as allelochemicals in the soil and therefore can be used to evaluate whether the interspecific interactions we observe are mediated by allelochemicals. In addition, at Fort McCoy the four seed mixes and two activated carbon treatments (1 kg m^{-2} of activated carbon and the tilled control) were crossed with two insecticide treatments (insecticide and a control), to examine the effects of biocontrol organisms that were released at Fort McCoy on interactions between spotted knapweed or leafy spurge and the native species. At both sites (Fort McCoy and YTC), the experiments were arranged in a

randomized complete block design, with five replicate blocks per site. Data from these experiments were collected from 2006 through 2009.

The experiments were initiated at each site in November 2005. At Fort McCoy, five, 13-m x 16-m areas (i.e., blocks) with relatively uniform vegetation dominated by spotted knapweed and leafy spurge were selected. Twenty, 2-m x 2-m plots were established in a 4 x 5 plot grid in each block, with 1-m buffer strips between plots. At the Yakima Training Center, five, 10-m x 16-m areas (i.e., blocks) with relatively uniform vegetation dominated by Russian knapweed were selected. Twelve, 2-m x 2-m plots were established in a 3 x 4 plot grid in each block, with 1-m buffer strips between plots.

At both sites, treatments were arranged at random among the plots in each block using a random number table. To establish the treatments, all plots were first mowed to approximately 10 cm above the soil surface, and raked to remove the mowed vegetation. Next, 4 kg of activated carbon was spread evenly across the surface of each activated carbon plot. The activated carbon was then incorporated into the soil to a depth of 15 cm with a garden tiller. The tilled control plots were also tilled to a depth of 15 cm. All plots were raked after tilling to create an even seedbed. Then the plots were seeded with the appropriate seed mixes (Tables 12 and 13). Seeds of each species in each plot were applied separately and were spread evenly across the plot surface by hand. All plots were then lightly raked to incorporate the seeds into the soil.

Table 12. Composition of the seed mixes applied in the field experiment at Fort McCoy. To ensure that the species selected were native to the site, they were selected from compiled lists of species that occurred at Fort McCoy prior to the experiment. Allelochemical-resistant species were selected based on laboratory tests of (±)-catechin resistance for species native to Wisconsin. Allelopathic native species were selected based on evidence for allelopathy in the literature. Growth forms: G = grass, F=forb. Life history: P = perennial, A = annual.

Seed Mix	Species	Common Name	Growth Form	Life History	Rate (PLS m^{-2})
Standard	*Bouteloua curtipendula*	sideoats grama	G	P	80
	Schizachyrium scoparium	little bluestem	G	P	80
	Symphyotrichum oolentangiense	skyblue aster	F	P	125
	Rudbeckia hirta	blackeyed Susan	F	P	125
	Verbena stricta	hoary verbena	F	A	125
Resistant	*Andropogon gerardii*	big bluestem	G	P	50
	Panicum virgatum	switchgrass	G	P	50
	Grindelia squarrosa	curlycup gumweed	F	P	190
	Lupinus perennis	sundial lupine	F	P	190
	Symphyotrichum laeve	smooth blue aster	F	P	125
Allelopathic	*Ambrosia artemisiifolia*	annual ragweed	F	A	160
	Asclepias syriaca	common milkweed	F	P	110
	Solidago canadensis	Canada goldenrod	F	P	110
	Solidago gigantea	giant goldenrod	F	P	160

Table 13. Composition of the seed mixes applied in the field experiment at the Yakima Training Center. To ensure that the species selected were native to the site, they were selected from compiled lists of species that occurred at the Yakima Training Center prior to the experiment. Allelochemical-resistant species were selected based on published studies of native grasses that compete well with Russian knapweed and of (±)-catechin-resistant forbs. Allelopathic native species were selected based on evidence for allelopathy in the literature. Growth forms: G=grass, F=forb. Life history: P = perennial, A = annual.

Seed Mix	Species	Common Name	Growth Form	Life History	Rate (PLS m^{-2})
Standard	*Pascopyrum smithii*	western wheatgrass	G	P	80
	Pseudoroegneria spicata	bluebunch wheatgrass	G	P	80
	Coreopsis tinctoria	golden tickseed	F	A	125
	Oenothera pallida	pale evening-primrose	F	P	125
	Sphaeralcea munroana	Munro's globemallow	F	P	125
Resistant	*Achnatherum hymenoides*	Indian ricegrass	G	P	55
	Hesperostipa comata	needle and thread	G	P	55
	Poa canbyi	Canby bluegrass	G	P	55
	Gaillardia aristata	common gaillardia	F	P	180
	Lupinus sericeus	silky lupine	F	P	190
Allelopathic	*Antennaria microphylla*	littleleaf pussytoes	F	P	135
	Helianthus annuus	common sunflower	F	A	135
	Solidago canadensis	Canada goldenrod	F	P	270

To monitor vegetation changes in 2006, 2007, 2008, and 2009, we measured vegetation cover by species, species richness, and the density of the invasive plants of interest in four permanent $0.19m^2$ subplots within each treatment plot. Vegetation biomass was destructively sampled at the end of the experiment in 2009 from each subplot. Vegetation cover estimates were made using the point intercept method within each subplot. Estimates of vegetation cover provided important information about the composition of the vegetation community; however, these estimates were not designed to catalogue all species present in the community. Because we were interested in not only the response of seeded species and target invasives, but also the native plant community, we recorded a complete species list for each subplot to estimate species richness at the whole plot level. We also estimated the density of the invasive species of interest within each subplot by counting the number of rosettes and bolting/flowering individuals of spotted knapweed, and the number of stems of leafy spurge. Similarly, at Yakima Training Center, we counted the number of Russian knapweed stems.

In 2009, at the end of the experiment, we analyzed the main effects of seed mix treatment, activated carbon treatment, and at Fort McCoy the insecticide treatment, and their interactions, on the vegetation response variables of density and cover using repeated-measures ANOVAs on a completely randomized block design. Tukey's means separation test was used to identify significant differences within treatments or treatment combinations. Vegetation biomass was recorded only in 2009, and was analyzed with a mixed model with seed mix, activated carbon, and insecticide treatments as fixed effects and block as a random effect. We also used simple

correlation to investigate the relationship between two species of interest in an effort to explain the patterns uncovered these analyses. Response variables that did not meet the assumption of normality were transformed appropriately.

Biocontrol Studies at Fort McCoy

The presence of biological control agents in spotted knapweed seedheads and roots at Fort McCoy was assessed in spring and fall of 2006 and 2007. In addition, we measured impacts of the insects by counting seeds and carefully monitoring invasive plant abundance in plots at Fort McCoy treated with systemic insecticides compared to untreated plots. This work was performed by Dr. Lincoln Smith of the USDA ARS using methods described by Paschke et al. (2008). In addition, root collections were made of leafy spurge and dissected to determine presence and quantity of biological control agents feeding on roots. In order to ensure an adequate population of insects to study at Fort McCoy, we worked with Fort McCoy staff and arranged releases of *Larinus minutus*, *Cyphocleonus achates*, *Agapeta zoegana*, adjacent to the study site in 2006.

At Fort McCoy, an insecticide treatment was included in the experimental design to evaluate the effects of biocontrol insects on the plant community and on allelochemical production. A systemic insecticide (Acephate Pro 75, 75% active ingredient) was applied to spotted knapweed and leafy spurge individuals in the insecticide-treated plots monthly from late April to August in 2006. In 2007, Acephate Pro 75 was applied again from late April to late September, and in addition, a soil-drench insecticide (Admire Pro, 43% active ingredient) was applied twice (June and September) during the 2007, 2008 and 2009 growing seasons. This second insecticide was applied to gain increased control over biological control agents, in particular, those that attack the roots of leafy spurge. Based on our observation of biocontrol insect densities in 2007, we increased the Acephate application to biweekly during the 2008 and 2009 growing seasons.

The root-feeding weevil, *Cyphocleonus achates*, occurred at very low densities in 2006 and 2007 at the Fort McCoy study plots. Insecticide treatment did not affect infestation rate of roots in spring 2007 (samples in 2006 preceded application of insecticides). The root-feeding moth, *Agapeta zoegana*, was not observed either year. Infestation of seedheads by introduced insects continued to increase, damaging 94% of unsprayed plants in 2007.

Based on dissections of seedheads collected in Sept. 2007 in six pairs of plots, insecticide treatments reduced attack by the *Urophora* flies (infesting 13% of insecticide and 69% of control seedheads). However, attack rates by the weevil *Larinus minutus* were actually higher on sprayed plants (infesting 88% of insecticide and 72% of control seedheads). The insecticide treatments did not significantly affect mortality of *Larinus minutus* developing within seedheads; however, it did kill more *Urophora* flies (65% in insecticide vs. 23% in control). These patterns were also observed in the regular experimental plots.

While the insecticide applications appeared to have impacts on insect populations, the treatments did not appear to have any effects on spotted knapweed or leafy spurge populations during the course of the study (see the following sections) indicating that the biocontrols were likely not impacting knapweed populations as has been observed in other locations (Story et al. 2006).

Vegetative Cover in Study Plots at Fort McCoy and YTC

Vegetation Cover at Fort McCoy, WI: Results from a repeated measures ANOVA for the response of spotted knapweed cover at Fort McCoy, WI to the main effects of year, seed mix treatment, activated carbon treatment, and insecticide treatment, as well as their interactions, are presented in Table 14. Hereafter, given that the analysis is identical for all response variables analyzed within the Fort McCoy cover data set, only those factors resulting in significant *p*-values ($\alpha < 0.05$) will be discussed.

Table 14. Repeated measures ANOVA results showing the response of spotted knapweed cover at Fort McCoy, WI over time to the main treatment effects of seed mix, activated carbon (AC), and insecticide (*p*-values in bold are significant at $\alpha < 0.05$).

Vegetation Category	Source of Variation	*F*-statistic (df)	*p*-value
spotted knapweed	Year	7.20 (3,190)	**0.0001**
	Seed Mix	2.29 (3,74)	0.0856
	Year * Seed Mix	8.50 (9,197)	**<0.0001**
	AC	0.22 (1,74)	0.6388
	Year * AC	0.63 (3,190)	0.5941
	Seed Mix* AC	0.17 (3,74)	0.9149
	Year * Seed Mix * AC	1.46 (9,197)	0.1646
	Insecticide	0.01 (1,74)	0.9310
	Year * Insecticide	0.22 (3,190)	0.8838
	Seed Mix* Insecticide	2.46 (3,74)	0.0691
	Year * Seed Mix * Insecticide	0.75 (9,197)	0.6653
	AC * Insecticide	0.86 (1,74)	0.3581
	Year * AC * Insecticide	1.14 (3,190)	0.3333
	Seed Mix * AC * Insecticide	0.28 (1,74)	0.8399
	Year * Seed Mix * AC * Insecticide	0.94 (9,197)	0.4941

For spotted knapweed cover at Fort McCoy, WI, only the main effect of year and the interaction between year and seed mix treatment was significant (Table 14, Figure 15). Specifically, the cover of spotted knapweed was significantly higher in 2008 (15 ± 1%, mean ± SE) than in 2006 or 2009 (10 ± 1% and 12 ± 1%, mean ± SE, respectively) (Figure 15). The impact of seed mix was dependent on year (Table 14), with plots seeded with the allelopathic seed mix having significantly lower spotted knapweed cover in 2006 compared to plots receiving the other seed mixes. This pattern shifted in 2007 and 2008 with significantly higher spotted knapweed cover in plots receiving the allelopathic seed mix than those plots receiving the resistant seed mix. No differences among spotted knapweed cover attributed to seed mix treatment were observed in 2009. The transient suppression of spotted knapweed in 2006 was likely due to the good establishment of annual ragweed in the allelopathic seed mix (discussed below).

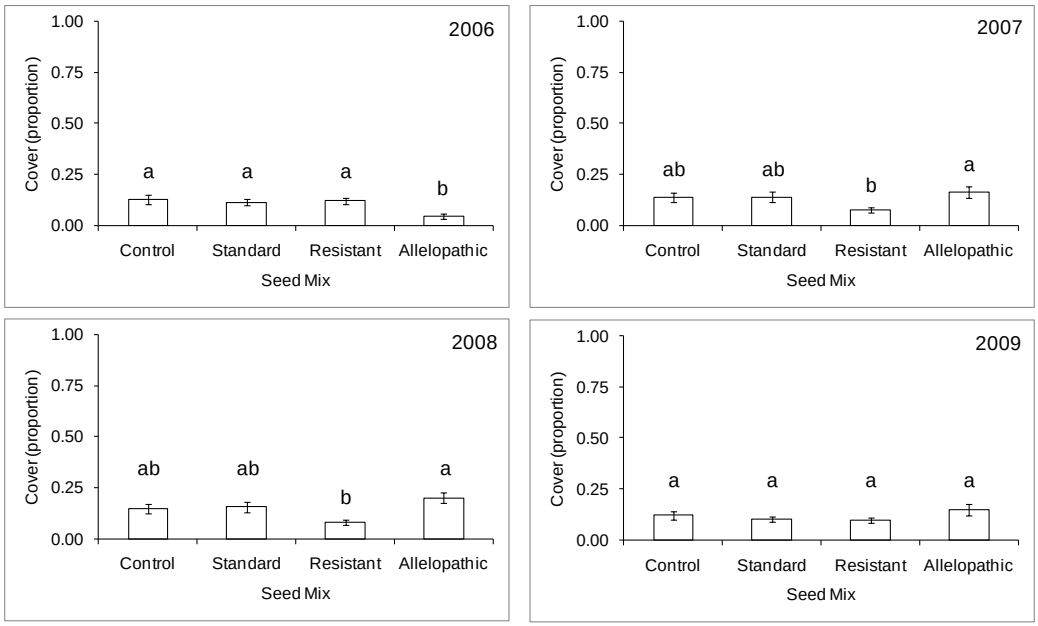

Figure 15. Mean cover of spotted knapweed (± SE) at Fort McCoy, WI in 2006, 2007, 2008, and 2009 among seed mix treatments. Within a given year, different letters indicate significant differences between seed mix treatments based on Tukey's tests ($\alpha < 0.05$).

For leafy spurge cover at Fort McCoy, WI, the main effect of year and the interaction between year and seed mix treatment had a significant impact (Figure 16). Specifically, the cover of leafy spurge was significantly higher in 2006 (12 ± 1%, mean ± SE) than in 2007, 2008, or 2009 (7 ± 1%, 7 ± 1%, and 3 ± 1%, respectively, mean ± SE; $F_{(3,185)} = 35.97$, p-value < 0.0001) (Figure 20). The impact of seed mix was dependent on year ($F_{(9,188)} = 6.43$, p-value < 0.0001), with plots receiving the allelopathic seed mix having significantly lower leafy spurge cover in 2006 than those plots receiving the control or resistant seed mix (Figure 16). Again, this is likely due to good establishment of annual ragweed in these plots. No differences among seed mix treatments were observed in subsequent years (Figure 16) after ragweed lost dominance. Also, the main effect of activated carbon had a significant impact on cover of leafy spurge, with cover of leafy spurge higher in tilled plots with activated carbon than without ($F_{(1,61)} = 4.65$, p-value = 0.0350; Figure 17).

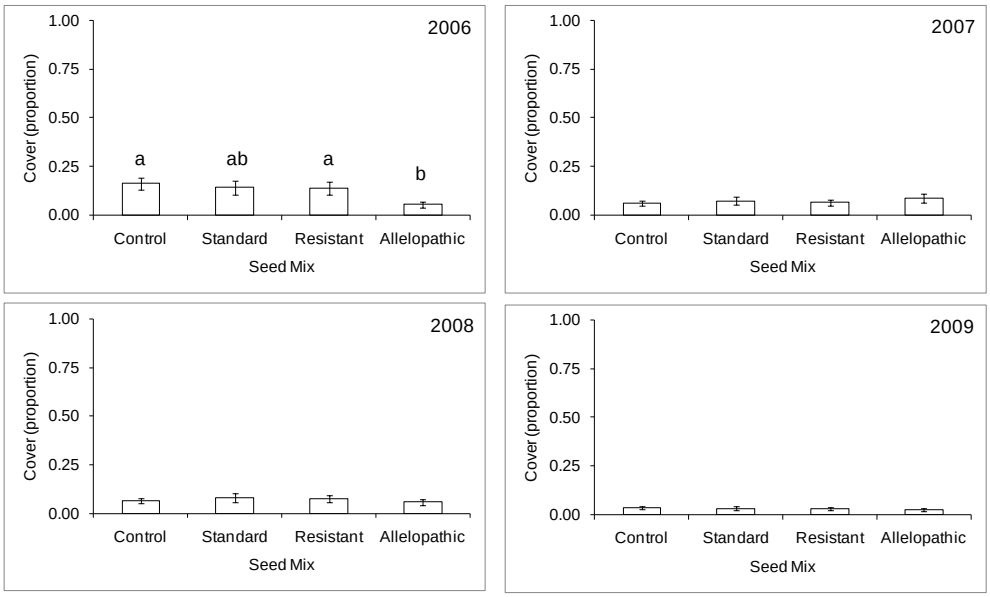

Figure 16. Mean cover of leafy spurge (± SE) at Fort McCoy, WI in 2006, 2007, 2008, and 2009 among seed mix treatments. Within a given year, different letters indicate significant differences between seed mix treatments based on Tukey's tests ($\alpha < 0.05$).

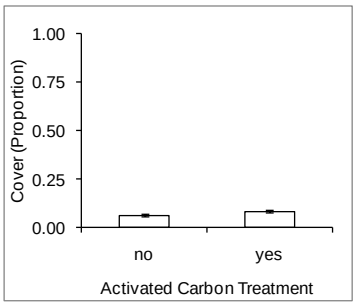

Figure 17. Mean cover of leafy spurge (± SE) at Fort McCoy, WI in plots treated with and without activated carbon.

Given that the cover of spotted knapweed and leafy spurge were impacted by the seed mix treatment in certain years, we analyzed the cover of seeded and unseeded species at Fort McCoy, WI in a repeated measures ANOVA. The cover of seeded species was significantly impacted by year ($F_{(3,180)} = 76.39$, p-value < 0.0001; Figure 22) and by the interaction of year and seed mix treatment ($F_{(9,188)} = 34.05$, p-value = < 0.0001; Figure 18). Cover of seeded species was significantly higher in 2008 (42 ± 2%) compared to all other years (27 ± 3%, 21 ± 1%, 26 ± 2% in 2006, 2007, and 2009, respectively) (Figure 18). In addition, in 2006, the cover of seeded species was much greater on plots receiving the allelopathic seed mix than any other seed mix (Figure 18). In subsequent years, the cover of seeded species was significantly greater on plots receiving the resistant seed mix than those receiving the allelopathic seed mix (Figure 18). The response of unseeded species was similar to that of seeded species, with the cover of unseeded species significantly impacted by year ($F_{(3,184)} = 20.45$ p-value < 0.0001; Figure 18), seed mix ($F_{(3,78)} = 4.12$, p-value = 0.0092; Figure 22), and the interaction of year and seed mix ($F_{(9,194)} = 20.00$, p-value < 0.0001; Figure 18). Cover of unseeded species was significantly higher in 2006

and 2009 (55 ± 3% and 57 ± 2%, respectively) compared to 2007 and 2008 (49 ± 2% and 44 ± 2%, respectively) (Figure 18). Of particular note, the cover of unseeded species was significantly reduced in plots seeded with the allelopathic seed mix in 2006 compared to all other seed mixes (Figure 22); however, this pattern was not present in subsequent years. The cover of unseeded species was also significantly greater in tilled plots without activated carbon than those tilled plots with activated carbon (53 ± 1% vs. 49 ± 1%, respectively; $F_{(1,78)} = 4.62$, p-value = 0.0347; Figure 19).

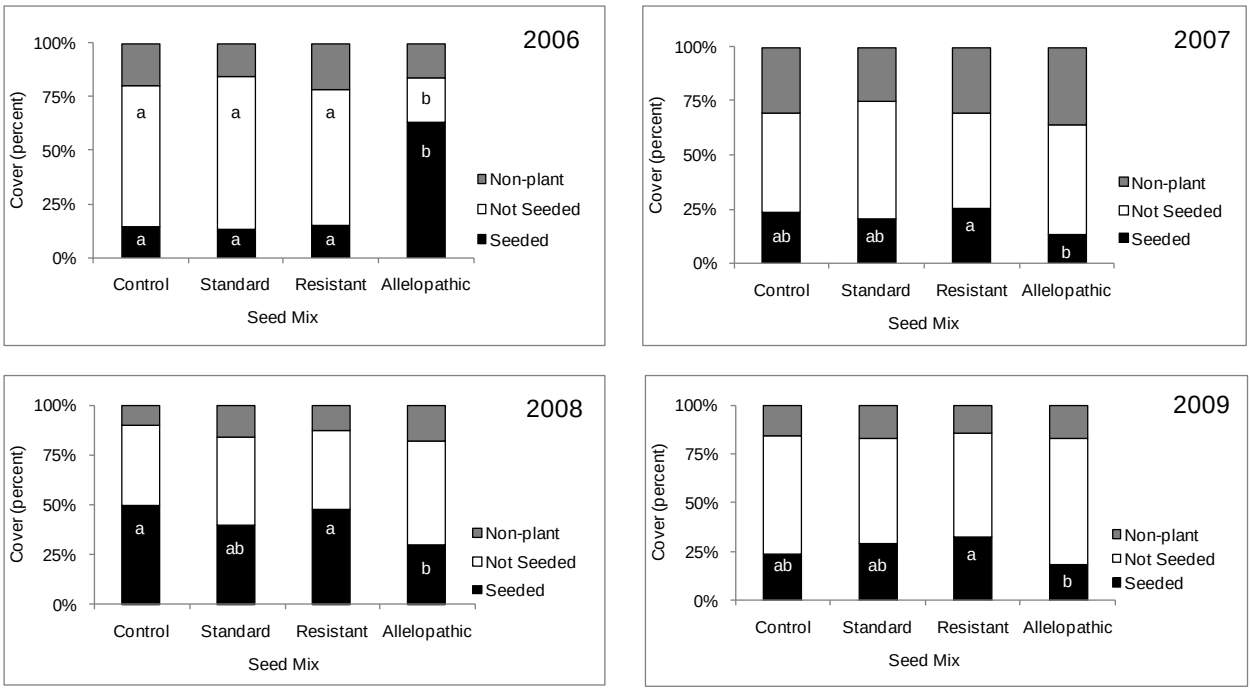

Figure 18. Mean cover of species that were seeded as part of a seed mix at Fort McCoy, WI, as well as unseeded species and non-plant cover categories in 2006, 2007, 2008, and 2009 among seed mix treatments. Within a given year and seeded or unseeded cover category, different letters indicate significant differences between seed mix treatments based on Tukey's tests ($\alpha < 0.05$).

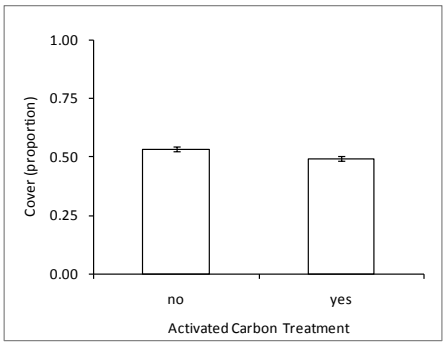

Figure 19. Mean cover (±SE) of unseeded species at Fort McCoy, WI in plots treated with and without activated carbon.

In an attempt to understand the response of invasives of interest to the seed mixes used in the field, we investigated simple correlations among the invasives and those species purposefully seeded into plots. In 2006, the allelopathic seed mix had a negative impact on the cover of both spotted knapweed and leafy spurge at Fort McCoy, WI. This pattern may be explained in part by a significant negative correlation in 2006 between annual ragweed (*Ambrosia artemisiifolia*) and spotted knapweed (r = -0.42, p-value = 0.0001), and annual ragweed and leafy spurge (r = -0.30, p-value = 0.0062). The cover of annual ragweed is largely responsible for the high cover values of seeded species in 2006 in the allelopathic seed mix; in 2006, seeded species represent 63 % of the vegetation cover on plots seeded with the allelopathic seed mix, and fully 95 % of that is annual ragweed. Annual ragweed represents only 8, 13, and 32 % of the seeded species in allelopathic plots in 2007, 2008, and 2009, and there is no significant correlation between spotted knapweed cover and annual ragweed cover in these years. In 2007, the cover of leafy spurge is actually positively correlated with the cover of annual ragweed (r = 0.32, p-value = 0.0043), and in 2008, the cover of leafy spurge is again negatively correlated with annual ragweed (r = -0.29, p-value = 0.0084). In 2009, there is no significant correlation between these two species.

Vegetation Cover at Yakima Training Center: At Yakima Training Center, the experimental design was more simplistic than that at Fort McCoy, WI, because it did not include an insecticide treatment to reduce biological control agents. Results from a repeated measures ANOVA for the response of Russian knapweed cover at YTC to the main effects of year, seed mix treatment, and activated carbon treatment, as well as their interactions, are presented in Table 15. Hereafter, given that the analysis is identical for all response variables analyzed within the YTC cover data set, only those factors resulting in significant p-values ($\alpha < 0.05$) are discussed.

Table 15. Results of a repeated measures ANOVA investigating the response of Russian knapweed cover at the Yakima Training Center, WA over time to the main effects of seed mix treatment and activated carbon (AC) treatment (p-values in bold are significant at $\alpha < 0.05$).

Vegetation Category	Source of Variation	F-statistic (df)	p-value
Russian knapweed	Year	3.56 (3,94)	**0.0173**
	Seed Mix	0.08 (3,32)	0.9684
	Year * Seed Mix	0.54 (9,96)	0.8409
	AC	0.26 (1,32)	0.6158
	Year * AC	0.03 (3,94)	0.9925
	Seed Mix * AC	0.99 (3,32)	0.4116
	Year * Seed Mix * AC	0.29 (9,96)	0.9772

For Russian knapweed at Yakima Training Center, only the main effect of year was significant ($F_{(3,94)}$ = 3.56, p-value = 0.0173; Table 7.8), with more Russian knapweed cover in 2008 compared to 2009 (36 ± 3% vs. 29 ± 3%, mean ± SE, respectively) (Figure 20). There was no significant effect of seed mix treatment (Figure 20) or activated carbon.

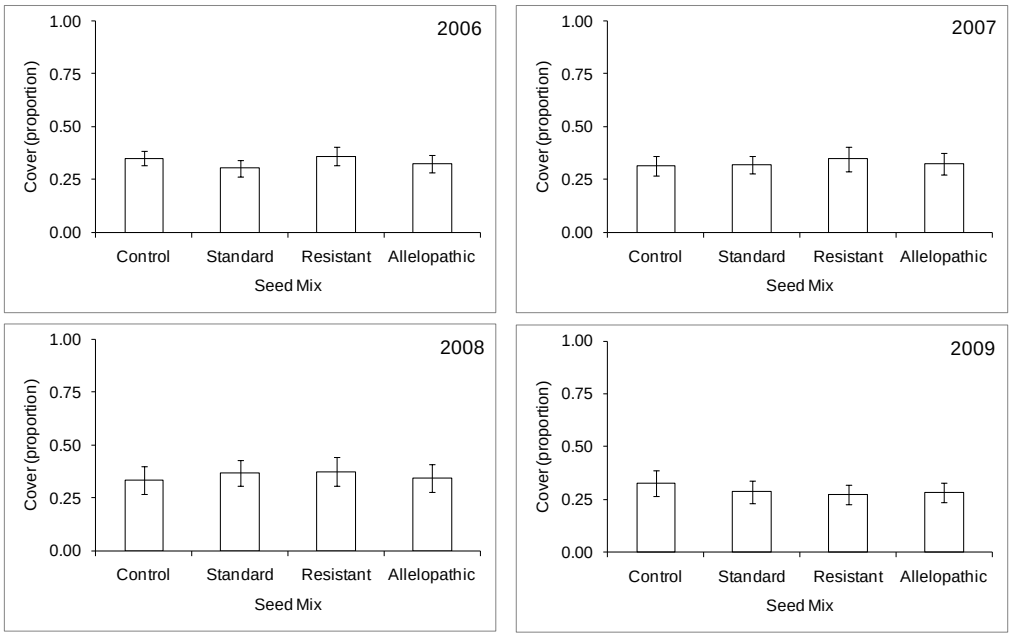

Figure 20. Mean cover (±SE) of Russian knapweed at Yakima Training Center in 2006, 2007, 2008, and 2009 among seed mix treatments.

We investigated the response of seeded and unseeded species at Yakima Training Center, because poor recruitment of species within our seed mixes might explain the lack of response from Russian knapweed cover. The cover of unseeded species was only significantly impacted by year ($F_{(3,95)}$ = 13.50, *p*-value < 0.0001), with higher cover of unseeded species in 2006 (72 ± 4%) compared to all other years (51 ± 4%, 46 ± 2%, 54 ± 2% in 2007, 2008, and 2009, respectively) (Figure 21). The cover of seeded species was significantly impacted by year ($F_{(3,90)}$ = 24.09, *p*-value < 0.0001), seed mix ($F_{(3,30)}$ = 7.00, *p*-value = 0.0010), and the interaction of year and seed mix treatment ($F_{(9,93)}$ = 29.67, *p*-value = < 0.0001) (Figure 21). The only seed mix that resulted in somewhat substantial cover of seeded species was the allelopathic seed mix, and then only in the year 2006 (Figure 21). And in 2009, the standard seed mix had significantly greater cover than the allelopathic seed mix (Figure 21). With the cover of seeded species so low, we would not expect Russian knapweed to respond strongly to seed mix, and accordingly, Russian knapweed cover was not strongly correlated with any species within our seed mixes (data not shown). In 2006, the only species that established within the allelopathic seed mix was common sunflower (*Helianthus annuus*), and this species represented 21 ± 3 % of the vegetation cover in 2006 in plots seeded with the allelopathic seed mix. Still, this species was not significantly correlated with the cover of Russian knapweed.

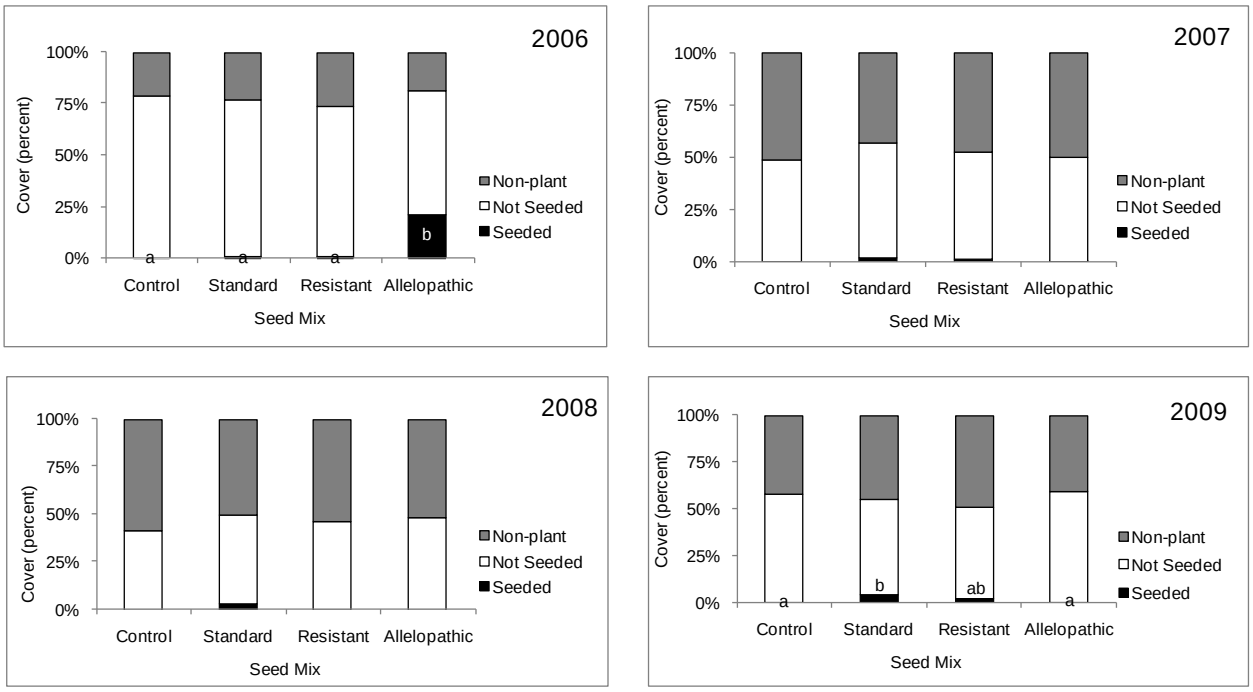

Figure 21. Mean cover of species that were seeded as part of a seed mix at Yakima Training Center, WA, as well as unseeded species and non-plant cover categories in 2006, 2007, 2008, and 2009 among seed mix treatments. For the seeded species, different letters within a given year indicate significant differences between seed mix treatments based on Tukey's means comparison tests ($\alpha < 0.05$).

In summary, the invasives of interest only showed a response to our seed mix treatment in the first year in Wisconsin, largely in response to the high cover of common ragweed. Activated carbon showed only very weak impacts on invasive species cover suggesting that allelopathy was not playing a strong role in plant community development. And the insecticide treatment had no significant impact on the cover of the invasives of interest. In addition to the response of the invasives of interest, we were also interested in the response of the plant community as a whole. Even if our seed mix treatments did not significantly reduce the cover of invasives, seeding may encourage the recruitment and establishment of desirable native species.

Vegetation Cover of Native versus Introduced Species

We investigated the response of the plant community, in terms of native and introduced species, to seed mix, activated carbon, and the insecticide treatments, and their interactions over time. These introduced species are defined as those that historically have not been found in Wisconsin or Washington, and therefore, are listed as introduced species by the USDA Plants Database (http://plants.usda.gov). These plants may or may not have weedy tendencies. Native species cover at Fort McCoy, WI was significantly affected by year ($F_{(3,180)} = 23.65$, p-value < 0.0001) and the interaction of year and seed mix treatment ($F_{(9,190)} = 14.14$, p-value < 0.0001) (Figure 22). In 2006, the cover of native species is significantly greater on plots seeded with the allelopathic seed mix compared to all others, yet this pattern disappears by 2007 and 2008, with plots receiving the resistant seed mix having significantly higher cover of native species in 2007

than plots receiving the allelopathic seed mix (Figure 22). A similar response is observed with introduced species, with the cover of introduced species significantly impacted by year ($F_{(3,180)}$ = 13.01, p-value < 0.0001) and the year by seed mix interaction ($F_{(9, 187)}$ = 11.54, p-value < 0.0001) (Figure 22). The cover of native species at Fort McCoy, WI is also significantly affected by activated carbon ($F_{(1,67)}$ = 6.82, p-value = 0.0111) (Figure 23), with tilled plots that did not receive activated carbon having significantly more cover of native species than those tilled plots with activated carbon (53 ± 1 % vs. 48 ± 1%, respectively; mean ± SE).

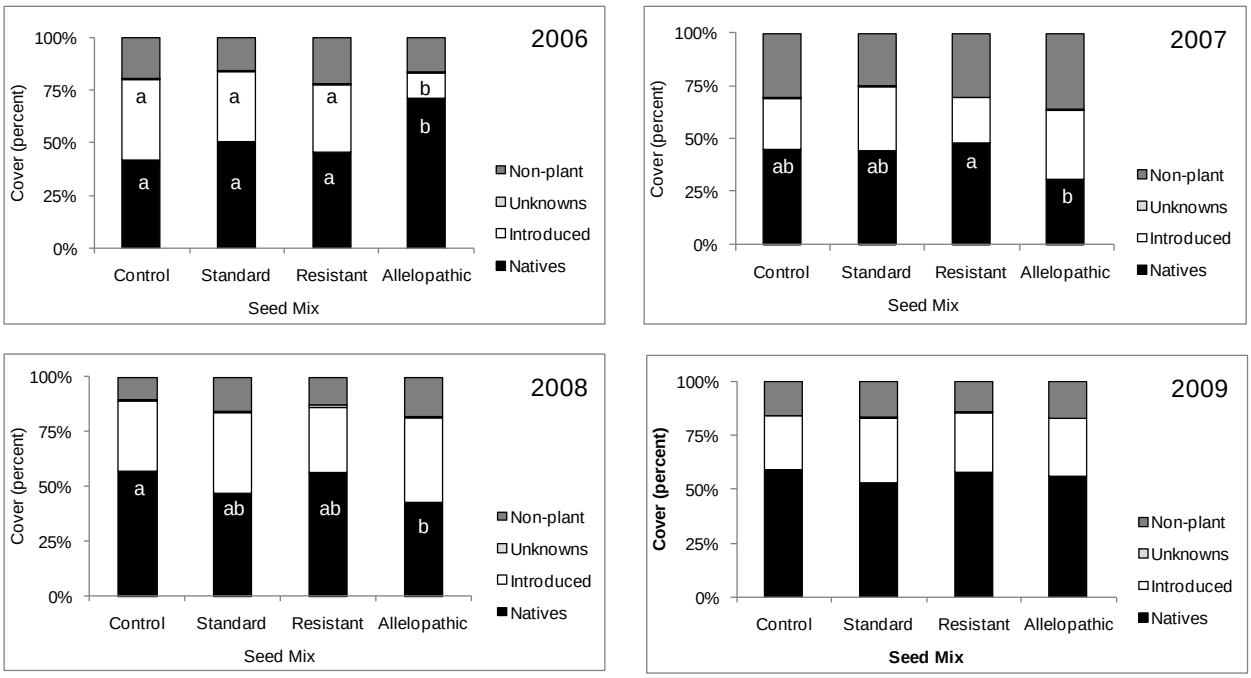

Figure 22. Mean cover of native species, introduced species, unknown species, and non-plant cover categories in 2006, 2007, 2008, and 2009 at Fort McCoy, WI among seed mix treatments. Within a given year and cover of native or introduced species, different letters indicate significant differences in cover between seed mix treatments based on Tukey's means comparison tests (α < 0.05).

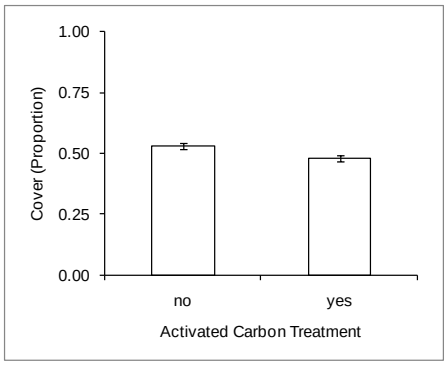

Figure 23. Mean cover (±SE) of native species at Fort McCoy, WI in plots treated with and without activated carbon.

The cover of native species at YTC was significantly affected by year ($F_{(3,93)}$ = 22.27, *p*-value < 0.0001), seed ($F_{(3,36)}$ = 3.46, *p*-value = 0.0261), and the interaction of year and seed mix treatment ($F_{(9,96)}$ = 8.03, *p*-value < 0.0001) (Figure 24). In 2006, the cover of native species was significantly higher on those plots receiving the allelopathic seed mix than any other seed mix, but this pattern disappears in 2007 and 2008 (Figure 24). And in 2009, the standard seed mix supported higher cover of native species than the control plots that were not seeded (Figure 28). The cover of introduced species was only significantly impacted by year ($F_{(3,96)}$ = 12.19, *p*-value < 0.0001) (Figure 24), with significantly more cover of introduced species in 2006 compared to subsequent years (70 ± 3% in 2006 compared to 51 ± 3%, 46 ± 2%, 54 ± 2% in 2007, 2008, and 2009, respectively; Figure 24).

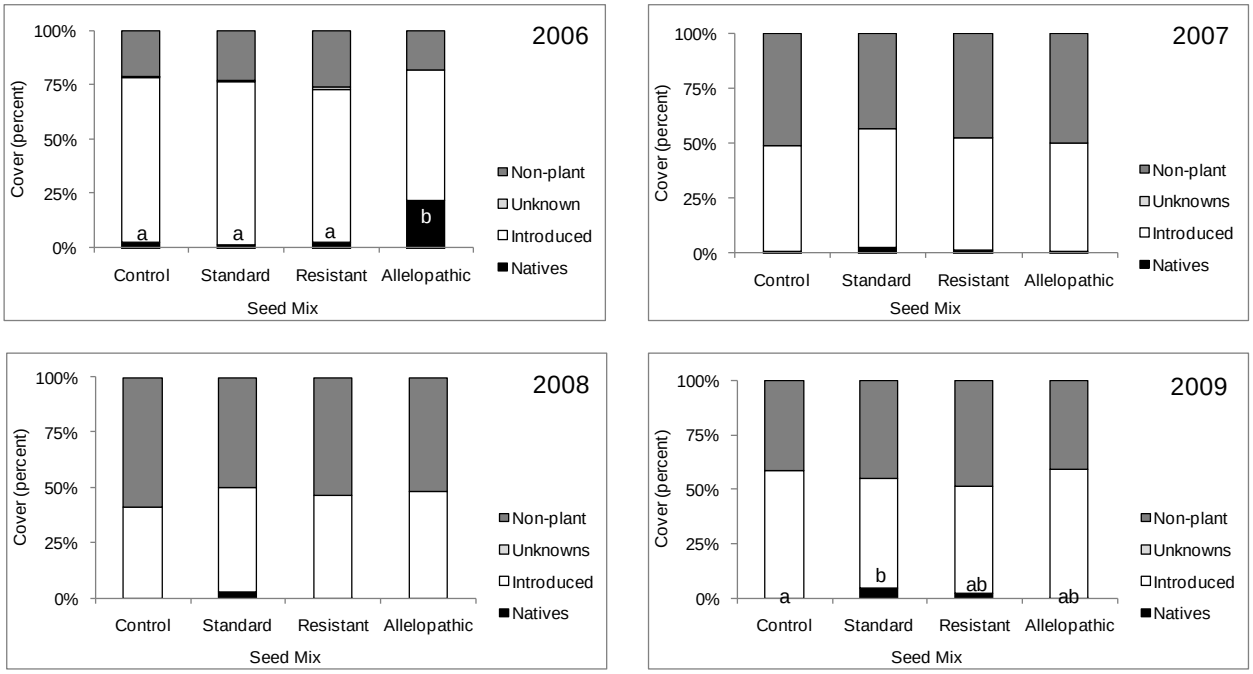

Figure 24. Mean cover of native species, introduced species, unknown species, and non-plant cover categories in 2006, 2007, 2008, and 2009 at Yakima Training Center, WA among seed mix treatments. Within a given year, different letters indicate significant differences between seed mix treatments for the cover of native plant species based on Tukey's means comparison tests (α < 0.05).

Invasive Plant Density

The density of Russian knapweed stems at Yakima Training Center was not significantly impacted by year, seed mix treatment, activated carbon treatment, or their interactions (data not shown).

Results from a repeated measures ANOVA for the response of spotted knapweed density at Fort McCoy, WI to the main effects of year, seed mix treatment, activated carbon treatment, and insecticide treatment, as well as their interactions, are presented in Table 16. Hereafter, given that the analysis is identical for all density response variables analyzed within the Fort McCoy density data set, only those factors resulting in significant *p*-values (α < 0.05) will be presented within the text.

Table 16. Results from a repeated measures ANOVA investigating the response of total density of spotted knapweed at Fort McCoy, WI over time to the main effects of seed mix treatment, activated carbon (AC) treatment, and insecticide treatment. Those p-values in bold are significant at $\alpha < 0.05$.

Invasive species	Source of Variation	*F*-statistic (df)	*p*-value
spotted knapweed	Year	70.05 (3,135)	**<0.0001**
	Seed Mix	4.31 (3,58)	**0.0082**
	Year * Seed Mix	1.57 (9,157)	0.1300
	AC	0.00 (1,58)	0.9879
	Year * AC	0.58 (3,135)	0.6274
	Seed Mix * AC	0.84 (3,58)	0.4788
	Year * Seed Mix * AC	1.25 (9,157)	0.2670
	Insecticide	0.11 (1,58)	0.7392
	Year * Insecticide	0.16 (3,135)	0.9242
	Seed Mix * Insecticide	4.19 (3,58)	**0.0095**
	Year * Seed Mix * Insecticide	0.70 (9,157)	0.7065
	AC * Insecticide	3.53 (1,58)	0.0654
	Year * AC * Insecticide	3.68 (3,135)	**0.0138**
	Seed Mix * AC * Insecticide	0.05 (1,58)	0.9847
	Year * Seed Mix * AC * Insecticide	0.60 (9,157)	0.7967

Total density of spotted knapweed (i.e., total number of spotted knapweed plants, regardless of developmental stage) at Fort McCoy, WI was significantly influenced by year, seed mix treatment, the two-way interaction of seed mix and insecticide, and the three-way interaction of year, activated carbon treatment, and insecticide (Table 16). In 2006, spotted knapweed density was highest in plots treated with insecticide but without activated carbon, and lowest in plots treated with both insecticide and activated carbon, but the difference in density was not significant between these two treatment types (Table 16, Figure 25). Furthermore, in subsequent years, the ranking of treatment combinations from highest to lowest density of spotted knapweed observed in 2006 was not maintained in subsequent years, resulting in a significant three-way interaction among year, activated carbon treatment, and insecticide treatment (Table 16, Figure 25). There was, however, a clear, significant impact of year on density of spotted knapweed; the density of spotted knapweed plants was significantly lower in 2006 (10.18 ± 1.79 plants/m^2, means ± SE) than in 2007, 2008, or 2009 (25.20 ± 2.22, 28.83 ± 2.16, 27.00 ± 2.29 plants/m^2, respectively; means ± SE) (Table 16, Figure 25). Spotted knapweed density was also significantly impacted by the seed mix treatment, but this was dependent on the insecticide treatment, with plots receiving the resistant seed mix having significantly lower density of spotted knapweed than those receiving the allelopathic seed mix, but only on plots that were not treated with insecticide (Table 16, Figure 26). Density of spotted knapweed, however, was not significantly different within a given seed mix when comparing plots with and without insecticide (Figure 26). This same pattern of lower total density of spotted knapweed in plots receiving the resistant seed mix compared to those receiving the allelopathic seed mix is further supported by the significant main effect of seed mix (Table 16, Figure 27).

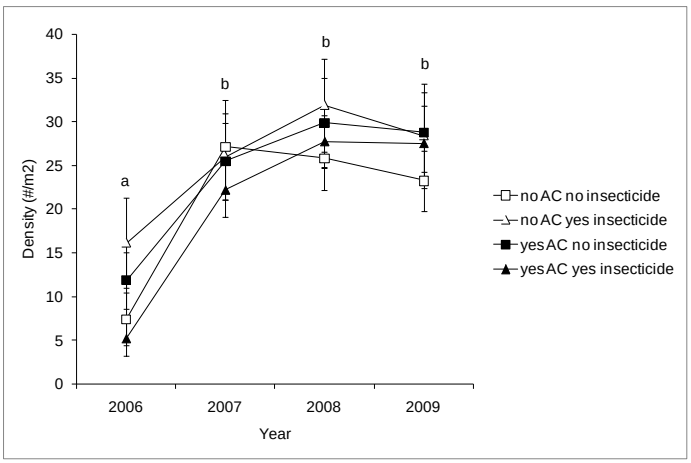

Figure 25. Mean density (±SE) of spotted knapweed over time at Fort McCoy, WI in response to activated carbon and insecticide treatments. The three-way interaction among year, activated carbon treatment, and insecticide treatment was significant; however, within a given year, no significant differences among treatment combinations were observed. The main effect of year was significant, and different letters indicate significant differences between years based on Tukey's means comparison tests ($\alpha < 0.05$).

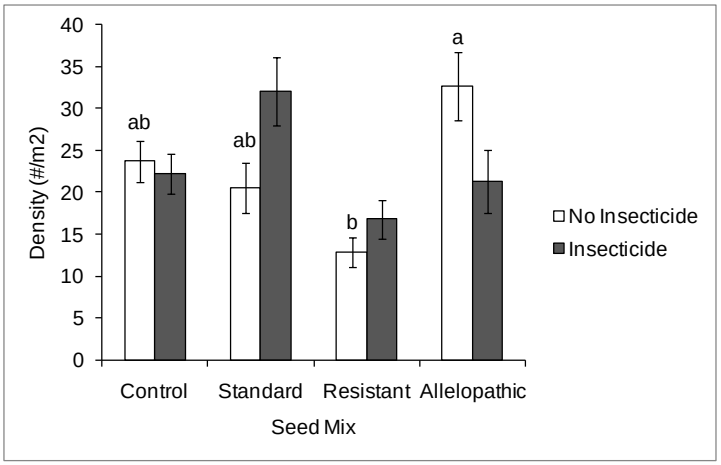

Figure 26. Mean density (±SE) of spotted knapweed at Fort McCoy, WI in response to seed mix treatment and insecticide treatment. Within the no insecticide treatment, different letters indicate significant differences between seed mix treatments based on Tukey's means comparison tests ($\alpha < 0.05$).

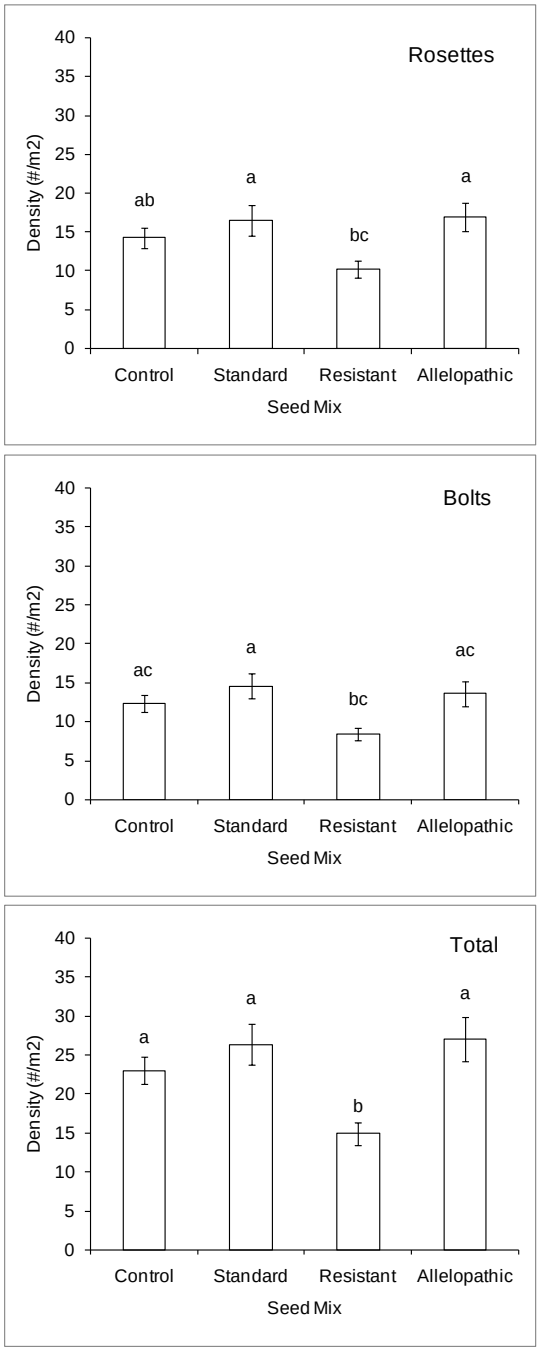

Figure 27. Mean density (±SE) of spotted knapweed at Fort McCoy, WI in response to seed mix treatment. Density was measured for plants in the rosette or bolting stage, and summed for total density of spotted knapweed. Within a given density metric (rosettes, bolts, or total count of spotted knapweed), different letters indicate significant differences between seed mix treatments based on Tukey's means comparison tests ($\alpha < 0.05$).

Density of spotted knapweed was also recorded based on two lifestages: rosette and bolting plants. Density of spotted knapweed rosettes ($F_{(9,160)} = 5.83$, p-value < 0.0001) and bolts ($F_{(9,155)} = 3.71$, p-value $= 0.0003$) were both significantly impacted by the interaction of year and seed

mix treatment. Plots receiving the allelopathic seed mix had a significantly higher density of spotted knapweed rosettes than those plots receiving any other seed mix, but only in 2007 (Figure 28). And in 2008, plots receiving the allelopathic seed mix had a significantly higher density of spotted knapweed bolts than those plots receiving the resistant seed mix (Figure 29). In addition, both spotted knapweed rosettes ($F_{(3,59)} = 3.33$, p-value = 0.0254) and bolts ($F_{(3,62)} = 4.33$, p-value = 0.0078) were significantly impacted by the interaction of seed mix and insecticide, showing a similar pattern to that observed for total count of spotted knapweed (Figure 32), with the plots receiving the resistant seed mix having significantly lower density of spotted knapweed than those receiving the allelopathic seed mix, but only on plots that were not treated with insecticide (data not shown). For both rosettes and bolts of spotted knapweed, the main effects of year and seed mix treatment significantly impacted density. Density of rosettes was significantly higher in 2006 (21.47 ± 1.93 plants/m^2, means ± SE) than in 2007, 2008, or 2009 (9.87 ± 1.42, 10.63 ± 0.85, 15.94 ± 1.69 plants/m^2, means ± SE, respectively) ($F_{(3,141)} = 33.26$, p-value < 0.0001). In contrast, density of bolting plants was significantly lower in 2006 (2.48 ± 0.34 plants/m^2, means ± SE) compared to 2007, 2008, or 2009 (15.33 ± 1.33, 18.20 ± 1.59, 12.81 ± 1.05 plants/m^2, means ± SE, respectively), with density significantly highest in 2008 ($F_{(3,132)} = 95.79$, p-value < 0.0001). The pattern of high numbers of rosettes but few bolts of spotted knapweed in 2006 may be representative of recovery after tilling of plots during the installation of the experiment. The main effect of seed mix also had a significant impact on the density of rosettes and bolts of spotted knapweed ($F_{(3,59)} = 4.36$, p-value =0.0077; $F_{(3,62)} = 4.10$, p-value = 0.0103, respectively), with both life stages showing a similar pattern to that of the total count of spotted knapweed plants, with plots seeded with the resistant mix having significant fewer plants than some of the other seed mixes (Figure 29).

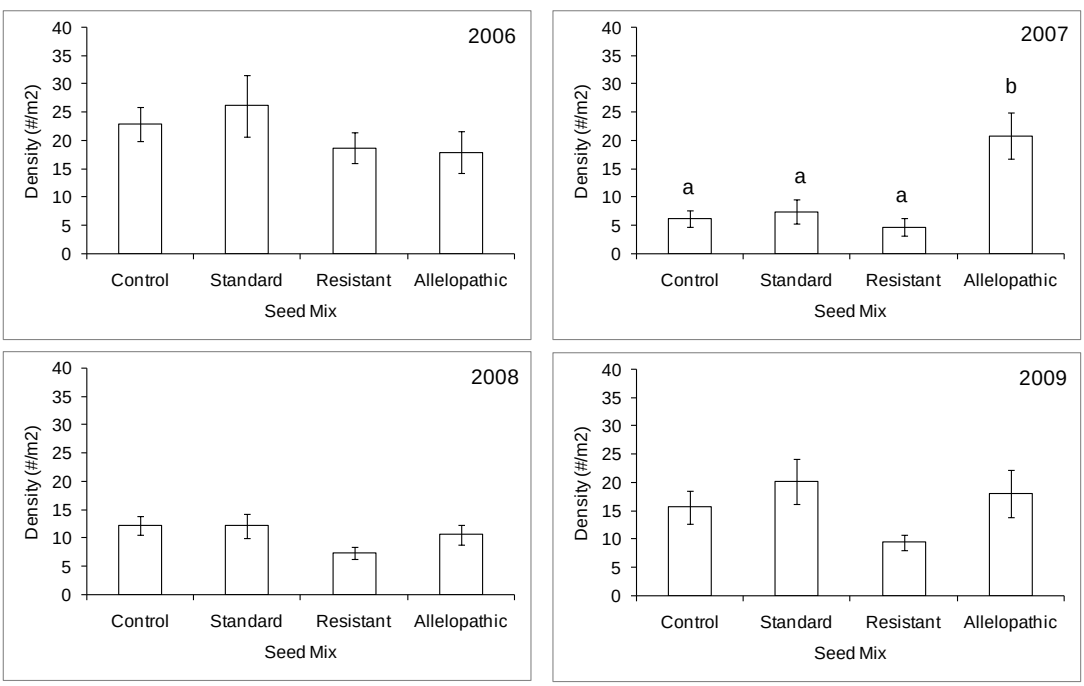

Figure 28. Mean density (±SE) of spotted knapweed rosettes at Fort McCoy, WI in 2006, 2007, 2008, and 2009 among seed mix treatments. Different letters indicate significant differences between seed mix treatments within a given year based on Tukey's means comparison tests ($\alpha < 0.05$).

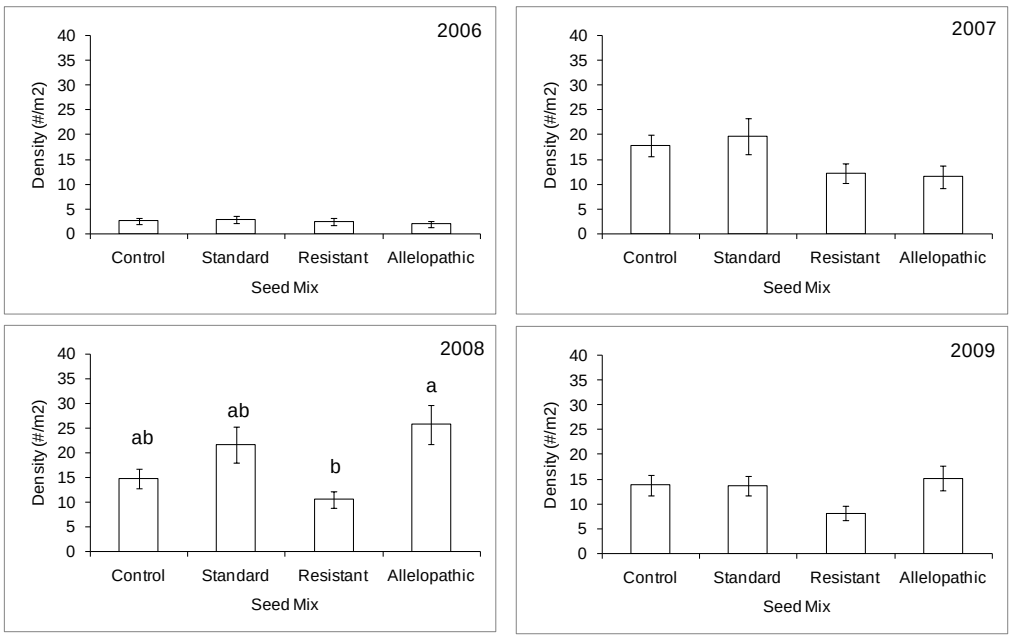

Figure 29. Mean density (±SE) of spotted knapweed bolts at Fort McCoy, WI in 2006, 2007, 2008, and 2009 among seed mix treatments. Different letters indicate significant differences between seed mix treatments within a given year based on Tukey's means comparison tests ($\alpha < 0.05$).

The density of leafy spurge stems at Fort McCoy, WI was significantly influenced by year, and the two-way interaction of year and seed mix, and the two-way interaction of seed mix and insecticide. Although within a given year, the response of the density of leafy spurge stems is not significantly different among seed mix treatments, the response of density of leafy spurge stems is not consistent year to year to seed mix, resulting in a significant year and seed mix interaction ($F_{(9,157)} = 2.47$, p-value = 0.0117) (Figure 30). There were significant differences in the density of leafy spurge from year to year ($F_{(3,134)} = 21.96$, p-value < 0.0001), with leafy spurge density significantly highest in 2006 (46.65 ± 5.09, mean ± SE) compared to 2007, 2008, or 2009 (34.60 ± 3.83, 33.35 ± 3.64, and 23.80 ± 2.79, respectively, mean ± SE). Density of leafy spurge stems was also significantly impacted by the interaction of seed mix and insecticide ($F_{(3,61)} = 3.18$, p-value = 0.0302), with the density of leafy spurge stems being only marginally different in plots with and without insecticide that were seeded with the standard mix (p-value = 0.0967) (Figure 31).

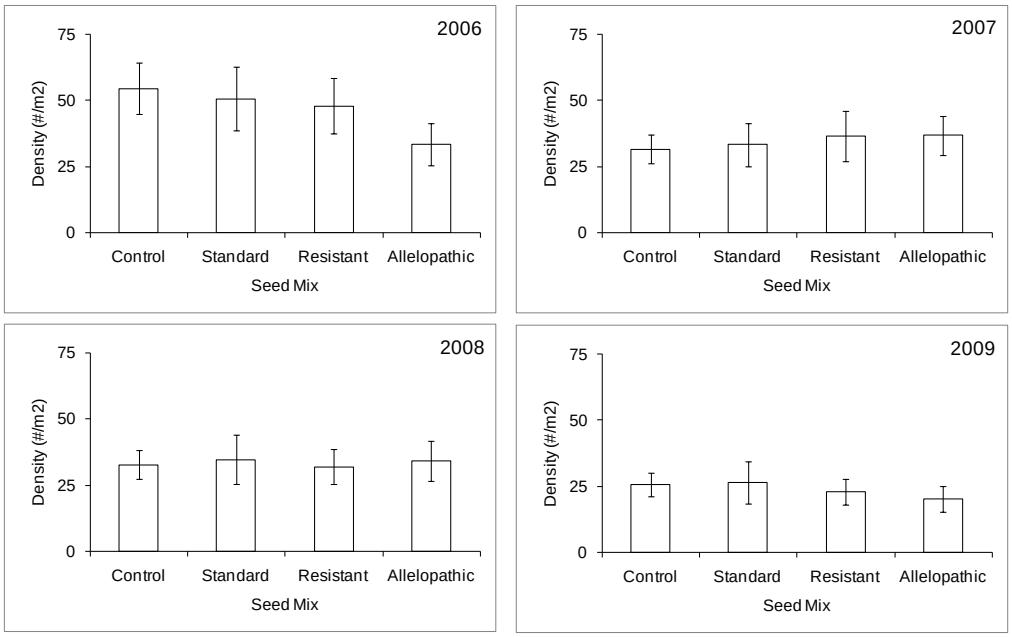

Figure 30. Mean density (±SE) of leafy spurge stems at Fort McCoy, WI in 2006, 2007, 2008, and 2009 among seed mix treatments.

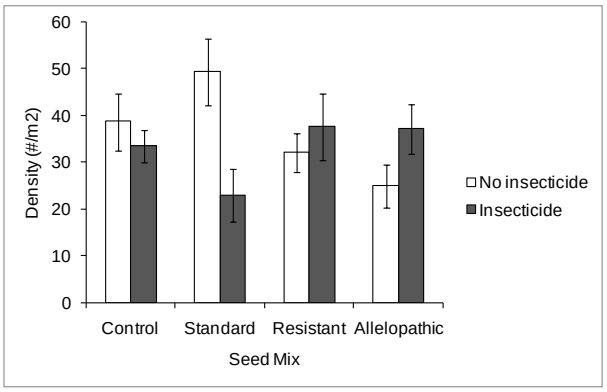

Figure 31. Mean density (±SE) of leafy spurge stems at Fort McCoy, WI in response to seed mix and insecticide treatments.

Vegetation Biomass in Study Plots

At Fort McCoy, WI, the biomass of spotted knapweed in 2009 was not significantly impacted by any of the main effects (seed mix treatment, activated carbon treatment, or insecticide treatment) or their interactions (Figure 32).

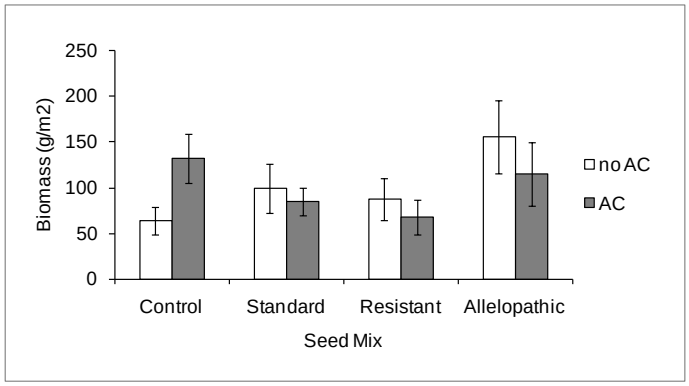

Figure 32. Mean biomass (±SE) of spotted knapweed at Fort McCoy, WI in 2009 in response to seed mix and activated carbon treatments.

Results from a mixed model ANOVA for the response of leafy spurge biomass at Fort McCoy, WI to the main effects of seed mix treatment, activated carbon treatment, and insecticide treatment, as well as their interactions, are presented in Table 17. Hereafter, given that the analysis is identical for all response variables analyzed within the Fort McCoy biomass data set, only those factors resulting in significant *p*-values ($\alpha < 0.05$) will be presented within the text.

Table 17. Results from a mixed model ANOVA investigating the response of 2009 leafy spurge biomass at Fort McCoy, WI to the main effects of seed mix treatment, activated carbon (AC) treatment, and insecticide treatment, and their interactions. Those *p*-values in bold are significant at $\alpha < 0.05$.

Invasive species	Source of Variation	*F*-statistic (df)	*p*-value
Leafy spurge	Seed Mix	0.41 (3,60)	0.7431
	AC	4.74 (1,60)	**0.0333**
	Seed Mix * AC	0.40 (3,60)	0.7568
	Insecticide	2.51 (1,60)	0.1187
	Seed Mix * Insecticide	1.38 (3,60)	0.2571
	AC * Insecticide	0.04 (1,60)	0.8510
	Seed Mix * AC * Insecticide	0.56 (3,60)	0.6464

For 2009 biomass of leafy spurge at Fort McCoy, WI, only the main effect of activated carbon had a significant impact (Table 17), with plots treated with activated carbon having significantly more biomass than those plots without activated carbon (Figure 33). The opposite pattern was observed for native species biomass, with plots treated with activated carbon having significantly less native species biomass than those plots without activated carbon ($F_{(1,60)} = 4.16$, *p*-value = 0.0458, Figure 34). No other main effects or interactions significantly impacted native species biomass in 2009, and introduced species biomass was not significantly affected by any of the main effects or interactions (Figure 35).

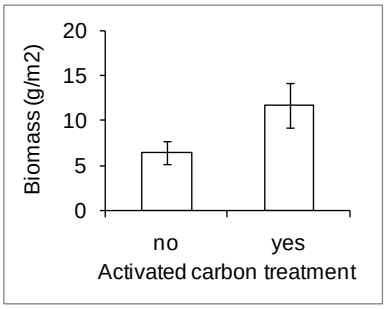

Figure 33. Mean biomass (±SE) of leafy spurge at Fort McCoy, WI in 2009 in response to activated carbon treatment.

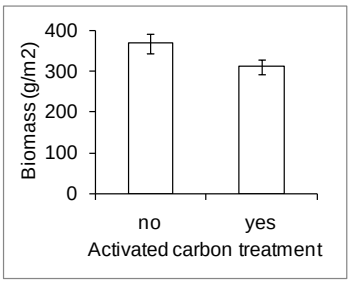

Figure 34. Mean biomass (±SE) of native species at Fort McCoy, WI in 2009 in response to activated carbon treatment.

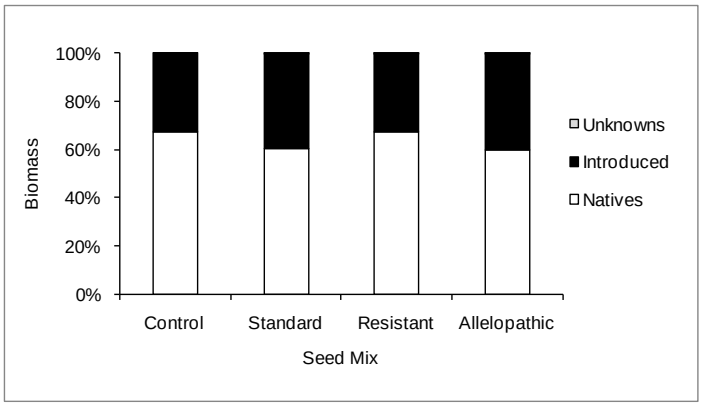

Figure 35. Mean biomass of native, introduced, and species of unknown origin at Fort McCoy, WI in 2009 in response to seed mix treatment.

Biomass of seeded species at Fort McCoy, WI in 2009 was significantly greater on plots seeded with the resistant seed mix than those seeded with the allelopathic seed mix ($F_{(3,60)} = 9.13$, p-value < 0.0001) (Figure 36). Biomass of seeded species was also significantly affected by the interaction between activated carbon and insecticide treatments ($F_{(1,60)} = 4.60$, p-value $= 0.0361$) (Figure 37), although no significant differences among means were observed with Tukey means comparison tests.

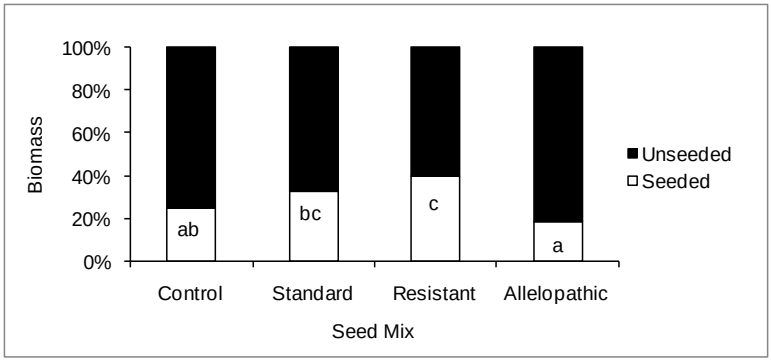

Figure 36. Mean biomass of seeded and unseeded species at Fort McCoy, WI in 2009 in response to seed mix treatment. Different letters indicate significant differences between seed mix treatments for seeded species based on Tukey's means comparison tests ($\alpha < 0.05$).

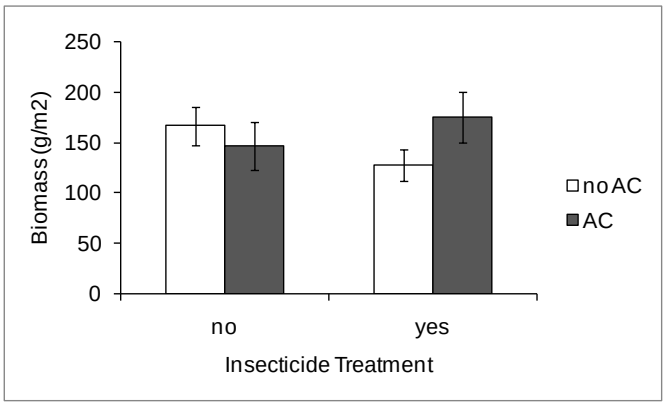

Figure 37. Mean biomass (±SE) of seeded species at Fort McCoy, WI in 2009 in response to the activated carbon and insecticide treatments.

Biomass of unseeded species at Fort McCoy, WI in 2009 was significantly lower on plots with activated carbon than those without activated carbon, but only when treated with insecticide ($F_{(1,60)} = 7.30$, p-value = 0.0090) (Figure 38). This pattern is supported by the significant main effects of activated carbon ($F_{(1,60)} = 4.64$, p-value = 0.0353) and insecticide ($F_{(1,60)} = 5.46$, p-value = 0.0228) on biomass of unseeded species.

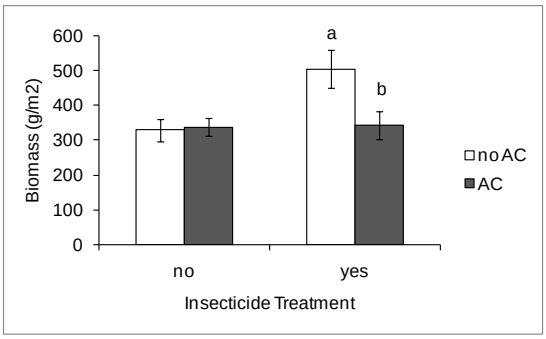

Figure 38. Mean biomass (±SE) of unseeded species at Fort McCoy, WI in response to the activated carbon and insecticide treatments. Different letters indicate a significant difference between activated carbon treatments within an insecticide treatment based on Tukey's means comparison tests ($\alpha < 0.05$).

At Yakima Training Center, WA, the biomass of Russian knapweed in 2009 was not significantly impacted by any of the main effects (seed mix treatment, activated carbon treatment) or their interactions (Figure 39).

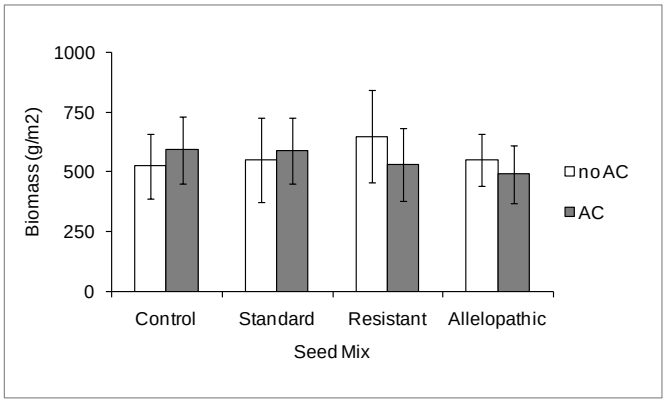

Figure 39. Mean biomass (±SE) of Russian knapweed at Yakima Training Center, WA in response to seed mix treatment and activated carbon treatment.

Results from a mixed model ANOVA for the response of native species biomass at Yakima Training Center, WA to the main effects of seed mix treatment and activated carbon treatment, as well as their interaction, are presented in Table 18. Hereafter, given that the analysis is identical for all response variables analyzed within the Yakima Training Center biomass data set, only those factors resulting in significant *p*-values ($\alpha < 0.05$) will be presented within the text.

Table 18. Results from a mixed model ANOVA investigating the response of 2009 native species biomass at Yakima Training Center, WA to the main effects of seed mix treatment and activated carbon (AC) treatment, and their interaction. Those *p*-values in bold are significant at $\alpha < 0.05$.

Vegetation Category	Source of Variation	*F*-statistic (df)	*p*-value
Native Species	Seed Mix	5.19 (3,28)	**0.0056**
	AC	0.22 (1,28)	0.6421
	Seed Mix * AC	0.29 (3,28)	0.8313

The biomass of native species was significantly greater in plots receiving standard and resistant seed mixes than the control plots (Table 18, Figure 40). Biomass of introduced species was not significantly affected by any of the main effects or their interactions (Figure 40). The biomass of seeded species was significantly greater on plots receiving the standard and resistant seed mixes than the plots receiving the allelopathic seed mix or control plots ($F_{(3,28)} = 11.51$, *p*-value < 0.0001) (Figure 41). Biomass of unseeded species was not significantly affected by any of the main effects or their interactions (Figure 41).

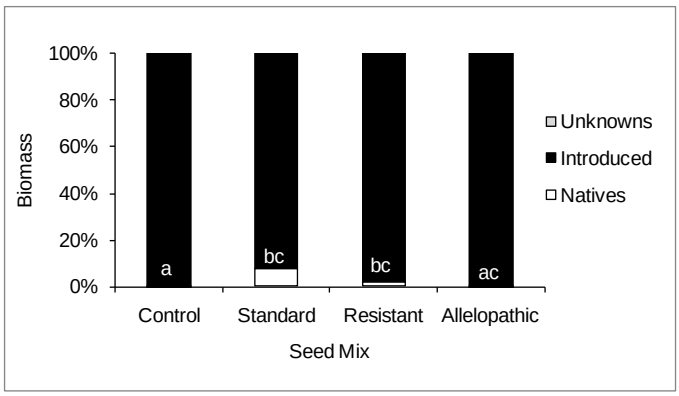

Figure 40. Mean biomass of native, introduced, and species of unknown origin at Yakima Training Center, WA in 2009 in response to seed mix treatment. Different letters indicate significant differences between seed mix treatments for native species based on Tukey's means comparison tests ($\alpha < 0.05$).

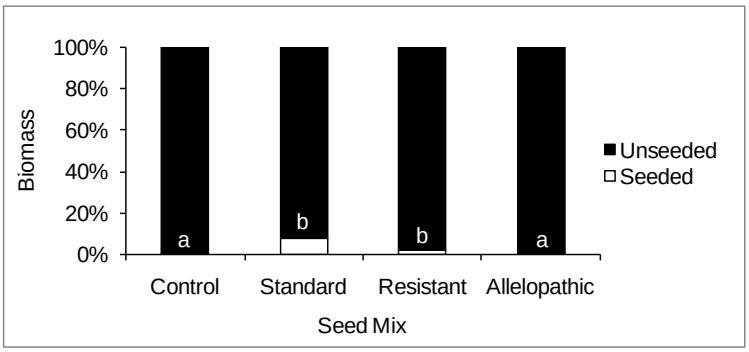

Figure 41. Mean biomass of seeded and unseeded species at Yakima Training Center, WA in 2009 in response to seed mix treatment. Different letters indicate significant differences between seed mix treatments for seeded species based on Tukey's means comparison tests ($\alpha < 0.05$).

Additional Studies of Native Seeding in Russian Knapweed at YTC

In October 2005 we established a second field experiment at the Yakima Training Center (YTC) with a paired study in Eurasia. This experiment further examined seeding native species into Russian knapweed stands. The international part of these studies was funded by the US-Russian Civilian Fund.

Acroptilon repens (hereafter *Acroptilon*) appears to be highly competitive and casual observations suggest it can form nearly pure monocultures in its invaded range. Such monocultures do not occur in at least two parts of *Acroptilon's* native range, Uzbekistan and Turkey (U. Schaffner, *unpublished data*) suggesting that *Acroptilon* may have lower impacts on its neighbors at home. We compared the biomass attained by *Acroptilon* in its native range of Uzbekistan to that attained in its non-native range in northwestern North America. Furthermore, we also compared the diversity and biomass of native species associated with *Acroptilon* in each range and conducted multi-year field experiments to test the effects of disturbance and seed addition on *Acroptilon* and associated native species.

In the non-native range, *Acroptilon* reached a maximum mean cover value of 86.8±1.4% for all treatments combined (Figure 42). The mean biomass at YTC site at the end of the multi-year experimental period was 339 g/m^2. In the native range, with both sites combined (hereafter we refer to these combined sites as the "Kattakurgan/Urgut site") at the end of the experimental period the mean cover of *Acroptilon* across all treatments at the Uzbek sites was 24.2±1.8% with a mean total biomass at the end of the experiment for both sites and all treatments combined of 170 g/m^2. At the Kattakurgan/Urgut site in Uzbekistan, *Acroptilon* reached maximum mean cover values of 46.2±4.0% and 45.7±5.1% for the disturbance-no seeding and the disturbance-seeding treatments, respectively, (Figure 42).

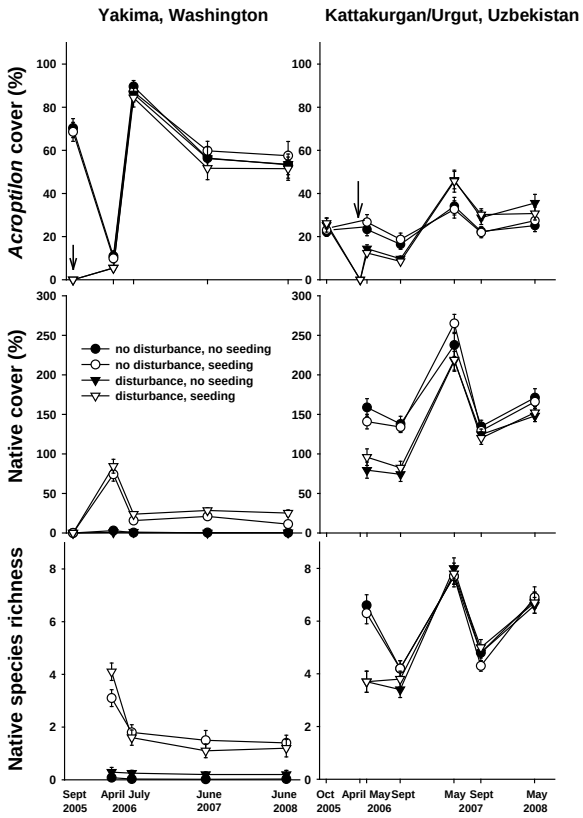

Figure 42. Native species richness and cover, and *Acoptilon* cover, in Washington and Uzbekistan, respectively.

There were no effects of disturbance at YTC on either *Acroptilon* or native cover (Figure 42). In Uzbekistan, disturbance in April 2006 decreased *Acroptilon* cover in September 2006 from 23.3±2.9% and 26.7±2.9% in the no disturbance-seeding and no disturbance-no seeding treatments, respectively, to 14.4±1.8% and 12.4±1.3% in the same treatments. One year after disturbance these relationships switched so that *Acroptilon* cover in the disturbance treatments was higher than in the no disturbance treatments; 46.2±4.0% and 45.7±5.1% for seeding and no seeding treatments, versus 34.4±4.1% and 32.6±4.0% in the same treatments. Disturbance reduced the cover of all Uzbek native species soon after the disturbance event, and disturbed plots sustained slightly lower native cover than undisturbed plots for the duration of the experiment, suggesting that in contrast the absence of disturbance in its non-native range, *Acroptilon* may have acquired a slight relative advantage from disturbance in its native range.

Seeding had very strong effects on the cover of native North American species, increasing the mean cover at the end of the experimental periods from 0.3±0.3% and 2.4±0.4% in the no disturbance-seeded and disturbance-seeded plots to 11.2±2.4% and 25.2±4.0% in the same treatment combinations. However, surviving native species were very small in size; usually existing beneath a canopy of *Acroptilon*, and this is reflected in the dramatic differences in biomass between the native and non-native ranges. Seeding increased mean species richness of North American natives from 0.03 and 0.2 species per m^2 to 1.4 and 1.2 species per m^2.

The most striking results from our biogeographic comparison of the community-scale impacts of *Acroptilon* was the 15-20-fold difference in the ratio of *Acroptilon* to natives between the non-native and native ranges. Put another way, the biomass (a close estimate of annual productivity) of *Acroptilon* was about twice as high in the non-native range as in the native range. However, the biomass of native species was roughly 30 times lower in the non-native range than in the native range of *Acroptilon*. This correlative pattern could have been caused by any number of mechanistic processes that we have not separated: e.g. unknown historical events, enemy release, or differences in the fundamental competitive interactions between *Acroptilon* and the natives of the different regions. However, our results clearly document field patterns that indicate biogeographic differences in the fundamental ecology and impact of a highly invasive plant species; differences that do not correlate with only an increase in biomass by the invader in its non-native range. Such different behavior in the native and non-native ranges suggests that powerful biotic controls are important for the distribution and abundance of *Acroptilon* in its native communities and release from these biotic controls is likely to lead to equally powerful biological effects in non-native ranges. Our results suggest that native species in North America are at an inherent competitive disadvantage, much as we have found for spotted knapweed, emphasizing the importance of identifying strong North American competitors.

We compared the competitive and allelopathic effects of *Acroptilon repens* on native North American species to effects on related species from the native range of Acroptilon in Uzbekistan. We also compared the competitive interactions between these North American and Eurasian species, in the absence of *Acroptilon*, examining the hypothesis that particular regional species pools may show differences in competitive ability. The results showed that *Acroptilon* had stronger competitive effects against native North American species than against species native to Uzbekistan. However, there was no difference in the competitive effects among Eurasians and North Americans (Figure 43).

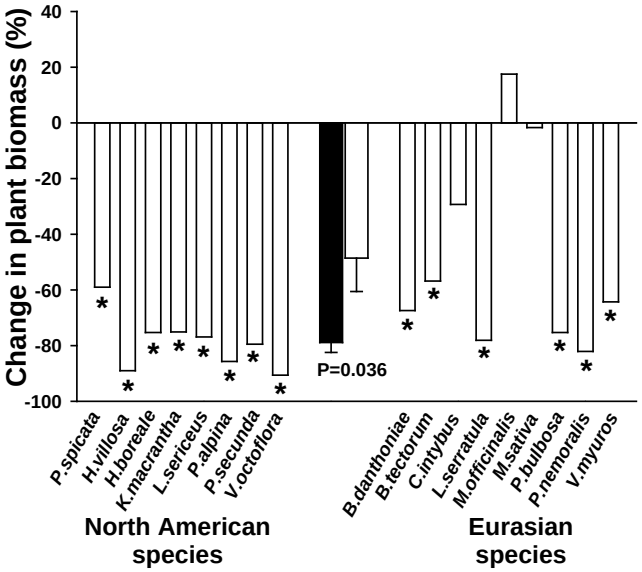

Figure 43. Change in total plant biomass, relative to plants grown alone, for North American and Eurasian species grown with Acroptilon repens and related species. Thicker bars in the center are the means for each region computed using the means of the species from each region. Error bars represent 1 SE. Asterisks indicate significant competitive effect of A. repens for each species in separate t-tests significant at P=0.05.

The effects of leachates collected from *Acroptilon* roots were weak but more negative on species from North America than on species from Uzbekistan. Our results suggest that inherently stronger competitive and allelopathic effects of *Acroptilon* on North American plants than on plants from its native range may contribute to its invasive success. In this experiment, all North American natives were highly suppressed by Acroptilon, and none demonstrated substantial resistance to the invader. Of all North American species, *Pseudoroegneria spicata* was suppressed the least by *Acroptilon* (Figure 44) suggesting this species as a potential good competitor in other environmental conditions.

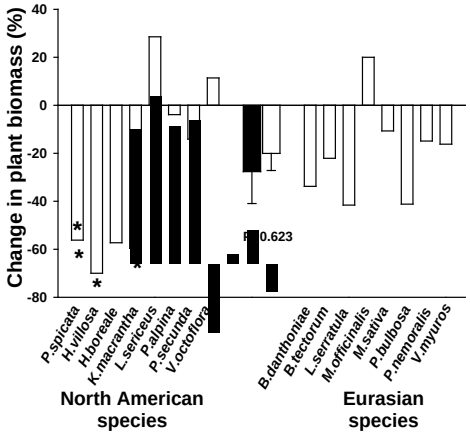

Figure 44. Change in total plant biomass, relative to plants grown alone, for North American and Eurasian species grown in pairwise experiments with related species. Thicker bars in the center are the means for each region computed using the means of the species from each region. Error bars represent 1 SE. Asterisks indicate significant competitive effect of closely related species from the other continent for each species in separate t-tests significant at P=0.05.

DISCUSSION

The conditionality of the presence of catechin in soils needs further discussion. First, to our knowledge, no other published studies of any other allelochemical have incorporated a season or diurnal component as indicated by Perry et al. (2007) and Tharayil and Treibwasser (2010). Second, to our knowledge our finding of highly variable effects of different soil metals on the toxicity of catechin is unique in the literature. Third, our finding that native plant species appear to be able to adapt to spotted knapweed (Callaway et al. 2005) was the first to document the potential for natives to evolve resistance to the effects of invaders, a finding that has lead to other such results for other invasive species. Fourth there is strong evidence that spotted knapweed is allelopathic (Ridenour & Callaway 2001; Callaway et al. 2005), produces catechin, and that catechin has allelopathic potential (Buta et al. 1986; Blair et al. 2005; Perry et al. 2007; Inderjit et al. 2008ab; Simoes et al. 2008; He et al. 2009; Pollock et al. 2009; Thorpe 2009; Tharayil and Treibwasser 2010); however, it is much less clear whether or not spotted knapweed produces enough catechin for it to be the main allelochemical. It is also quite possible that other phytotoxins might be released by spotted knapweed, and that the presence of these allelochemicals in the soil might not be constant. Similar observations were made for other allelochemicals from difuse and Russian knapweeds (Quintana et al. 2008, 2009; Tharayil et al. 2008). In other words, allelopathy is not a black or white situation but as with any other ecological phenomena it is conditional. The only implication of these findings on the results reported in this project is that we screened native plants for their resistance to catechin as a technique to find successful competitors. In retrospect, we subjected our plants to an allelochemical that they might not encounter constantly in nature. However, following this approach we discovered very strong native competitors in general that were successfully tested.

We have learned a great deal of information that is depicted throughout the report. Key findings include:

1. Allelopathy is conditional.
2. Different metals in soils determine at least one aspect of the conditional effect of catechin.
3. Plants may use mixtures of compounds as allelochemicals rather that single compounds as we have found for Russian knapweed.
4. The defense and aggressive strategies of invasive plants are not entirely determined by intrinsic physiology and biochemistry but also by the presence of specific plant neighbors surrounding the invasive species.
5. Native plant species have the potential to evolve resistance to invaders.
6. Genomics and metabolomics will contribute to the future of understanding plant invasion and this project has generated seminal information on this topic.

7. Screening for successful competitors by direct application of allelochemicals or by direct competition with a given invasive plant taxa is a cost-effective way to find native competitors to revegetate areas invaded by invasives.

On a different subject, it is important to note here that the release of spotted knapweed from specialist enemies has been considered an important factor its invasive success, and this has spurred the introduction of a number of biological control species to North America over the past thirty years (Muller-Scharer et al. 2004; Maddox 1979; Maddox 1982; Smith and Story, 2003). Although many of these specialist herbivores have become established and widespread, spotted knapweed densities have only been reduced in a few specific areas (e.g. Story et al. 2006), and the invasive continues to expand its range at other sites (Muller-Scharer et al. 2004; Sheley et al. 1998). Interestingly, field observations in North America suggest that introduced spotted knapweed experiences little pressure from generalist herbivores and pathogens (RM Callaway and WM Ridenour, personal communication), indicating that spotted knapweed currently experiences a partial release from both specialist and generalist enemies in the introduced range.

In order to better understand defense responses in spotted knapweed, future studies should monitor gene expression and physiological responses in tetraploid geo-cytotpyes when exposed to pathogens and herbivores. This would help determine if expression of genes involved in constitutive defenses are good predictors of pathogen and herbivore susceptibility. In addition, it would be interesting to test the response of spotted knapweed geo-cytotypes to a variety of generalist and specialist enemies at the level of gene expression.

Although plant ploidy is often unaccounted for in comparisons of native and introduced populations, we found it to be a necessary and essential component for gene expression analyses. In native populations, we found lower expression of PAL2a, PAL2b and the transposable element in diploids compared to tetraploids, and all other genes examined showed similar relative expression (Table 11). The literature suggests that gene expression rates in polyploids tend to vary depending on plant species, ploidy, genetic background, and the genes examined; however, the phenomenon of gene dosage compensation appears to be common (Chen and Ni, 2006; Albertin et al. 2005; Guo et al. 1996; Wang et al. 2006). This dosage effect results in gene or protein expression patterns in polyploids that are similar to their diploid progenitors. We did not necessarily expect to see this phenomenon in our plant populations because other studies involving ploidy and gene or protein expression have traditionally utilized plants with the same genetic background, whereas evidence suggests that spotted knapweed plants within the native range harbor different genetic backgrounds (Hufbauer and Sforza, 2008; Mars et al. 2008). However, it appears that gene dosage compensation may be occurring to some extent in the native cytotypes of spotted knapweed. Additionally, we observed increased expression of two PAL transcripts in native tetraploids compared to diploids, which may reflect increases in secondary compounds due to polyploidy as is seen in other plants (Dhawan and Lavania, 1996). Interestingly, native diploids exhibited similar expression profiles for nine of the ten total genes analyzed when compared to introduced tetraploids (Table 10), also suggesting gene dosage compensation. This result was rather surprising in that the diploid appears to be extremely rare (i.e., unsuccessful) in the introduced range, whereas the introduced tetraploid is a very problematic weed. Therefore, it is likely that other factors, such as plant performance

characteristics, life cycle traits and the expression of other genes, are of greater importance in determining the success of tetraploids over diploids in the introduced range. Overall, the observed differences in gene expression between and within ploidies highlight the importance of using appropriate plant types when examining a particular species in both the native and introduced range.

In our field studies treatments applied to Russian knapweed at YTC had little effect on the native plant community, which was composed mostly of Russian knapweed despite seeding natives or disturbance. Due to the clonal nature of this species, it is likely that the competitive ability of the resprouting Russian knapweed was too much for any of the seeded species to overcome. However, yarrow and common sunflower in the seed mix, made a relatively strong showing in the first year and resulted in a significant increase of native species cover relative to control plots and other seed mixtures. However, the annual sunflower quickly dropped out of the system and the allelopathic seed mix plots quickly returned to being dominated by re-sprouting and/or re-colonizing Russian knapweed. On the other hand, the perennial yarrow appeared to be able to retain a foothold over the entire experimental period in the companion experiment. Nevertheless, in 2009 there was a native component of the plant community biomass present in plots that received the standard and resistant seed mixes, which was not present in the control plots. The presence of these native plants in the understory of the Russian knapweed suggests that seeding can increase diversity in monotypic stands of Russian knapweed, but the very small amount of native biomass relative to Russian knapweed is unacceptable from a management perspective. For a seeding approach to work, aggressive Russian knapweed control measures would need to be combined with seeding.

Spotted knapweed insect biocontrol agents were present in our study plots at Fort McCoy and we also observed biocontrol insects on leafy spurge, which had been released by Fort McCoy personnel. Our insecticide applications seemed to reduce insects but had little impact on the knapweed population, indicating that the biocontrols may not have been exerting strong control over the knapweed population. Twelve species of insects have been approved for introduction into the United States for biological control of spotted and diffuse knapweed (Rees et al. 1996). Although several of these agents have become well established there is little quantitative data on their efficacy for invasive plant control.

At Fort Mc Coy, leafy spurge cover and biomass increased with activated carbon (AC) with a concomitant decrease in native species biomass. The mechanism(s) behind AC promotion of leafy spurge are unclear, as AC is known to have varying effects on plant competitive interactions (Lau et al. 2008). One possibility is that AC binds or deactivates a compound (or compounds) in the soil that is inhibitory to leafy spurge.

Initially (2006) the allelopathic smother crop that we seeded in some study plots seemed to suppress spotted knapweed and leafy spurge and also significantly reduced other non-seeded species, while at the same time increasing native species cover. These effects seem to be due to the success of annual ragweed in this seed mix. However, this effect was short lived and as the annual ragweed dropped out of the community in 2007, both invasive species resurged. We speculate that the initial establishment of the annual ragweed in the allelopathic seed mix was followed by an ecological void the second year when this previously dominant ruderal species

dropped out of the plant community perhaps due to poor seed production or unfavorable conditions for germination of offspring. With poor establishment of other seeded species, this ecological void was filled by other introduced species emerging from an abundant soil seed bank in subsequent years. Therefore it would be worth exploring the potential of combining or following the transient allelopathic seed mix, with a perennial seed mix that could fill this ecological void and compete with emerging invasive species. The use of annual ragweed as a smother crop for combating invasive species appears to be promising as indicated by the Fort McCoy results as well as our previous greenhouse studies (Perry et al. 2009). Higher seeding rates for annual ragweed and other native species are worthy of further study for competing with invasives that have high densities in soil seed banks.

Because of the problems we experienced with catechin as a viable allelochemical, we focused much more intensely on testing native species responses to spotted knapweed and other invaders in the contest of revegetation efforts in the additional studies. In this context we were highly successful. First, our biogeographic comparisons clearly demonstrate that these invaders are likely to experience far greater competition from species native to their home range than they do in their non-native ranges. Furthermore, in experiments with dozens of native species we found many that exert strong competitive effects on invaders. This is a highly novel and important contribution. We have established several long-term field experiments with different seeding treatments in which we will be testing these strong competitive effects in the context of real invasive resistance for years into the future.

In conclusion successful seeding of natives in natural systems invaded by knapweeds was variable. It is ideal to establish native plants by seed, and, once these plants are established, they should resist the invasion of unwanted invasive plants. In some situations, such as highly disturbed sites the seeding efficacy can be high and the desired outcome (resistance to invasion) is promising. However, in established grassland sites our seeding efforts had mixed success, but generally the establishment of seeded species was low. The limited establishment of seeding in some situations could be due to rodent pressure on the propagules, poor seed to soil contact, poor climate conditions (most of our trials occurred during record heat and low moisture conditions and we did not augment with water), historic spraying regimes with residual herbicide legacies, or because the plant communities were already well established and native plant recruitment could be naturally low. Spotted knapweed seems to be able to take advantage of disturbance (e.g. herbicide spray, rodent mounding) and recruit far more rapidly than natives. Seeding with native species should be conducted soon after any disturbance that compromises community diversity. Once knapweed is the dominant plant or a system has experienced a spray regime, seeding natives may have limited success. However, immediately after physical disturbance seeding with natives, especially ruderal natives can be effective.

Finally, the project developed ample molecular and genomics tools for our model invasive species *C. maculosa*. The information related to this technology was explained in the appropriate systems and it will be ideal to utilize these tools to further understand the genetic basis of invasion. As with any other biological process, genetics and the environment are likely to determine the outcome of invasions. Information in the literature is pointing to environmental and ecological data that favors this occurrence and the tools developed in this project will

facilitate the understanding of the missing genetic link. The next 5-10 years are likely to play a crucial role in the understanding of plant invasiveness using a holistic approach.

CONCLUSIONS AND IMPLICATIONS FOR FUTURE RESEARCH/IMPLEMENTATION

- *Isolation and characterization of allelochemicals from invasive plants.* We successfully isolated and characterized allelochemicals from several invasive plants indicating that allelopathy may potentially play a role in the invasion biology of these species. Some of these allelochemicals constitute tools to screen for successful plant competitors. Knowledge of these compounds will help guide future studies into the potential ecological significance of allelopathy in invasions. Future research questions in this area include: How many different allelochemicals can be produced by a single invasive species? And most importantly, does such multi-allelochemical potential affect other plants and how is it physiologically orchestrated?

- *Using allelopathy for the control of invasive plants.* Allelopathy remains a controversial topic in ecology. We have provided vital information regarding the conditionality of allelopathy in invasive knapweed species. We have also provided evidence that some native species, which are either resistant to allelochemicals, or superior competitors, can be used to compete with invasive allelopathic plants. Specifically, planting annual ragweed and common sunflower as cover crops in western grassland restorations may reduce cheatgrass, Japanese brome, Canada thistle, and whitetop invasion and may improve desired species growth in competition with cheatgrass and Japanese brome. Planting Canada goldenrod and littleleaf pussytoes as cover crops may improve desired species success, but may not inhibit invasive species. Using native plant diversity as a weapon against invasion is potentially powerful, yet underutilized, in restoration contexts. We believe that these studies warrant further experimentation and development at DOD facilities.
- *Biological degradation of allelochemicals.* Our research group and others have found that catechin is produced and secreted by spotted knapweed. However, its secretion and persistence in the soil seems to be conditional. At this point, the cause of the conditionality is not clear. Catechin appears to undergo rapid transformation in soils making it difficult to detect under natural conditions. Other allelochemicals are likely to be produced by spotted knapweed and it is not clear if those compounds are more stable and/or phytotoxic than catechin. Allelochemicals from other species may be similar in this regard. These observations make conclusions regarding the ecological role of allelochemicals less certain and difficult to interpret. Future work on allelopathy needs to consider the fate of chemicals in complex soil matrixes. Clearly, technology to measure allelopathy and or allelochemicals under field conditions is needed. 7,8-benzoflavone does not appear to be the main allelopathic compound used by Russian knapweed as evidenced by its persistence in greenhouse soils combined with lack of detection in field soils. Other compounds from Russian knapweed were identified in the soil but further testing is needed. From our studies it is clear that additional research on understanding the conditionality of allelopathy is needed. This point could encompass a variety of research questions such as: Are allelochemicals more stable in the soil more stable at certain times of the year? And if so what are the processes that drive this? What are potential triggering mechanisms that enable the plant to secrete more allelochemicals at certain times of the year or under certain environmental conditions? And, what is the

effect of allelopathy on soil microbial communities and how is this effect translated into improved plant health for the invasive species at the detriment of native plants?

- *Identify native plants that are resistant to allelochemicals.* We identified native species and particular ecotypes of native species that are good competitors with allelopathic weeds, which could be used to reclaim infestations of allelopathic weeds. Results of subsequent field trials are promising but incomplete. Additional work is needed in this promising area.
- *Knapweed control impacts on allelochemicals.* Various controls measures may injure spotted knapweed, but it is usually able to recover from mechanical treatments, responds by producing more allelochemicals under biological control, or be the primary recolonizer after herbicide treatment. Given the difficulty of detecting allelochemicals in soils as we encountered here, this is an area in need of further study.
- *Understanding the mechanisms of allelochemical detoxification.* The phytoxicity of catechin was investigated with the intent of eventually turning the invasive weapon used by spotted knapweed against itself as well as harnessing it for broad-spectrum weed control strategies. When this approach did not work we turned to developing broad-based genomics and metabolomics understanding of the invasion capabilities of spotted knapweed. We have advanced the field of invasive plant biochemistry and genetics in ways that were not conceived before the start of the project. Our work has promoted similar types of biochemical and genetic work on other invasive species besides spotted knapweed. It is expected that this type of research will advance our understanding of why some plants become invasive.
- *Integrating allelochemical control of invasive species with other proven control strategies.* The information and products from all of our studies were tested in various field studies addressing the control and ecology of allelopathic weeds. The use of native ruderal species (early seral annuals) and perhaps native allelopathic species are approaches worthy of further study. In our studies, recruitment of seeded species was low pointing towards the need to evaluate higher seeding rates in restoration activities where there are established invasive plant populations. Diversity matters; when revegetating a highly disturbed site use as diverse a native seed mix as possible. Sites with more diversity will typically limit the invasion of unwanted invasive plants. Many invasive species may be successful in part due to the allelochemicals they produce. However, not all native species are susceptible to these allelochemicals, and this again suggests that using diverse mixes of native species to restore disturbed or degraded sites is important. Insecticide treatments to control biocontrol insects did not result in measurable effects on invasive plant populations indicating that biocontrol may not be having the desired effect at Fort McCoy.

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Qin, B., Lau J.A., Kopshever, J., Callaway, R.M., McGray, H., Perry, L.G., Weir, T.L., Paschke, M.W., Hierro, J.L., Yoder, J., Vivanco J.M., and Strauss, S. (2007) No evidence for root-mediated allelopathy in Centaurea solstitialis, a species in a commonly allelopathic genus. Biological Invasions 9:897-907

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APPENDIX A - SCIENTIFIC PUBLICATIONS

PEER-REVIEWED JOURNAL ARTICLES

Abhilasha, D., Quintana, N., and Vivanco, J.M., and Joshi J. (2008) Do allelopathic compounds in invasive Solidago canadensis s.l. restrain the native European flora? Journal of Ecology 96: 993-1001

Alford, É.R., J.M. Vivanco and M.W. Paschke. 2009. The effects of flavonoid allelochemicals from knapweeds on legume - rhizobia candidates for restoration. Restoration Ecology 17:506-514.

Alford, É.R., L.G. Perry, B. Qin, J.M. Vivanco, M.W. Paschke. 2007. A putative allelopathic agent of Russian knapweed occurs in host soils. Soil Biology and Biochemistry 39: 1812-1815

Broeckling, C.D., Vivanco, J.M. (2008) A selective, sensitive and rapid in field-assay for soil catechin, an allelochemical of Centaurea maculosa. Soil Biology and Biochemistry 40: 1189-1196

Brooker, R.W., R.M. Callaway, L. Cavieres, Z. Kikvidze, C. Lortie, R. Michalet, F.I. Pugnaire, A. Valiente-Banuet, T.G. Whitham. 2009. Hunting the ghost of Clements? A comment on Ricklefs' disintegrating communities. American Naturalist.

Broz AK, Broeckling CD, De-la-Peña C, Lewis MR, Greene E, Callaway RM, Sumner LW, Vivanco JM (2010) Plant neighbor identity influences plant biochemistry and physiology related to defense. BMC Plant Biology 10:115 doi:10.1186/1471-2229-10-115

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Broz, A.K., Manter, D.K., Bowman, G. Muller-Scharer, H., Vivanco, J.M. (2009) Plant origin and ploidy influence gene expression and life cycle characteristics in an invasive weed. BMC Plant Biology 9:33 doi:10.1186/1471-2229-9-33

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Callaway, R.M. and T.G. Howard. 2007. Competitive networks, indirect interactions, and allelopathy: a microbial viewpoint on plant communities. Progress in Botany 68: 317-335

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He, W., Y.Feng, W.M. Ridenour, G.C.Thelen, J.L.Pollock, A.Diaconu, and R.M.Callaway. 2009. Novel weapons and invasion: biogeographic differences in the competitive effects of Centaurea maculosa and its root exudate (±)-catechin. Oecologia 159:803-815.

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Inderjit, Jarrod L. Pollock, Ragan M. Callaway, William Holben. 2008. Phytotoxic Effects of (6)-Catechin In vitro, in Soil, and in the Field. PLoS ONE 3: e2536. doi:10.1371/journal.pone.0002536

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Ni, G. U. Schaffner, S. Peng, and R.M. Callaway. In press. Acroptilon repens, an Asian invader, has stronger competitive effects on species from America than species from its native range. Biological Invasions.

Perry, L.G., Johnson, C., Alford, E.R., Vivanco, J.M., and Paschke, M.W. (2005) Screening of grassland plants for restoration after spotted knapweed invasion. Restoration Ecology 13:725-735

Perry, L.G., S.A. Cronin and M.W. Paschke. 2009. Native cover crops suppress exotic annuals and release native perennials in a greenhouse competition experiment. Plant Ecology 204:247-259.

Perry, L.G., Thelen, G.C., Ridenour, W.M., Callaway, R.M., Paschke, M.W., Vivanco, J.M. (2007) Concentrations of the allelochemical catechin in Centaurea maculosa soils. Journal of Chemical Ecology 33:2337-2344

Pollock, J.L., R.M. Callaway, G.T. Thelan and W.E. Holben. 2009. Catechin-metal interactions as a mechanism for conditional allelopathy by the invasive plant Centaurea maculosa. J. Ecology 97:1234-1242.

Prithiviraj, B., Perry, L.G., Badri, D., Vivanco, J.M. (2007) Chemical facilitation and induced pathogen resistance mediated by a root-secreted phytotoxin. New Phytologist 173: 852-860

Qin, B., Lau J.A., Kopshever, J., Callaway, R.M., McGray, H., Perry, L.G., Weir, T.L., Paschke, M.W., Hierro, J.L., Yoder, J., Vivanco J.M., and Strauss, S. (2007) No evidence for root-mediated allelopathy in Centaurea solstitialis, a species in a commonly allelopathic genus. Biological Invasions 9:897-907

Qin, B., Perry, G.L., Broeckling, C.D., Du, J., Stermitz, F.R., Paschke, M.W., and Vivanco, J.M. (2006) Phytotoxic allelochemicals from roots and root exudates of Leafy Spurge (Euphorbia esula L.). Plant Signaling and Behavior 1: 323-327

Quintana, N., Weir, T.L., Du, J., Broeckling, C.D., Rieder, J.P., Stermitz, F.R., Paschke, M.W. and Vivanco, J.M. (2008) Phytotoxic polyacetylenes from roots of Russian knapweed (Acroptilon repens (L) DC.). Phytochemistry 69:2572-2578

Ridenour, W.M., J.M. Vivanco, Y. Feng, J. Horiuchi and R.M. Callaway. 2008. No evidence for tradeoffs: Centaurea plants from America are better competitors and defenders than plants from the native range. Ecological Monographs 78:369-386.

Thorpe, A.S., G.C. Thelen, A. Diaconu and R.M. Callaway. 2009. Root exudate is allelopathic in invaded community but not in native community: field evidence for the novel weapons hypothesis. Journal of Ecology 97:641-645.

Weir, T.L., Bais, H.P., Stull, V.J., Callaway, R.M., Thelen, G.C., Ridenour, W.M., Bhamidi, S., Stermitz, F.R., and Vivanco, J.M. (2006) Oxalate contributes to the resistance of Gaillardia grandiflora and Lupinus sericeus to a phytotoxin produced by Centaurea maculosa. Planta 223:785-795

THESES AND DISSERTATIONS

Alford, E.A. 2006. Soil ecological interactions of invasive knapweeds. M.S. Thesis. Colorado State University. Fort Collins, CO.

Schultz, M.J. 2008. Soil ecological interactions of spotted knapweed and native plant species. M.S. Thesis. Colorado State University. Fort Collins, CO.

Broeckling, C.D. 2008. Primary and secondary metabolism in *Centaurea maculosa* and their potential roles in invasion biology. Ph.D. Thesis. Colorado State University. Fort Collins, CO.

Broz, A.K. 2009. Development and utilization of molecular tools to understand invasion biology in Centaurea maculosa (Spotted knapweed). Ph.D. Thesis. Colorado State University. Fort Collins, CO.

PRESENTATIONS

By Mark Paschke and his Students:

Busby, R., M. Paschke, C. Herron, J. Rieder. 2010. Utilization of native annuals for restoration. Native Plant Materials Development, Production & Use in Habitat Restoration. The National Native Seed Conference. Snowbird, Utah, May 17 - 21, 2010

Grant, T.A., M.W. Paschke, J.P. Rieder, B.H. Wolk and J.M. Vivanco. 2009. Can native weedy plants be used to control invasive exotic weeds on military training grounds? The 2009 SERDP and ESTCP Partners in Environmental Technology Technical Symposium, December 2009, Washington, D.C

Grant, T.A. III and M.W. Paschke. 2009. Use of native soil inoculum in the restoration of lands invaded by exotic weeds. 19th Conference of the Society for Ecological Restoration International, Perth, Western Australia, Australia. August 2009**.**

Grant, T.A. III and M.W. Paschke. 2009. Influences of plant-soil feedbacks on the intra- and inter-specific competition of several invasive knapweeds. Ecological Society of America 94th Annual Meeting. August 2009, Albuquerque, NM. Oral Presentation.

Paschke, M.W, J.P. Rieder, L.G. Perry, S.A. Cronin, M.J. Schultz and J.M. Vivanco. 2008. Potential use of native weedy plants for controlling invasive exotic weeds on military training grounds. The 2008 SERDP and ESTCP Partners in Environmental Technology Technical Symposium, December 2008, Washington, D.C.

Rieder, J.P., M.W. Paschke, L.G. Perry, S.A. Cronin, R.M. Callaway and J.M. Vivanco. 2007. Allelopathy and the control of exotic weeds on military training grounds. The 2007 SERDP and ESTCP Partners in Environmental Technology Technical Symposium, December 2007, Washington, D.C.

Schultz, M.J., L.G. Perry and M.W. Paschke. 2007. Importance of establishment order in competitive interactions between native plant species and *Centaurea stoebe*. ESA/SER Joint meeting, August 2007, San Jose, CA.

Smith, L., M.W. Paschke, E.F. Redente, S.D. Warren, and D.A. Klein. 2007. Integration of biological control with other methods to restore rangeland infested with spotted and diffuse knapweed. ESA/SER Joint meeting, August 2007, San Jose, CA.

Cronin, S.A., M.W Paschke, L.G Perry, E.F Redente, and J.M Vivanco. 2007. Using native allelopathic species to combat exotic species. ESA/SER Joint meeting, August 2007, San Jose, CA.

Rieder, J.P., M.W. Paschke, L.G. Perry, S.A. Cronin, R.M. Callaway, J.M. Vivanco. 2006. Novel weapons for controlling exotic plant species. The 2006 SERDP and ESTCP Partners in Environmental Technology Technical Symposium, November 2006, Washington, D.C.

Alford, É.R, L.G. Perry, M.W. Paschke and J.M. Vivanco. Tough enough? Identification of allelochemical resistant plants for revegetation following spotted knapweed invasion. The 2005 SERDP and ESTCP Partners in Environmental Technology Technical Symposium, December 2005, Washington, D.C.

Perry, L.G. C. Broeckling, M.W. Paschke and J.M. Vivanco. Variation in production of the phytotoxin (±)-catechin by the invasive spotted knapweed, effects on knapweed and implications for management. The 2005 SERDP and ESTCP Partners in Environmental Technology Technical Symposium, December 2005, Washington, D.C.

Perry, L.G., M.W. Paschke and J.M. Vivanco. 2005. Dual role for an allelochemical: (±)-catechin from *Centaurea maculosa* root exudates regulates conspecific seedling establishment. In an Organized Oral Session: Allelopathy - Biochemical interactions among plants affecting community structure, exotic invasions and evolutionary theory. 90th Ecological Society of America Annual Meeting, August 7-12, 2005, Montreal, Canada.

Alford, É.R., L.G. Perry, B. Qin, M.W. Paschke, J.M. Vivanco. Underground Chemical Warfare: A Root-Exuded Allelopathic Secondary Metabolite from *Acroptilon repens*. Gordon Research Conference, Plant Metabolic Engineering. July 10 - 15, 2005, Tilton, NH.

Perry, L.G., M.W. Paschke, and J.M. Vivanco. Root exudation and rhizosphere biology: allelochemicals and cell death. The First Symposium on Plant Neurobiology, May 17-20, 2005, Florence, Italy.

By Jorge Vivanco:

Syngenta Research Center, Stein, Switzerland (October 28, 2010)

Institute of Plant Sciences, University of Zürich, Switzerland (October 27, 2010)

Enza Zaden Inc., Enkhuizen, The Netherlands (October 22, 2010)

All-Russia Research Institute for Agricultural Microbiology, St. Petersburg, Russia (September 22, 2010)

Syngenta Biotechnology, Inc., Research Triangle Park, NC (October 28, 2009)
2009 SACNAS (Society for Advancing Hispanics/Chicanos and Native Americans in Science) National Conference, Dallas, TX (October 16, 2009)
Plant Biology Program, University of Rouen, Rouen, France (September 29, 2009)
6th International Symbiosis Society Congress, Madison, WI (August 12, 2009)
Biology Department, Brookhaven National Laboratory, Upton, NY (January 30, 2009)
Department of Horticulture and Landscape Architecture, Colorado State University (November 19, 2008)
Post Graduate School, Universidad Nacional Agraria La Molina, Lima, Perú (July 1st, 2008)
Pan American Study Institute (PASI) "Chemical Ecology of the Tropics", Tambopata, Peru (May 29, 2008)
Department of Chemistry, Pontificia Universidad Católica del Perú, Lima, Perú (November 29, 2007)
IV Congreso Peruano de Ecología, Arequipa, Perú (November 21, 2007)
Graduate Program in Molecular Plant Sciences, Washington State University (April 11, 2007)
Plenary Lecture, "Root-Microbe Interactions" Meeting, Albrecht-von-Haller-Institut, Göttingen, Germany (March 7-9, 2007)
Department of Microbiology, University of Washington-Seattle (February 13, 2007)
Institute of Plant Sciences, University of Zürich, Switzerland (January 26, 2007)
Department of Biology, Ecology and Evolution, University of Fribourg, Switzerland (January 25, 2007)
Institute of Environmental Sciences, University of Zürich, Switzerland (January 26, 2007)
Department of Plant Biology and Pathology, Rutgers University (December 15, 2006)
Department of Biology, Texas State University (October 2, 2006)
52nd Brazilian Genetics Congress, Foz do Iguaçu, Brazil (September 3-6, 2006)
Centro de Investigaciones Bioquímicas y Fisiológicas del CONICET-Facultad de Agronomía, Universidad de Buenos Aires, Argentina (May 18, 2006)
Department of Chemistry, University of Texas at San Antonio (April 14, 2006)
Distinguished Chemical Ecologist Lectureship, Institute of Chemical Ecology, The Pennsylvania State University, State College, PA (March 3, 2006)
Distinguished Biologist Lectureship, Department of Biology, University of Massachusetts, Boston, MA (February 3, 2006)
Plant Pathology Program, Universidade Federal Rural de Pernambuco, Recife, Brazil (December 7, 2005)
4th Brazilian Meeting on Chemical Ecology, Piracicaba, Brazil (November 29-December 2, 2005)
Gordon Research Conference on Plant Metabolic Engineering, Tilton, NH (July 10-15, 2005)
16th International Conference on Arabidopis Research, Madison, WI (June 14-19, 2005)
LeTourneau Memorial Lectureship, University of Idaho, Moscow, ID (April 21, 2005)
Biology Department, Duke University, Durham, NC (February 7, 2005)

By Ragan Callaway
Callaway, R.M. 2005. Exotic invasions and the Novel Weapons Hypothesis. Iowa State University, Ames, Iowa. October 7, 2005. Invited talk.

Callaway, R.M. 2005. Herbivory and the response of exotic invaders. Symposium on plant responses to below ground herbivory. Reading, United Kingdom. October 23-25. Invited presentation.

Callaway, R.M. 2005. Exotic invasions and the Novel Weapons Hypothesis. Purdue University, Lafayette, Indiana. October 7, 2005. Invited seminar

Callaway, R.M. 2005. Exotic invasions and the Novel Weapons Hypothesis. University of California, Santa Barbara. December 12, 2005. Invited seminar

Callaway, R.M. 2005. Facilitation and climatic conditions. Symposium: Integrating species interactions with global climate change, Aberdeen, Scotland. January 7-11, 2005. Invited seminar

Callaway, R.M. 2005. Exotic invasions and the Novel Weapons Hypothesis. University of California, Davis, CA. January 24, 2005. Invited talk.

Callaway, R.M. 2005. Soil biota and invasions: taking a biogeographic perspective. University of California, Davis, CA. January 26, 2005. Invited talk.

Callaway, R.M. 2005. Exotic invasions: evolution of invaders and the invaded. University of California, Davis, CA. January, 28 2005. Invited talk.

Callaway, R.M. 2005. Exotic invasions and the Novel Weapons Hypothesis. Georgia Tech University, Atlanta, Georgia. February 24, 2005. Invited talk.

Callaway, R.M. 2005. Exotic invasions and the Novel Weapons Hypothesis. Ohio University, Athens, Ohio. February 24, 2005. Invited talk.

Callaway, R.M. 2005. Exotic invasions and the Novel Weapons Hypothesis. Utah State University, Logan, Utah. March 10, 2005. Invited talk.

Callaway, R.M. 2005. Soil microbes and exotic invasions. Utah State University, Logan, Utah. March 11, 2005. Invited talk.

Callaway, R.M. 2005. Exotic invasions and the Novel Weapons Hypothesis. University of Texas, Arlington. April 22, 2005. Invited talk.

Callaway, R.M. 2005. Exotic invasions and the Novel Weapons Hypothesis. Duke University, Durham, North Carolina. April 7, 2005. Invited talk.

Callaway, R.M. 2005. Allelopathy and exotic invasions. Symposium on Plant Neurobiology, Florence, Italy. May 17-20, 2005. Invited talk.

Callaway, R.M. 2005. The role of allelopathy in exotic invasions. The International Botanical Congress, Vienna, Austria, July 17-23. Keynote Address

Reinhart, K.O. and Callaway, R.M. 2005. Soil microbes and exotic invasion: taking a biogeographical approach. The International Botanical Congress, Vienna, Austria, July 17-23. Invited Symposium Presentation.

Callaway, R.M. and J.M Vivanco. 2005. Invasion of plants into native communities using the underground information superhighway The International Allelopathy Symposium. Keynote Address. Wagga Wagga, Australia. August 22, 2005.

Callaway, R.M. 2005. Invasion Allelopathy and exotic plant invasion: from genes to communities: synopsis, updates, and implications. The International Allelopathy Symposium. Grodzinsky Award Oration. Wagga Wagga, Australia. August 23, 2005.

Callaway, R.M. 2005. Exotic invasions and the Novel Weapons Hypothesis. The University of Wisconsin, Eau Claire, September 15, 2005. Invited talk Callaway, R.M. 2005. Exotic invasions and the Novel Weapons Hypothesis. The University of Minnesota, Mankato, September 16, 2005. Invited talk

Callaway, R.M. 2005. Exotic invasions and the Novel Weapons Hypothesis. The Institute of Ecosystem Studies, White Plains, New York. October 7, 2005. Invited talk.

Callaway, R.M. 2005. Exotic invasions and the Novel Weapons Hypothesis. The University of Tennessee. October 7, 2005. Invited talk.

Callaway, R.M. 2005. Exotic invasions and the Novel Weapons Hypothesis. The University of Indiana. November 11, 2005. Invited talk.

Callaway, R.M. 2006. Exotic invasions and the Novel Weapons Hypothesis. University of British Columbia. January 10, 2006. Invited talk.

Callaway, R.M. 2005. Evolution and exotic invasion. University of British Columbia. January 11, 2006. Invited talk.

Callaway, R.M. 2006. Exotic invasions and the Novel Weapons Hypothesis. Archbold Biology Station. Florida. February 9, 2006. Invited talk.

Callaway, R.M. 2006. Exotic invasions and the Novel Weapons Hypothesis. Halle University, Halle Germany. February 28, 2006. Invited talk.

Callaway, R.M. 2006. Exotic invasions and the Novel Weapons Hypothesis. Lakehead University, Thunder Bay, Ontario, Canada. March 9. Invited talk.

Callaway, R.M. 2006. E Soil biota and invasions: a biogeographic perspective. Lakehead University, Thunder Bay, Ontario, Canada. March 10. Invited talk.

Callaway, R.M. 2006. Exotic invasions and the Novel Weapons Hypothesis. University of Zurich, Zurich Switzerland, March 30. Keynote presentation; Swiss National Graduate Student Conference.

Callaway, R.M. 2006. Why is facilitation important? International Symposium on Facilitation, Madrid, Spain. June 22. Keynote presentation.

Callaway, R.M. 2006. Exotic invasions and the Novel Weapons Hypothesis. University of Nevada, Las Vegas. September 15. Invited talk.

Callaway, R.M. 2006. Exotic invasions and the Novel Weapons Hypothesis. University of York, Toronto, Ontario. October 16. Invited talk.

Callaway, R.M. 2006. Exotic invasions and the Novel Weapons Hypothesis. Guelph University Guelph, Ontario. October 18. Invited talk.

Callaway, R.M. 2007. Exotic invasions and the Novel Weapons Hypothesis. University of Idaho, Moscow, ID. January 17. Invited talk.

Callaway, R.M. 2007. Exotic invasions and the Novel Weapons Hypothesis. University of Georgia, Athens, GA. February 27. Invited talk.

Callaway, R.M. 2007. Exotic invasions and the Novel Weapons Hypothesis. University of New York at Stony Brook, Stony Brook, NY. March 14. Invited talk.

Callaway, R.M. 2007. The evolution of invaders: selection for competitive ability and defense against herbivores. University of New York at Stony Brook, Stony Brook, NY. March 15. Invited talk. Slobodkin Award.

Callaway, R.M. 2007. Exotic invasions and the Novel Weapons Hypothesis. Virginia Tech University, Blacksburg, Virginia. March 23. Invited talk.

Callaway, R.M. 2007. Exotic invasions and the Novel Weapons Hypothesis. Cornell University Cornell, NY. April 4. Invited talk.

Callaway, R.M. 2007. Exotic invasions and the Novel Weapons Hypothesis. University of California, Irvine, CA. April 13. Invited talk.

Callaway, R.M. 2007. Exotic invasions and the Novel Weapons Hypothesis. Montana State University, Bozeman, MT. April 26. Invited talk.

Callaway, R.M. 2007. Exotic invasions and the Novel Weapons Hypothesis. University of Amsterdam, The Netherlands. May 22. Invited talk.

Callaway, R.M. 2007. Exotic invasions and the Novel Weapons Hypothesis. University of Nimegen, The Netherlands. May 23. Invited talk.

Callaway, R.M. 2007. Exotic invasions and the Novel Weapons Hypothesis. NIOO-KNAWCentre for Terrestrial Ecology, The Netherlands. May 24. Invited talk.

Callaway, R.M. 2008. Novel biochemistry and exotic plant invasions. Syracuse University. October 7. Invited seminar.

Callaway, R.M. 2008. Exotic plant invasions: how does a rare species at home become dominant away from home? Syracuse University. The Annual Jack and Pat Bryan Award. Invited seminar.

Callaway, R.M. 2008. Centaurea species, invasions, and novel biochemistry. International 5th International Weed Science Congress, Vancouver British Columbia, June. Invited talk.

Callaway, R.M. 2008. Conditional effects of an allelopathic root exudate: The toxicity of (±)-catechin is affected by interactions with different metals. Ecological Society of America Annual Meeting, August, Milwaukee, Wisconsin.

Callaway, R.M. 2008. Allelopathic effects of Centaurea species. International Allelopathy Society Meeting, Saratoga Springs, New York. Invited talk.

Pollock, J., W. Holben and R.M. Callaway. 2008. Conditionality in the allelopathic effects of Centaurea maculosa. International Allelopathy Society Meeting, Saratoga Springs, New York. Invited talk.

Callaway, R.M. 2008. Novel biochemistry and exotic plant invasions. University of Montreal. May. Invited seminar.

Callaway, R.M. 2008. Novel biochemistry and exotic plant invasions. University of California, Santa Cruz. April 9. Invited seminar.

Callaway, R.M. 2008. Novel weapons and exotic plant invasions. University of Alberta, March. Invited seminar.

Callaway, R.M. 2008. Novel weapons and exotic plant invasions. NSF workshop on ecology and biochemical interactions, Lima. Peru. May. Keynote presentation.

Callaway, R.M. 2008. Novel biochemistry and exotic plant invasions. Montana Tech University. November 30. Invited seminar.

Callaway, R.M. 2008. Novel biochemistry and exotic plant invasions. University of Illinois. October. Invited seminar.

Callaway, R.M. 2009. Facilitation in plant communities – the current state of play and future challenges. Annual British Ecological Society Symposium, Aberdeen, Scotland. January 20-22, 2009. Keynote presentation.

Callaway, R.M. 2009. Biochemical interactions in plant communities. Otago University, New Zealand. Invited Seminar.

Callaway, R.M. 2009. Does competition run the world? Positive interactions among plant species. Wichita State University, September 21, 2009. Invited seminar.

Callaway, R.M. 2009. Novel biochemistry and exotic plant invasions. Wichita State University, September 22, 2009. Invited seminar. Watkins Visiting Professor.

Callaway, R.M. 2009. Novel biochemistry and exotic plant invasions. Instituto Florestal de São Paulo, October 15, 2009. Invited seminar.

Callaway, R.M. 2009. Positive interations and interdependence in plant communities. University of São Paulo, October 17, 2009. Invited seminar.

Callaway, R.M. 2009. Novel biochemistry and exotic plant invasions. Universidad Autonoma, Mexico City, November 21, 2009. Invited seminar.

Callaway, R.M. 2009. Novel biochemistry and exotic plant invasions. University of Texas, San Antonio, December 16, 2009. Invited seminar.

Callaway, R.M. 2010. Positive interations and interdependence in plant communities. Ohio State University. January 21, 2010. Invited seminar.

Callaway, R.M. 2010. Novel biochemistry and exotic plant invasions. Clemson University March 22, 2010. Invited seminar.

Callaway, R.M. 2010. Novel biochemistry and exotic plant invasions. University of Arizona, March 29, 2010. Invited seminar.

Callaway, R.M. 2010. Novel biochemistry and exotic plant invasions. Montana Tech University, Butte, April 22, 2010. Invited seminar.

Callaway, R.M. 2010. Novel biochemistry and exotic plant invasions. Southern Denmark University, May 17, 2010. Invited seminar.

Callaway, R.M. 2010. Novel biochemistry and exotic plant invasions. Tartu University, Estonia. May 20, 2010. Invited seminar.

Callaway, R.M. 2010. Positive interations and interdependence in plant communities. Tartu University, Estonia. May 20, 2010. Invited seminar.

Callaway, R.M. 2010. Impacts of invaders in native and non-native ranges. Ecological Society of America, Pittsburgh, August 2010. Invited session.

Callaway, RM. 2010. Novel biochemistry and exotic plant invasions. University of Mississippi, September, 2010. Invited seminar

Callaway, RM. 2010. Novel biochemistry and exotic plant invasions. University of Wyoming, October, 2010. Invited seminar.

Callaway, RM. 2010. Novel biochemistry and exotic plant invasions. University of Kentucky, November, 2010. Invited seminar

Callaway, RM. 2010. Soil biota and exotic plant invasions. University of Sao Carlos, Brazil, December, 2010. Invited seminar