DISSERTATION

DEVELOPMENT AND UTILIZATION OF MOLECULAR TOOLS TO UNDERSTAND INVASION BIOLOGY IN *CENTAUREA MACULOSA* (SPOTTED KNAPWEED)

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY AMANDA K BROZ ENTITLED DEVELOPMENT AND UTILIZATION OF MOLECULAR TOOLS TO UNDERSTAND INVASION BIOLOGY IN *CENTAUREA MACULOSA* (SPOTTED KNAPWEED) BE ACCEPTED AS FULLFILING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

DEVELOPMENT AND UTILIZATION OF MOLECULAR TOOLS TO UNDERSTAND INVASION BIOLOGY IN *CENTAUREA MACULOSA* (SPOTTED KNAPWEED)

My doctoral research at Colorado State University was designed to create and utilize molecular tools to help understand ecological phenomena in the invasive weed, spotted knapweed (*Centaurea maculosa* Lam.). In this dissertation, I first introduce the need for research at multiple scales and the potential benefits of collectively examining molecular, physiological and ecological phenomena in an invasive plant. I then give a brief overview of the life history characteristics of spotted knapweed and report on the development and characterization of a spotted knapweed gene library. By utilizing sequence information from the gene library, I determined that both ploidy (diploid or tetraploid) and origin (native or invasive) influence expression of genes that may be important for plant defense in spotted knapweed populations. I found that spotted knapweed can differentially respond to strong or weak competitors at the level of gene expression by using existing molecular tools from a model plant coupled with sequence information from the gene library. In addition, I found that plant neighbor identity, simulated herbivory and resource availability are all important factors that influence accumulation of biomass and secondary metabolites in both spotted knapweed and a

native grass species. I utilized molecular tools to demonstrate that spotted knapweed infestation alters the composition of North American soil fungal communities; however, the ecological ramifications of this observation remain undetermined. The major goal of my research was to better understand spotted knapweed invasion biology by utilizing molecular tools. I believe this approach was successful in that it led to a variety of interesting results. However, more research is required to fully link these molecular findings with ecological and physiological aspects of spotted knapweed invasion biology. It is my hope that the chapters of this dissertation highlight both the opportunities and limitations associated with using molecular tools to understand invasion biology in this system.

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I lived in peace and quiet, until IT came along
IT started out like a jingle and transformed into a song

I knew at that moment, beyond a shadow of a doubt
I had to close my windows and just try to keep IT out!

But glass and metal were no match, and so IT got inside
I looked around in vain to find some sort of place to hide

But just about the time that I was diving 'neath my bed
IT pounced upon my shoulder and IT crawled inside my head…

That tune has never left me, IT disturbs me more and more
IT reverberates the questions that I don't have answers for.

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RESEARCH SUMMARY

My doctoral research at Colorado State University is designed to create and utilize molecular tools to help understand ecological phenomena in the invasive weed, spotted knapweed (*Centaurea maculosa* Lam., also known as *Centaurea stoebe* L. spp. *micranthos*). In this dissertation, I first introduce the need for research at multiple scales and the potential benefits of collectively examining molecular, physiological and ecological phenomena in an invasive plant. I give a brief overview of the life history characteristics of spotted knapweed, as these characteristics are essential in understanding the species in a biological and ecological framework. I also present a brief discussion of developed hypotheses of plant invasion into new environments, and evidence for these hypotheses in regards to spotted knapweed.

In the second chapter of this dissertation, I report on the development and characterization of a spotted knapweed gene library and discuss some ways in which this molecular tool could be used to investigate invasion biology. The library contains 4423 unique gene sequences, seventy seven percent of which showed significant similarity to genes in the National Center for Biotechnology Information (NCBI) database and could be categorized using gene ontology assignments.

The third chapter concerns a study in which I utilized sequence information from the spotted knapweed gene library to investigate expression of genes that may be important in plant defense. I studied both native and invasive populations of spotted knapweed and found that both plant origin (native or invasive) and ploidy (diploid or

teterapolid) influence relative rates of gene expression. Interestingly, invasive plants exhibited reduced expression of defense related genes compared to their native tetraploid counterparts. This finding may give some support to ecological hypotheses of plant invasion that predict plants in the invaded range will be more poorly defended than those in the native range, due to factors such as release from natural enemies and rapid evolution.

The fourth chapter covers the use of existing molecular tools from a model plant to investigate gene expression changes that may be important in plant-plant interactions. In this study, I used cross-species hybridization of spotted knapweed cDNA to an *Arabidopsis thaliana* microarray, in an attempt to identify changes in gene expression provoked by different plant neighbors. Spotted knapweed was grown with either a weak competitor (the native grass Idaho fescue), a strong competitor (the native forb Indian blanket flower), or alone (control), and spotted knapweed gene expression was characterized in these different competitive settings. I found that some genes were up or down regulated due to the mere presence of a plant neighbor, whereas other genes were differentially regulated based on the identity of the plant neighbor. These findings suggest that spotted knapweed can respond differently to different plant neighbors at the level of gene expression.

In the fifth chapter, I utilize an existing chemical assay to investigate the relative influence of plant neighbor identity, resource availability, and simulated herbivory on secondary metabolite production in both spotted knapweed and the native grass Idaho fescue. In this experiment I consistently identified an interaction between plant neighbor identity and simulated herbivory that impacted both secondary metabolite production and

plant biomass. When plants were elicited with jasmonic acid to simulate herbivory plants grown with a neighbor of the same species always produced more secondary metabolites (total phenolics) than plants that were grown with a neighbor of a different species. This suggests that plants are able to modify their metabolic processes in response to the plant community in which they grow.

The sixth chapter describes the use of existing molecular tools to identify changes in native North American soil microbial communities that result from spotted knapweed invasion. In this experiment I collected soils from rangelands that were infested with either high or low densities of spotted knapweed. I isolated DNA from these field samples and used quantitative PCR and capillary electrophoresis to identify changes in microbial community structure. I found that high densities of spotted knapweed reduced fungal biomass and altered fungal community structure. In addition, I found that spotted knapweed altered fungal community composition not only within its own rhizosphere, but also within the rhizosphere of native North American grasses.

The experiments described in the seventh chapter were designed to investigate the ecological relevance of the findings described in chapter six. I planted spotted knapweed and Idaho fescue in the soils collected from high and low density spotted knapweed stands to determine if there were any feedback effects on plant biomass accumulation. In general, I found that both spotted knapweed and Idaho fescue accumulated more biomass in soils that had been sterilized versus soils containing active microbial communities; but the original density of spotted knapweed in the soil had no impact on biomass. This could suggest the presence of plant pathogens in the soils; however, my results differed between experimental conditions, making it difficult to draw robust conclusions. I

manipulated spotted knapweed planting density in the greenhouse, and found that soil fungal biomass increased with increased planting density of spotted knapweed. These results suggest that factors other than, or in combination with knapweed density are driving the observed reductions in fungal community biomass in field collected soils.

The final chapter of this dissertation provides some general conclusions in regards to the utilization of molecular tools to understand biological and ecological phenomena. I present a brief discussion of the opportunities and limitations I have encountered over the course of my research with spotted knapweed, and discuss a few ideas for future research work. Generally, I have found that the use of molecular tools can compliment more traditional research approaches, but will by no means be able to replace classical techniques associated with plant biology and ecology.

CHAPTER 1

Introduction

Parts of this work are awaiting publication.

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"The Genomics of Plant Invasion; a Case Study in Spotted Knapweed"

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1.1 WHY USE MOLECULAR TOOLS TO STUDY INVASION BIOLOGY?

It is estimated that over 25,000 non-native plant species have been introduced into the United States (Pimentel et al., 2000). Although some of these species represent valuable food crops, such as corn, rice and wheat, other introduced plants have become some of the nation's worst weed problems, threatening the viability of food production and the community structure of native ecosystems. Weeds are responsible for agricultural losses of nearly $20 billion per year in the United States alone (Basu et al., 2004), and it is estimated that the economic cost of all invasive plant species exceeds $34.5 billion per year (Pimentel et al., 2000). Although the monetary cost of these weeds is astounding, their negative impact on the biodiversity of native ecosystems may be of greater concern. It is estimated that introduced plant species invade over 700,000 hectares per year of United States wildlife habitat (Pimentel et al., 2000). In addition, hundreds of native species are being threatened by exotic invasive plants. For instance, nearly half of Hawaii's 1750 native plant species are endangered, and over 200 species endemic to Hawaii are thought to have become extinct due to displacement by invasive species (Pimentel et al., 2000).

Although the economic and biological issues associated with invasive weeds are recognized internationally, the majority of research efforts have focused on ecological consequences of plant invasion, leaving the genomic basis of these consequences relatively unexplored. This is due at least in part to the paucity of molecular tools available for invasive plants and other weedy species (Basu et al., 2004). Most genomics efforts have focused to a greater extent on crop plants, as opposed to their weedy relatives. However, as genomics resources become more affordable and available to

scientists, an understanding of the genetic basis of invasiveness becomes increasingly possible.

It has been widely observed that many weedy species possess certain characteristics that enhance their ability to succeed in the environment. These traits include high output of seeds, long distance seed dispersal, competitive ability, discontinuous dormancy and rapid growth, to name a few (Basu et al., 2004). Although these traits are often associated with weeds, many of them are actually desirable for breeding in crop plants. Thus, plant breeders and geneticists have begun to utilize genomics resources in order understand agronomically important phenotypes, including increased yield, pathogen resistance and competitive ability of crops. In addition, the sequencing of both the *Arabidopsis* and rice genomes have led to the identification and characterization of many biochemical pathways involved in growth, fecundity, defense, and other important plant phenotypes. As many agronomic weeds are close relatives of crop plants, these studies are likely to provide clues concerning weed phenotypes and their underlying gene networks. Because genes conferring important traits are often conserved throughout plant lineages, it is possible that these studies of crops and their close relatives could provide insight into the biology of other weeds. However, the overlap of gene sequence and gene function between plant species is often not reliable, and thus it will be important to develop genomics resources for multiple plant families (Shimamoto and Kyozuka, 2002).

Invasive species present an interesting case study in both weed research and evolutionary biology. Although members of a given species will, in theory, possess the same capacity to exhibit fundamental 'weedy' traits, in their native range these plants are

generally seen as benign, whereas in the invasive or introduced range they are extremely problematic pests. As many of the plants currently listed as invasive species were originally introduced as ornamentals or food and fiber crops (Pimentel et al., 2000), these plants likely underwent years of human selection in the native range that may have conferred an advantageous genotype to founding populations in the invasive range. However, many other invasive plant species were not purposefully introduced to North America and do not appear to have undergone human selection for hardiness, competitive ability or other traits. There is some evidence to suggest that environmental adaptation and evolution play an important role in the success of these invasive species, and ecological hypotheses of plant invasion have been developed based on this evidence (Blossey and Notzold, 1995; Callaway and Aschehoug, 2000; Lee, 2002). Molecular marker studies have revealed differences in population structure and diversity between the native and introduced ranges for many invasive plant species (Lee, 2002; Bossdorf et al., 2005). However, there has been little work examining how the genetic diversity of these populations influences the gene expression and protein accumulation that are ultimately responsible for the phenotypic characteristics that allow for invasive success. These questions could begin to be addressed by creating genomics resources for invasive species, and coupling them with molecular markers, mapping studies, and characterization of important phenotypic traits. Genomics investigations may then be able to provide links between plant gene expression and ecological hypotheses of plant invasion into new environments.

Investigations into the ecological consequences of plant invasion may benefit from genomics resources as well. New molecular tools are being developed that can be

used to assess the impacts of plant invasion on other components of ecological communities. For example, the role of soil microbial communities in exotic plant invasion has gained recent interest, particularly due to observations of plant-soil feedback effects that tend to favor invasive species (Klironomos, 2002; Callaway et al., 2004; Callaway et al., 2004; Wardle et al., 2004; Wolfe and Klironomos, 2005; Stinson et al., 2006). Traditional culture-based techniques used to assess soil microbial community composition are thought to capture only a small percentage of the microbial diversity in soils (Manter and Vivanco, 2007). However, new PCR and DNA sequencing techniques provide for a wider of view of soil microbial community composition, and may allow researchers to better identify microbial community changes that are related to plant invasion. Large scale sequencing efforts have provided a wealth of information on microbial community composition in soils, bodies of water, and even on microbes that reside inside plant tissues as endophytes. This sequence information, in combination with other molecular techniques, could be used to identify the impact of plant invasion on native microbial communities, and may lead to important insights that link above and below ground community composition.

By integrating molecular techniques into ecological research, it is possible to generate interesting new hypotheses that can be tested at molecular, physiological and ecological levels. It is important to note that, although molecular techniques will compliment and enhance studies of plant invasion, they will by no means be able to replace classical measures of plant physiological or ecological characteristics. What characterizes invasive plants to the greatest extent is a weedy phenotype, and this phenotype is the consequence of both genetics and the environment. Thus, molecular

studies of invasive weeds must be interpreted within a context of both plant biology and plant community dynamics to have truly meaningful relevance.

Some exciting advances have already been made in the area of ecological genomics regarding plant-water relations, plant-herbivore and host-parasite interactions, temperature acclimation in ectothermic (cold-blooded) vertebrates, social interaction in honeybees and microbial community ecology in marine and terrestrial systems (Hofmann et al., 2005; Travers et al., 2007). These studies highlight the potential utility of using molecular tools to understand physiological problems and ecological phenomena. Studies of this type can help identify the genetic mechanisms that underlie an organism's response to environmental change and natural selection, which will provide a deeper understanding of the interplay between phenotype, genotype and the environment. This may be especially useful in identifying genetic and environmental factors that dictate the spread and success of invasive organisms around the world. Understanding the process of exotic invasion at ecological *and* molecular levels will enhance our current 'tool box', and may result in better techniques for predicting invasive capacity of organisms and managing the spread of invasive exotics. In this dissertation I attempt to create and utilize molecular tools in order to better understand invasion biology of spotted knapweed.

1.2 SPOTTED KNAPWEED LIFE HISTORY

Spotted knapweed (*Centaurea maculosa* Lam.) is a particularly aggressive invasive weed in the northwestern United States, infesting over 4.7 million acres in Montana alone (Mauer et al., 2001). A Eurasian native, spotted knapweed is proposed to have been introduced on both coasts of North America in the late 1800s as a contaminant of alfalfa seed. The weed has since expanded its range to all but three states in the continental US (plants.usda.gov), and is prominent in western and central Canadian provinces. Spotted knapweed inhabits a variety of environments in the native and invaded ranges, and is common in disturbed areas, pastures, prairies, rocky slopes, and rangeland. In Eurasia it is not considered very weedy or problematic; however, in many areas of western North America spotted knapweed has invaded native ecosystems, displacing native species and forming near monocultures. In addition, the weed increases water runoff leading to erosion (Lacey et al., 1989), and reduces forage for livestock and wildlife (Thompson, 1996).

In the native range, taxonomists have identified at least two forms of the weed, although the nomenclature is often confusing and inconsistent. The diploid form (*Centaurea stoebe* L. spp. *stoebe*, synonyms: *C. maculosa* L. spp *maculosa*) and the tetraploid form (*Centaurea stoebe* L. spp. *micranthos* (Gugler) Hayek, *C. biebersteinii,* commonly known as *C. maculosa* Lam), occur in separate and mixed stands in the native range (Treier et al., in press). Recently a mixed stand of diploids and tetraploids was identified in Canada (Treier et al., in press), but otherwise only the tetraploid form has been found in North America ((Oschmann, 2001), Treier et al., in press). Karyotyping and other molecular techniques have been used to identify the two forms as they are indistinguishable based on morphological characters (Oschmann, 2001). The diploid

form of the weed contains 18 chromosomes (Powell et al., 1974) with a 2C DNA content near 3.6 pg, based on measures from closely related *Centaurea* species of the same chromosome number (Grime et al., 1985). This translates to an estimated genome size of 1,800 Mbp, over 10 times larger than the genome of the model species *Arabidopsis thaliana* (125 Mbp), which contains over 25,000 genes (www.arabidopsis.org).

Both diploid and tetraploid forms of spotted knapweed are tap-rooted, short-lived perennials of the aster family, but the diploid is monocarpic and the tetraploid polycarpic (Oschmann, 2001). Both are capable of remaining in rosette form for many years before bolting (Freville et al., 1998; Mauer et al., 2001). In addition to flowering multiple years, the tetraploid is capable of producing multiple flowering stems with up to 15 capitula each (Mauer et al., 2001), whereas the diploid produces only one stem (Oschmann, 2001). Flowers bloom from late summer to fall, producing up to 35 seeds per capitula (Mauer et al., 2001). Compared to native populations, introduced tetraploids exhibit the highest proportion of polycarpic plants and have the greatest number of stems per plant (Treier et al., in press), which may increase their reproductive capacity. It is hypothesized that this perennial polycarpic lifestyle is selected for in environments lacking natural enemies (Muller-Scharer et al., 2004), which may help explain why the tetraploid form became predominate in the introduced range (Treier et al., in press). Seeds of spotted knapweed germinate in both the spring and early fall and are capable of remaining viable in the soil for up to ten years (Mauer et al., 2001). Gravity is the major plant mechanism for seed dispersal; however, human and animal dispersal also play an important role in seed transport.

Both forms of the weed are insect pollinated and predominately, if not entirely, out-crossing. Genetic diversity studies of plants in the native and invaded range suggest that there have been multiple introductions of spotted knapweed (Hufbauer and Sforza, 2008; Marrs et al., 2008). A large amount of allelic richness was found in both native and invasive populations using chloroplast haplotype sequence data, and there was some evidence to indicate possible introgression of chloroplasts between taxa. Spotted knapweed was found to share multiple haplotypes with another *Centaurea* species (diffuse knapweed, *C. diffusa*) that also occurs as a diploid and tetraploid in the native range, suggesting hybridization between the two species may have occurred. In addition, studies of *Centaurea* species in southern France suggest that the rare, endemic *C. corymbosa* and the more widespread *C. maculosa* spp *albida* are likely derived from spotted knapweed (*C. maculosa* spp *maculosa*), and have undergone ecological specialization (Freville et al., 1998). These studies provide an interesting context for studying spotted knapweed in both the native and invasive ranges, in that the species contains a range of genetic diversity from which new taxa are able to evolve.

Spotted knapweed is able to tolerate a wide variety of soil types and precipitation amounts in both Eurasia and North America (Sheley et al., 1998; Oschmann, 2001). The range of the native tetraploid in Eurasia has expanded over the range of the native diploid within the past 100-150 years (Oschmann, 2001), with a general migration towards lower latitudes (Treier et al., in press). Introduced tetraploids appear to have a higher ecological tolerance, or niche breadth, than either of the native forms (Treier et al., in press; (Broennimann et al., 2007)). This apparent post-invasion climatic niche shift may be due to rapid evolution in the introduced range; however, more studies are needed to

rule out other alternatives (Treier et al., in press). In addition, the tetraploid can withstand dense vegetation (Oschmann, 2001) and drier environments (Broennimann et al., 2007), Treier et al., in press), which is presumed to be the main reason why only the tetraploid form is invasive in North America, while neither form is considered invasive or even predominant in its native Eurasian habitat.

1.3 SPOTTED KNAPWEED AND ECOLOGICAL HYPOTHESES OF PLANT INVASION

Although differences in life history characteristics may be important in spotted knapweed invasion, there are a variety of other hypotheses that may help explain the success of this weed in North America. Ecologists have long been interested in identifying key factors involved in the invasive success of exotic plant species, but the mechanisms of invasion are still not well understood. Multiple non-exclusive hypotheses have been developed to explain plant invasion of new environments, all of which are partially supported by empirical evidence. A few of these hypotheses are discussed below in regards to spotted knapweed invasion.

The enemy release hypothesis, originally developed by Darwin (1859) and expanded by Elton (1958), suggests that plants escape their co-evolved pathogens upon introduction to a new environment, which allows them to increase in numbers as their population growth continues, unchecked by native enemies (Darwin, 1859; Elton, 1958). Blossey and Notzold (1995) expanded on enemy release, developing the evolution of increased competitive ability hypothesis (EICA), which suggests that after escaping their enemies, invaders would rapidly evolve to put fewer resources into defense and more

resources into growth and reproduction (Blossey and Notzold, 1995). Thus, introduced populations should be more poorly defended against herbivores and pathogens than their native counterparts. In addition, introduced populations are predicted to be faster growing, larger, and have a higher reproductive capacity than populations from the native range.

The release of spotted knapweed from specialist enemies has long been considered an important factor in the invasive success of the weed, and this has spurred the introduction of a number of biological control species to North America over the past thirty years (Maddox, 1979; Maddox, 1982; Smith and Story, 2003; Muller-Scharer et al., 2004). Although many of these specialist herbivores have become established, spotted knapweed has not been effectively controlled and the weed continues to expand its range (Sheley et al., 1998; Muller-Scharer et al., 2004). Interestingly, field observations in North America suggest that introduced *C. maculosa* experiences little pressure from generalist herbivores and pathogens (RM Callaway and WM Ridenour, personal communication). Thus, it appears that spotted knapweed currently experiences a partial release from both specialist and generalist enemies in the introduced range.

Evidence suggesting that spotted knapweed populations from the invaded range puts more resources in growth/fecundity and fewer resources into defense than populations from the native range is for the most part anecdotal. One study of native diploids and invasive tetraploids found greater compensatory rooting intensity of the latter (Muller, 1989). However, recent work by Ridenour and Callaway suggests that introduced plants are generally better competitors and better defended from herbivores than plants from native populations (Ridenour et al., 2008). A portion of my

investigations, particularly in Chapter 3 of this dissertation, are directed toward investigating the possibility of rapid evolution in introduced populations of spotted knapweed.

The Novel Weapons Hypothesis (NWH) suggests that some plants come to their new environment equipped with chemical or biochemical weapons that have a greater negative effect against plants in the invaded range than similar species in the native range (Callaway and Ridenour, 2004). These weapons give the invader a competitive advantage in the new environment, as they are proposed to exert strong phytotoxic effects against other plants. The NWH has also been expanded to encompass possibilities of rapid evolution; termed the allelopathic advantage against resident species hypothesis (AARS) (Callaway and Ridenour, 2004). AARS suggests that if plant invaders gain a competitive advantage through the use of novel weapons in the invaded range they will evolve to have greater concentrations of these weapons than populations in the native range. Thus, invasive populations should be better chemically defended and thus better competitors against other plant species then their native counterparts.

Allelopathic interactions have also been proposed to be responsible for the aggressive behavior of spotted knapweed (Suchy and Herout, 1962; Fletcher and Renney, 1963; Kelsey and Locken, 1987; Locken and Kelsey, 1987) in western North America. Extracts from leaves, stems, seeds and roots of both spotted and diffuse knapweed were found to have a negative impact on germination of barley and lettuce seeds (Fletcher and Renney, 1963). The sesquiterpene lactone, cnicin was later identified as the major chemical constituent of knapweed leaves and in bioassays inhibited growth of a variety of plant species (Kelsey and Locken, 1987; Locken and Kelsey, 1987). Previous analyses of

cnicin found it had strong antimicrobial activity (Cavallito and Bailey, 1949), and it was postulated that both of these properties could influence the invasive success of knapweed species. However, analysis of soil samples in knapweed-infested areas recovered only trace amounts of cnicin, suggesting that this compound was not biologically available at phytotoxic concentrations, and thus was unlikely to have ecologically relevant allelopathic effects (Locken and Kelsey, 1987). Although bioassays had suggested that other chemical compounds in knapweeds are phytotoxic (Fletcher and Renney, 1963), identification of these potential allelochemicals has not been pursued. However, a renewed interest in the potential allelopathic effects of knapweed species was initiated by experiments preformed by Ragan Callaway at the University of Montana, Missoula.

Spotted knapweed was grown in competition with Idaho fescue (*Festuca idahoensis*), a North American native bunchgrass, with and without activated carbon, which adsorbs organic compounds (Ridenour and Callaway, 2001). Biomass and root elongation of Idaho fescue were significantly inhibited when grown with a spotted knapweed competitor versus a conspecific competitor, and the addition of activated carbon reduced this inhibitory effect (Ridenour and Callaway, 2001). However, spotted knapweed outperformed Idaho fescue with and without carbon, suggesting that resource competition and allelopathy may both influence success of the invasive. Further laboratory experiments suggested a putative allelochemical, the flavanoid (±)-catechin was responsible for the inhibitory effects of spotted knapweed on native plants (Bais et al., 2003). This chemical was found to exuded from the roots of spotted knapweed in laboratory experiments; however, the ecological relevance of this allelochemical is currently under debate (Blair et al., 2005; Blair et al., 2006; Perry et al., 2007). Although

there is some evidence to suggest that allelopathy is involved in spotted knapweed invasion, I have chosen not to focus on allelopathic interactions in my research. I was more interested in exploring other potential mechanisms of knapweed invasion. However, the topic of allelopathy is generally discussed in relation to many of my findings.

1.4 REFERENCES

Bais HP, Vepachedu R, Gilroy S, Callaway RM, Vivanco JM (2003) Allelopathy and exotic plant invasion: From molecules and genes to species interactions. Science **301:** 1377-1380

Basu C, Halfhill MD, Mueller TC, Stewart CN (2004) Weed genomics: new tools to understand weed biology. Trends in Plant Science **9:** 391-398

Blair AC, Hanson BD, Brunk GR, Marrs RA, Westra P, Nissen SJ, Hufbauer RA (2005) New techniques and findings in the study of a candidate allelochemical implicated in invasion success. Ecology Letters **8:** 1039-1047

Blair AC, Nissen SJ, Brunk GR, Hufbauer RA (2006) A lack of evidence for an ecological role of the putative allelochemical (+/-)-catechin in spotted knapweed invasion success. Journal of Chemical Ecology **32:** 2327-2331

Blossey B, Notzold R (1995) EVOLUTION OF INCREASED COMPETITIVE ABILITY IN INVASIVE NONINDIGENOUS PLANTS - A HYPOTHESIS. Journal of Ecology **83:** 887-889

Bossdorf O, Auge H, Lafuma L, Rogers WE, Siemann E, Prati D (2005) Phenotypic and genetic differentiation between native and introduced plant populations. Oecologia **144:** 1-11

Broennimann O, Treier UA, Muller-Scharer H, Thuiller W, Peterson AT, Guisan A (2007) Evidence of climatic niche shift during biological invasion. Ecology Letters **10:** 701-709

Callaway RM, Aschehoug ET (2000) Invasive plants versus their new and old neighbors: A mechanism for exotic invasion. Science **290:** 521-523

Callaway RM, Ridenour WM (2004) Novel weapons: invasive success and the evolution of increased competitive ability. Frontiers in Ecology and the Environment **2:** 436-443

Callaway RM, Thelen GC, Barth S, Ramsey PW, Gannon JE (2004) Soil fungi alter interactions between the invader Centaurea maculosa and North American natives. Ecology **85:** 1062-1071

Callaway RM, Thelen GC, Rodriguez A, Holben WE (2004) Soil biota and exotic plant invasion. Nature **427:** 731-733

Cavallito C, Bailey J (1949) An antibacterial principle from Centaurea maculosa. Journal of Bacteriology **57:** 207-212

Darwin C (1859) On the Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life. John Murray, London

Elton C (1958) The Ecology of Invasions by Animals and Plants. Metheun, London

Fletcher R, Renney A (1963) A growth inhibitor found in Centaurea spp. Canadian Journal of Plant Science **43:** 475-481

Freville H, Colas B, Ronfort J, Riba M, Olivieri I (1998) Predicting endemism from population structure of a widespread species: Case study in Centaurea maculosa Lam. (Asteraceae). Conservation Biology **12:** 1269-1278

Grime JP, Shacklock JML, Band SR (1985) NUCLEAR-DNA CONTENTS, SHOOT PHENOLOGY AND SPECIES CO-EXISTENCE IN A LIMESTONE GRASSLAND COMMUNITY. New Phytologist **100:** 435-445

Hofmann GE, Burnaford JL, Fielman KT (2005) Genomics-fueled approaches to current challenges in marine ecology. Trends in Ecology & Evolution **20:** 305-311

Hufbauer RA, Sforza R (2008) Multiple introductions of two invasive Centaurea taxa inferred from cpDNA haplotypes. Diversity and Distributions **14:** 252-261

Kelsey RG, Locken LJ (1987) PHYTOTOXIC PROPERTIES OF CNICIN, A SESQUITERPENE LACTONE FROM CENTAUREA-MACULOSA (SPOTTED KNAPWEED). Journal of Chemical Ecology **13:** 19-33

Klironomos JN (2002) Feedback with soil biota contributes to plant rarity and invasiveness in communities. Nature **417:** 67-70

Lacey JR, Marlow CB, Lane JR (1989) INFLUENCE OF SPOTTED KNAPWEED (CENTAUREA-MACULOSA) ON SURFACE RUNOFF AND SEDIMENT YIELD. Weed Technology **3:** 627-631

Lee CE (2002) Evolutionary genetics of invasive species. Trends in Ecology & Evolution **17:** 386-391

Locken LJ, Kelsey RG (1987) CNICIN CONCENTRATIONS IN CENTAUREA-MACULOSA, SPOTTED KNAPWEED. Biochemical Systematics and Ecology **15:** 313-320

Maddox DM (1979) The knapweeds: their economics and biological control in the western states. USA Rangelands **1:** 139-141

Maddox DM (1982) BIOLOGICAL-CONTROL OF DIFFUSE KNAPWEED (CENTAUREA-DIFFUSA) AND SPOTTED KNAPWEED (C MACULOSA). Weed Science **30:** 76-82

Manter DK, Vivanco JM (2007) Use of the ITS primers, ITS1F and ITS4, to characterize fungal abundance and diversity in mixed-template samples by qPCR and length heterogeneity analysis. Journal of Microbiological Methods **71:** 7-14

Marrs RA, Sforza R, Hufbauer RA (2008) Evidence for multiple introductions of Centaurea stoebe micranthos (spotted knapweed, Asteraceae) to North America. Molecular Ecology **17:** 4197-4208

Mauer T, Russo M, Evans M (2001) Element Stewardship Abstract for Centaurea maculosa. *In*, Vol 2006. The Nature Conservancy, Arlington, VA

Muller-Scharer H, Schaffner U, Steinger T (2004) Evolution in invasive plants: implications for biological control. Trends in Ecology & Evolution **19:** 417-422

Muller H (1989) GROWTH-PATTERN OF DIPLOID AND TETRAPLOID SPOTTED KNAPWEED, CENTAUREA-MACULOSA LAM (COMPOSITAE), AND EFFECTS OF THE ROOT-MINING MOTH AGAPETA-ZOEGANA (L) (LEP, COCHYLIDAE). Weed Research **29:** 103-111

Oschmann J (2001) On the taxonomy of spotted knapweed (Centaurea stoebe L.) *In* Proceedings from The First International Knapweed Symposium of the Twenty First Century, pp 33-41

Perry LG, Thelen GC, Ridenour WM, Callaway RM, Paschke MW, Vivanco JM (2007) Concentrations of the allelochemical (+/-)-catechin in Centaurea maculosa soils. Journal of Chemical Ecology **33:** 2337-2344

Pimentel D, Lach L, Zuniga R, Morrison D (2000) Environmental and economic costs of nonindigenous species in the United States. Bioscience **50:** 53-65

Powell AM, Kyhos DW, Raven PH (1974) CHROMOSOME-NUMBERS IN COMPOSITAE X. American Journal of Botany **61:** 909-913

Ridenour WM, Callaway RM (2001) The relative importance of allelopathy in interference: the effects of an invasive weed on a native bunchgrass. Oecologia **126:** 444-450

Ridenour WM, Vivanco JM, Feng YL, Horiuchi J, Callaway RM (2008) No evidence for trade-offs: Centaurea plants from America are better competitors and defenders. Ecological Monographs **78:** 369-386

Sheley RL, Jacobs JS, Carpinelli MF (1998) Distribution, biology, and management of diffuse knapweed (Centaurea diffusa) and spotted knapweed (Centaurea maculosa). Weed Technology **12:** 353-362

Shimamoto K, Kyozuka J (2002) Rice as a model for comparative genomics of plants. Annual Review of Plant Biology **53:** 399-419

Smith L, Story JM (2003) Plant size preference of Agapeta zoegana L. (Lepidoptera : Tortricidae), a root-feeding biological control agent of spotted knapweed. Biological Control **26:** 270-278

Stinson KA, Campbell SA, Powell JR, Wolfe BE, Callaway RM, Thelen GC, Hallett SG, Prati D, Klironomos JN (2006) Invasive plant suppresses the growth of native tree seedlings by disrupting belowground mutualisms. Plos Biology **4:** 727-731

Suchy M, Herout V (1962) ON TERPENES .136. IDENTITY OF BITTER PRINCIPLE FROM CENTAUREA STOEBE (L.) SCH. ET THELL. WITH CNICIN. Collection of Czechoslovak Chemical Communications **27:** 1510-&

Thompson M (1996) Winter foraging responses of elk to spotted knapweed removal Northwest Science **70:** 10-19

Travers SE, Smith MD, Bai JF, Hulbert SH, Leach JE, Schnable PS, Knapp AK, Milliken GA, Fay PA, Saleh A, Garrett KA (2007) Ecological genomics: making the leap from model systems in the lab to native populations in the field. Frontiers in Ecology and the Environment **5:** 19-24

Treier UA, Broennimann O, Normand S, Guisan A, Schaffner U, Steinger T, Muller-Scharer H (in press, 2009) Shift in cytotype frequency and niche space in the invasive plant Centaurea maculosa. Ecology **90**: 1366-1377

Wardle DA, Bardgett RD, Klironomos JN, Setala H, van der Putten WH, Wall DH (2004) Ecological linkages between aboveground and belowground biota. Science **304:** 1629-1633

Wolfe BE, Klironomos JN (2005) Breaking new ground: Soil communities and exotic plant invasion. Bioscience **55:** 477-487

CHAPTER 2

A first step in understanding an invasive weed through its genes:

an EST analysis of invasive *Centaurea maculosa*

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A first step in understanding an invasive weed through its genes: an EST analysis of invasive *Centaurea maculosa.*

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Authors' contributions

AKB: designed and performed research, analyzed data, wrote manuscript
CDB: analyzed data, wrote manuscript
JH, XD, PXZ: provided new analytical techniques (PLAN database)
JMV: designed research, edited and approved manuscript

Data deposition

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2.1 ABSTRACT

The economic and biological implications of plant invasion are overwhelming; however, the processes by which plants become successful invaders are not well understood. Limited genetic resources are available for most invasive and weedy species, making it difficult to study molecular and genetic aspects that may be associated with invasion. As an initial step towards understanding the molecular mechanisms by which plants become invasive, we have generated a normalized Expressed Sequence Tag (EST) library comprising seven invasive populations of *Centaurea maculosa*, an invasive aster in North America. Seventy-seven percent of the 4423 unique transcripts showed significant similarity to existing proteins in the NCBI database and could be grouped based on gene ontology assignments. The *C. maculosa* EST library 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 *C. maculosa* and its native counterparts. To our knowledge, this is the first published set of ESTs derived from an invasive weed that will be targeted to study invasive behavior. Understanding the genetic basis of evolution for increased invasiveness in exotic plants is critical to understanding the mechanisms through which exotic invasions occur.

2.2 INTRODUCTION

Invasive weeds are regarded as major threats to biodiversity because they can spread through communities, displacing or even eradicating native species. Over 25,000 invasive plant species have been documented in the United States, invading nearly 700,000 hectares per year, with a cost exceeding 34.5 billion dollars per year (Pimentel et al., 2000). Multiple non-exclusive ecological hypotheses exist to explain plant invasion in new habitats (Hierro et al., 2005). The niche hypothesis suggests invaders are able to take advantage of unutilized resources in new environments (Elton, 1958). The natural enemy release hypothesis suggests invaders escape their natural enemies when moving to new environments, allowing them to obtain high population densities (Darwin, 1859). The novel weapons hypothesis suggests that invaders come equipped with an arsenal of chemical weapons that are detrimental to the resident community of the invaded habitat (Callaway and Aschehoug, 2000). The plant community in the invader's native habitat has had time to co-evolve defenses against these chemical weapons, whereas the invaded community has not, allowing invaders to obtain a competitive advantage in their new environment (Callaway and Aschehoug, 2000). The evolution of increased competitive ability (EICA) hypothesis expands on the idea of natural enemy release, suggesting that invaders have rapidly evolved in their new environment to direct more resources to competitive ability over defense (Blossey and Notzold, 1995). Similarly, the allelopathic advantages against resident species (AARS) hypothesis expands on the idea of novel weapons, suggesting that novel weapons which increase plant competitive ability are selected for in the invasive range (Callaway and Ridenour, 2004). All these hypotheses are supported at least in part by data from field experiments which are often coupled with

physiological and biochemical studies; however, it remains unclear why some plants become problematic invaders and others do not. One aspect that is rarely investigated in relation to invasive weeds and their native counterparts is the potential for modified gene regulation in the introduced range. As limited genetic resources are available for most invaders and other weedy species (Basu et al., 2004), defense and growth response genes cannot be effectively monitored at the molecular level to test hypotheses of plant invasion.

Centaurea maculosa Lam. (spotted knapweed) is a Eurasian native that has become a particularly problematic invasive weed in the northwestern United States, infesting over 4.5 million acres in Montana alone (Mauer T, 2001). Spotted knapweed often colonizes disturbed areas in North America, but also invades rangelands, pastures and prairies, where it displaces native species and establishes dense monocultures. Diploid and tetraploid forms of the weed exist in the native range, but only tetraploid plants have been identified in North America (Ochsmann, 2001). The diploid form contains 18 chromosomes (Powell et al., 1974), with a DNA content (2C) near 3.6 picograms based on measures from closely related species of the same chromosome number (Grime et al., 1985). This translates to an estimated genome size of approximately 1,800 Mbp. Molecular markers and karyotyping have been used to identify the two forms, as it is impossible to distinguish between them based on morphological characters (Ochsmann, 2001). Both forms are short-lived outcrossing perennials of the aster family; however, the diploid is monocarpic while the tetraploid is polycarpic. Also, the tetraploid can tolerate dense vegetation and thus may be a better competitor than the diploid. This is presumed to be the main reason why only the tetraploid form is invasive in North America, while it is not considered invasive or even

predominant in its native Eurasian habitat (Ochsmann, 2001). Ecological and greenhouse investigations suggest that invasive *C. maculosa* is a strong competitor against North American natives, even in the presence of biocontrol agents that have been introduced to limit the spread of the weed (Callaway et al., 1999; Bais et al., 2002; Bais et al., 2003; Weir et al., 2003; Perry et al., 2005). Also, common garden studies suggest that North American *C. maculosa* seeds germinate more readily than their Eurasian counterparts and the resulting individuals are larger, more robust, and better able to fend off and compensate for herbivore attack, under both greenhouse and field conditions (Ridenour *et al.*, unpublished data). These studies give partial support to the EICA hypotheses. There is also evidence that *Centaurea* species (*C. maculosa* and *C. diffusa*) produce allelopathic compounds that inhibit growth and germination of North American plant species more severely than their native congneners, lending support to the novel weapons hypothesis (Callaway and Aschehoug, 2000; Bais et al., 2002; Bais et al., 2003; Callaway and Ridenour, 2004), although the production of allelochemicals seems to be variable (Blair et al., 2005). It is not clear what combination of effects cause *C. maculosa* to become invasive in North America.

As an initial step towards understanding the molecular mechanisms by which plants become invasive, we have generated a normalized Expressed Sequence Tag (EST) library representing seven invasive populations of *C. maculosa*. Here we describe candidate genes that could be utilized in future experiments to correlate plant gene expression and ecological hypotheses proposed for invasive success. To the best of our knowledge, this is the first published set of ESTs derived from an invasive weed that will be targeted to study invasive behavior.

2.3 MATERIALS AND METHODS

PLANT MATERIAL

Seeds from seven invasive populations of *Centaurea maculosa* Lam were obtained from Ray Callaway (University of Montana, Missoula). Five populations originated from Montana, one from Washington, and one from Virginia. Six seeds from each of the seven populations were sterilized by heating at 50° C for ten minutes in distilled water. Seeds were cooled to ambient temperature, rinsed with sterile water and placed in Petri dishes containing moist germination paper. Plates were wrapped in parafilm and placed in a growth chamber with a photoperiod of 20 hours light/six hours dark at a constant temperature of 25° C. Upon the emergence of cotyledons, seedlings were planted in 2.5 cm pots in a mix of 70% sand, 10% perlite, and 20% autoclaved potting soil and transported to the greenhouse. Pots were placed in a flat and covered with plastic wrap for approximately one week until seedlings became established. Plants were given sufficient water and fertilized once per week with a dilute solution of Miracle Gro (Maryville, OH). After approximately two months, two to three plants per population were removed from pots and their roots were washed to remove soil particles. All plants were in the form of small rosettes, lacking stem and floral tissue. Entire plants, including roots, were wrapped in foil, frozen in liquid nitrogen, and stored at -80° C until processing.

CREATION OF NORMALIZED cDNA LIBRARY

Plant tissue was shipped in dry ice to Agencourt Bioscience Corporation (Beverly MA). Total RNA was extracted and optimized first strand cDNA synthesis was performed using a primer adapted with a rare enzyme cut site. cDNA fragments were size-selected by agarose gel electrophoresis, and directionally cloned into a pAGEN-1 vector. A positive control containing the Tet^R gene was used during construction of the primary library to ensure library quality. Single-stranded DNA was made from a portion of the primary library by phagemid production, and reactions were treated with DNase I to ensure the removal of double-stranded DNA. A second portion of the primary library was linearized and transcribed into anti-sense RNA with biotinylated dNTPs. Oligo dT and primer extension were used to pre-block the poly-A region prior to hybridization. The anti-sense RNA and single-stranded circular DNA were hybridized, and abundant clones were removed using streptavidin. To reduce the amount of empty vectors, a Not1 oligo and *Taq* polymerase were used to synthesize double stranded DNA from the single stranded normalized library prior to final transfection.

The normalized library was plated and 4969 clones were randomly selected for sequencing. Automated plasmid purification was achieved using the SPRI (SprintPrep™) technique, which harvests plasmid DNA directly from lysed bacterial cultures, trapping both plasmid and genomic DNA to functionalized bead particles and selectively eluting only the plasmid (Beckman Biomek FX robots and CCS Packard DNATraks).

SEQUENCING REACTIONS

DNA templates were sequenced in 384-well format using BigDye® Version 3.1 reactions on ABI3730 instruments at Agencourt Biosciences. Thermal cycling was performed using 384-well Thermal cyclers (ABI, MJ Research). Sequencing Reactions were purified using Agencourt's CleanSeq® dye-terminator removal kit. All reads are processed using Phred base calling software and constantly monitored against quality metrics using the Phred Q20. The quality scores for each run were monitored through the Oracle 9i driven Laboratory Information Management System (LIMS). *C. maculosa* ESTs were trimmed of vector sequence and the data was transferred to a secure site for download.

SEQUENCE ANALYSIS

To determine the number of unique transcripts in the library, an in-house pipeline program was used to cluster and assemble the trimmed EST sequences. The pipeline essentially utilizes TIGR Assembler with its default parameters (overlap of at least 40bp with 94% identity). The PLAN web system (Personal BLAST Navigator, Nobel foundation) was used to do a BLASTX search against the non-redundant protein (NR) database for functional annotation, and gene ontology (GO) sequence database for functional categorization (He et al., 2007). The BLASTX search considered translation of the assembled consensus (unigenes) in multiple reading frames. The top NR hit for each unigene sequence (E-value 10^{-4} or less) and top hits from GO assignment were deposited in PLAN and can be searched by keyword or unigene accession number (PLAN Project 30060, CENT_UG_00001-CENT_UG_04423). GO annotation was used

to categorize unigenes into functional categories by molecular function, cellular component and biological process. A customized in-house program was used to count the number of unigenes being grouped under different GO term categories in a hierarchical fashion (He et al., 2007). The vector trimmed EST sequences have also been deposited in GenBank (accession numbers EL930664-EL935630).

2.4 RESULTS AND DISCUSSION

LIBRARY CREATION AND SEQUENCE ANNOTATION

Seeds from seven invasive populations of *Centaurea maculosa* Lam were grown for one to two months, at which time two to three entire plants per population were harvested. Tissue was frozen in liquid nitrogen and shipped to Agencourt biosciences for normalized cDNA library construction. Single pass directional sequencing was performed on 4969 randomly selected clones from the *C. maculosa* normalized cDNA library (GenBank accessions EL930664-EL935630). These sequences were assembled into 4423 unique contigs (contiguous consensus sequences) or "unigenes" using Noble Foundation's in-house pipeline based on TIGR Assembler (He et al., 2007).

The library consisted of 894 contig-forming ESTs which created 348 unigenes and 4075 singlet ESTs, each representing a unique sequence. Of the 348 unigene contigs, the majority contained only two EST sequences (Figure 2.1). The largest unigene contig, which had high similarity to a chlorophyll a-b binding protein, contained 17 ESTs. Interestingly, the second largest unigene contig, a compilation of ten sequences, had extremely low similarity to known sequences and was not able to be annotated. Other large unigene contigs were annotated as polyphenol (catechol) oxidase, chloroplast ATP

synthase, photosystem I subunits II and XI, polygalacturonase (a pectinase), and the small subunit of RUBISCO. Normalization should remove most redundant transcripts and enrich for low abundant regulatory genes in the library. However, it is interesting that one of the most abundant transcripts found, polyphenol oxidase, is a potential defense-related protein (Shi et al., 2001).

Overall redundancy of the library was 18% (number of clustered ESTs/total ESTs) which suggests that the normalization process was effective, and that continued sequencing of the library has the potential to uncover many more unique transcripts. Sequence quality was high; over 80% of the unigenes were between 800-1100 bp in length (Figure 2.2), with an average size of 784 bp.

To annotate the *C. maculosa* ESTs, the 4423 unigenes were translated in all frames and searched for similarity against the NCBI non-redundant protein database using BLASTX (E-value of 10^{-4} or less). Of the entire unigene set, 77% (3392) had significant similarity to genes in the NCBI database, while the remaining 1031 sequences had low similarity and were not able to be annotated. In the group of annotated sequences, 35% (1177 unigenes) had top BLAST hits to transcripts from *Arabidopsis thaliana*, whereas only 10% (338 unigenes) had top hits to *Orzya* cultivars. Taxonomically, 64% (2182) of the annotated unigene top hits grouped into the rosids clade (which includes the families Brassicacea and Fabaceae), 20.6% grouped into the asterids clade (which includes the families Asteraceae and Solanacea), and 11.8% grouped into the commelinids clade (which includes members of the Poaceae family and other monocots) (Figure 2.3). Thirty four unigenes had top hits to non-plant sequences. Most of these non-plant sequences were annotated as hypothetical or unknown proteins.

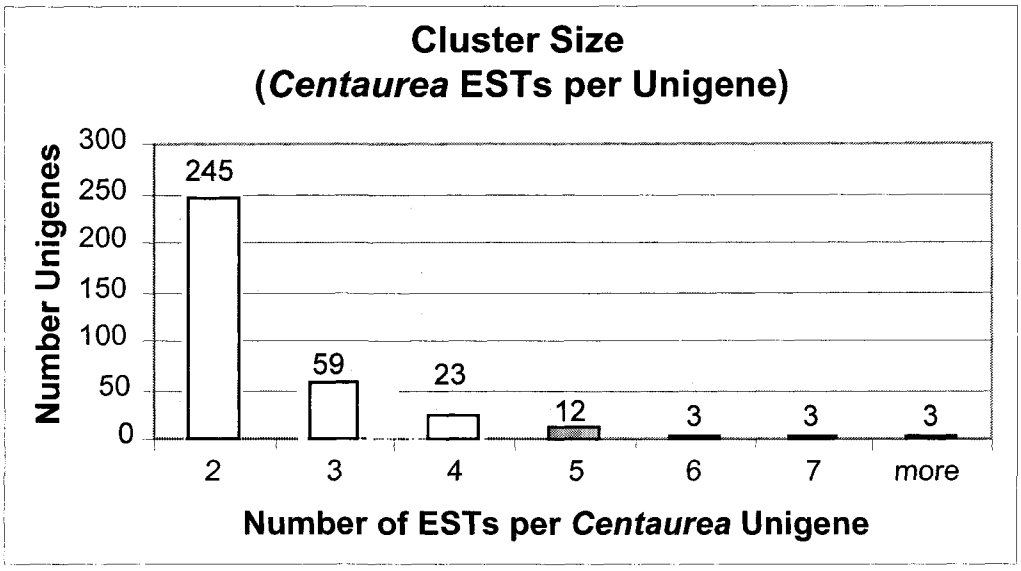

Figure 2.1. Distribution of assembled *Centaurea* ESTs by cluster size. The 4969 *Centaurea* ESTs were assembled into 4423 unique contigs or 'unigenes' using the PLAN database (Nobel foundation). In total, 4075 singlet ESTs were unique (not pictured on graph); 348 could be assembled into clusters containing one or more *Centaurea* unigene, and were plotted relative to their abundance in the EST library.

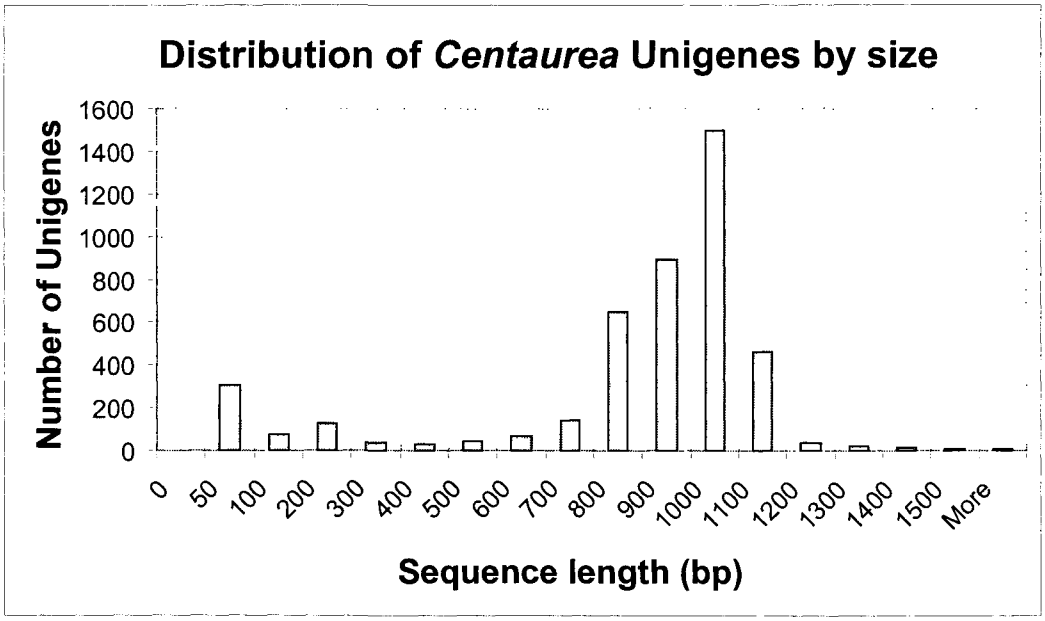

Figure 2.2. Distribution of *Centaurea* unigenes by sequence length. The 4423 *Centaurea* unigenes were plotted by their relative abundance based on sequence length in base pairs.

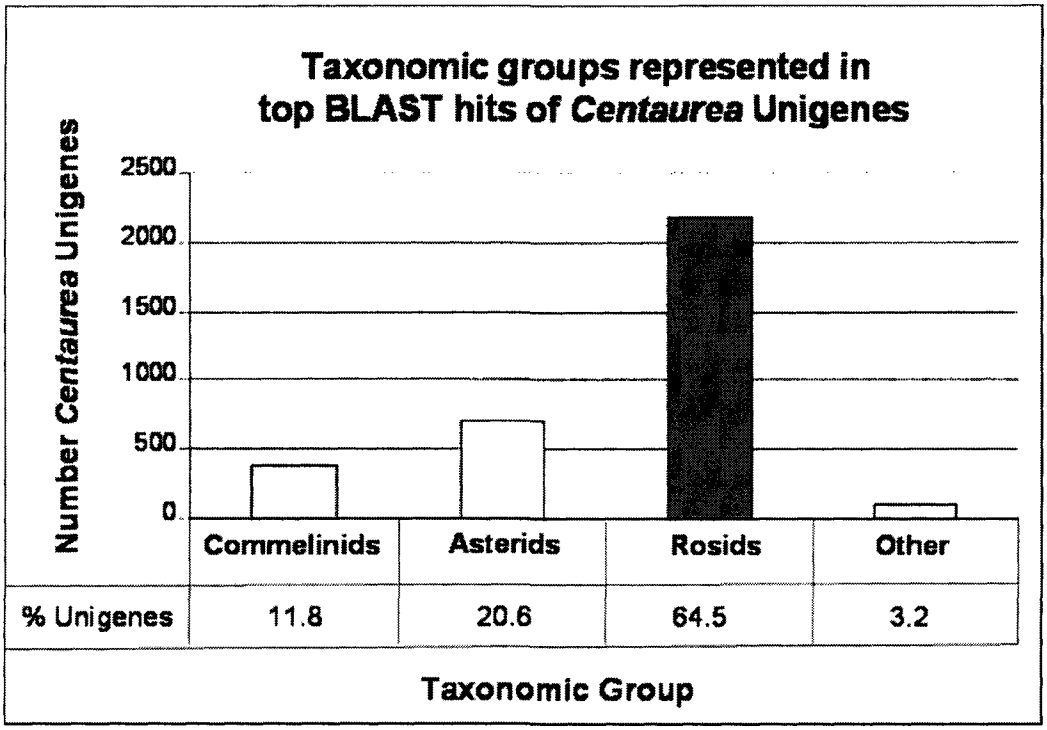

Figure 2.3. Taxonomic clades associated with *Centaurea* unigene top BLAST hits. *Centaurea* unigenes were used to query BLASTX nr database, and the top hit for each unigene was deposited in the PLAN database (3392 unigenes had significant top BLAST hits, others were unable to be annotated). These top hits were assembled by taxonomic group. Approximately 35% of the unigenes had top hits to *Arabidopsis*, which is part of the Rosid clade.

FUNCTIONAL CATEGORIZATION OF CENTAUREA UNIGENES

Gene ontology (GO) assignment programs were used to functionally categorize unigenes in the library. Unigene GO terms were counted and grouped in a hierarchical fashion into the major GO functional categories of Cellular Component, Biological Process and Molecular Function. The GO categories of cellular component and biological process contained over 3000 *Centaurea* unigene annotations, whereas the molecular function category contained only 2162 annotations. Each category contained approximately 16% unigenes that were annotated as "unknown," but this number does not account for the unigenes that were unable to be annotated in GO format (Figure 2.4).

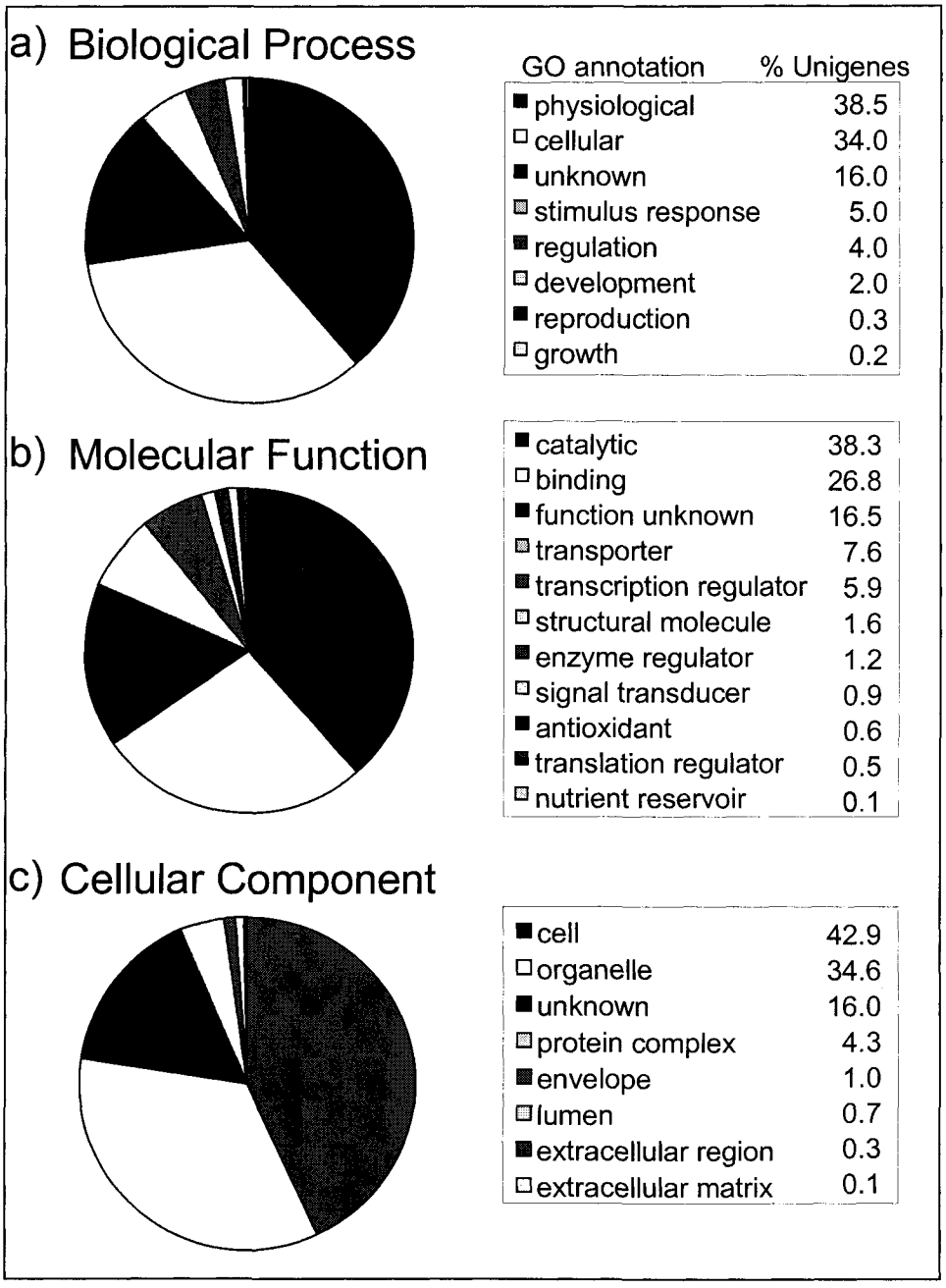

Figure 2.4. Gene Ontology annotation of *Centaurea* Unigenes. A normalized cDNA library was created from whole plants representing seven invasive populations of *Centaurea maculosa.* Five thousand ESTs were sequenced from the 5' end (Agencourt biosciences), and assembled into 4,423 contigs, or '*Centaurea* 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 were able to be annotated by GO programs. Computational analysis was done using the PLAN database (Noble Foundation).

Approximately 38% of the unigene annotations were grouped into the 'physiological process' category of the Biological Process GO (Figure 2.4), which includes subcategories such as metabolism, transport, photosynthesis, apoptosis, and homeostasis. The next largest category of unigene annotations (~34%), 'cellular process,' has some overlap of subcategories with physiological processes (i.e., metabolism, transport), but includes unique subcategories such as cell communication, recognition, and differentiation. Five percent of unigene annotations fell in the 'stimulus response' category which includes subcategories that relate to plant response to abiotic and biotic stresses, such as pathogen attack. Thirty unigenes were subcategorized as responding to hormone stimulus, and most of these fell into the ethylene and jasmonic acid signaling pathways. Ethylene- and jasmonic acid-mediated pathways have been implicated in the defense of plants against pathogens and insects (Ecker and Davis, 1987; Kunkel and Brooks, 2002), and these transcripts may be up-regulated in *C. maculosa* under biotic stress conditions. Only a small percentage of unigene annotations fell into the reproduction category of biological processes (0.3%), but this is not entirely surprising, as reproductive structures such as flowers were not used in the starting material for the library.

Over 38% (829) of the unigene annotations fell into the 'catalytic activity' category of the molecular function GO (Figure 2.4). Specific catalytic activities associated with these unigenes covered a range of GOs, with the largest amount of unigenes falling into the transferase and hydrolase categories (282 and 266 unigenes, respectively). These types of enzymes are involved in many intracellular processes including primary and secondary metabolism, signal transduction, and post translational

modification of proteins. The next largest category under molecular function was 'binding' (~27%, 580 unigenes) with the majority of unigenes being associated with nucleotide/nucleic acid binding (134 and 230 unigenes, respectively).

Transporters accounted for nearly 8% of all unigene annotations, the majority being ion transporters and transporters with carrier activity (54 and 48 unigenes). Eighteen unigenes fell into the 'ATP-ase coupled transporter' category, which includes transporters of xenobiotics, steroids, sugars, peptides and other small molecules. However, most unigenes could not be subcategorized with a specific transport role. Uptake and translocation of nutrients in plants differ, and regulated expression of specific transporters may allow increased competitive ability in different situations (e.g., increased expression of metal transporters may be beneficial in metal-limiting environments) (Lasat et al., 2000; Bereczky et al., 2003; Meerts et al., 2003). Depending on the specific transporter, these genes could be interesting targets for understanding root exudation or other release strategies for *Centaurea* secondary metabolites and uptake of nutrients, and may aid in understanding the role of transport in competitive ability of *Centaurea*.

One hundred twenty-seven unigene annotations were designated as transcriptional regulators, with the majority (118) being subcategorized as having 'transcription factor activity.' Transcription factors are responsible for modulating cellular responses to biotic and abiotic stimuli (Singh et al., 2002), and they may play important roles in plant invasion by up- or down-regulating the expression of genes involved in defense and growth responses. Transcription factors identified in the *Centaurea* library made up ~6% of the GO annotations for molecular function, whereas ~4% of the *Arabidopsis* genome

sequences are annotated as transcription factors (The *Arabidopsis* Information Resource, TAIR).

Over two-thirds of the unigenes were localized by cellular component to either cell or organelle, as shown in Figure 2.4. Of these, 164 unigenes were assigned to the nucleus, 357 to the mitochondrion, 52 to the chloroplast, and 37 to the cytosol. Thirty-five unigenes were assigned to the cell wall category and 290 unigenes were assigned to the membrane category, although only 27 could be further categorized to the plasma membrane. These membrane proteins may be interesting targets to investigate transport and/or signal transduction.

A wide variety of functional categories were represented in the *Centaurea* library. Many of these unigenes could be used as candidates for production of a microarray to visualize changes in global gene expression, or to look at more specific changes in regulation related to plant defense or stress.

UNIGENE CANDIDATES FOR TESTING ECOLOGICAL HYPOTHESES

Evolution and Plasticity

The EICA hypothesis suggests that when plants are introduced into a new range, they escape their enemies and rapidly evolve to put more resources into growth/reproduction and less into defense (Blossey and Notzold, 1995). Evolution through random mutation, movement of transposable elements, and genetic recombination may facilitate changes in plant genes or gene expression which give them a competitive and evolutionary advantage (Mooney and Cleland, 2001). In addition, novel environments can reveal genetic variants in a population that possess advantageous

phenotypes due to adaptive or developmental plasticity (Queitsch et al., 2002). Candidate unigenes from the *Centaurea* cDNA library potentially involved in genome evolution and plasticity are described below.

Mobile elements. Mobile, or transposable elements (transposons) have the ability to modify DNA sequences by 'jumping' in and out of places in the genome, and certain mobile elements carry gene fragments that, when transposed, lead to repetition or creation of new genetic material (Kazazian, 2004). Mobile elements can modify genome size, gene regulation, and gene function, all of which contribute to genome evolution (Kazazian, 2004; Morgante, 2006). In the invasive *Centaurea* cDNA library, six transposable element-related unigenes were identified (Table 2.1). Normally transposable elements are found in non-coding regions of their host genomes and are considered 'silent;' however, expressed transposable elements, such as those found in the *Centaurea* cDNA library, have been detected in plants at specific growth stages and under biotic stress conditions such as pathogen attack and wounding (Takeda et al., 1998; Kimura et al., 2001; Kwon et al., 2005). Evidence from such experiments support Barbara McClintock's idea that transposable elements may play a role in genome evolution through organismal adaptation to stress (McClintock, 1984), and it would be interesting to test this idea in relation to plant invasion.

Heat-shock proteins. Heat shock protein 90 (Hsp90), a stress-induced protein, has been shown to buffer genetic variation in morphogenic pathways in the fruit fly *Drosophila melanogaster* and the cruciferous plant *Arabiodpsis* (Queitsch et al., 2002).

As a chaperone of proteins that regulate growth and development, Hsp90 may allow for the storage and release of genetic variation, as well as allowing phenotypic plasticity in an organism's response to their environment (Queitsch et al., 2002; Liu et al., 2004). Eight heat shock-related unigenes were identified in the *Centaruea* cDNA library (Table 2.1). One unigene is closely related to Hsp90, one to Hsp60, one to Hsp70, and the others are annotated as 'putative heat-shock proteins' or 'heat shock factors.' Only two of these unigenes have top BLAST hits to sequences from *A. thaliana*, suggesting there may be a wide diversity of Hsp90 related sequences in *Centaurea*. Understanding the mechanism of these proteins in relation to plant genotype, environment and defense response throughout the native and invasive range of *C. maculosa* may give some clues to the plasticity of the species.

Table 2.1. Evolution/Plasticity-related sequences in *Centaurea* cDNA library. Evolution-plasticity related sequences from the *Centaurea* cDNA library are represented by *Centaurea* unigene identification number. Accession number, organism, functional description, and E value of the top BLAST hit for each unigene is listed.

Centaurea ID	Top BLAST hit ID	Organism	Function	E value
(A) Mobile element-related sequences				
CENT_UG_01296	AAX92763	*O. sativa*	transposon protein, putative	3e-78
CENT_UG_02640	ABA99201	*O. sativa*	transposon protein, putative, mutator sub-class (class II)	7e-21
CENT_UG_00681	ABB46630	*O. sativa*	transposon protein, putative, CACTA, En/Spm sub-class (CATCA)	8e-55
CENT_UG_03112	BAA22788	*V. faba*	retrotransposon-like gene (class I)	2e-06
CENT_UG_03716	AAX92941	*O. sativa*	retrotransposon protein, putative, Ty1-copia sub-class (class I)	8e-13
CENT_UG_01089	ABA97402	*O. sativa*	retrotransposon protein, putative, unclassified (class I)	3e-15
(B) Heat-shock-related sequences				
CENT_UG_03940	AAR12194	*N. benthamiana*	Molecular chaperone Hsp90-2	2e-98
CENT_UG_03246	AAF23074	*T. aestivum*	heat shock protein 70	4e-99
CENT_UG_00169	AAL38353	*A. thaliana*	putative heat-shock protein	1e-82
CENT_UG_01316	AAM67147	*A. thaliana*	putative heat shock protein	2e-38
CENT_UG_01530	AAN63805	*P. dulcis*	heat shock protein 60	4e-111
CENT_UG_00637	AAS57912	*V. radiata*	70 kDa heat shock cognate protein 1	8e-74
CENT_UG_00352	BAA83710	*N. tabacum*	heat shock factor	8e-44
CENT_UG_02194	CAA47345	*P. vulgaris*	70 kDa heat shock protein	9e-100

Secondary metabolism

As explained above the AARS hypothesis proposes that novel weapons, often in the form of secondary metabolites, are selected for in invasive plants in the introduced ranges. Members of the aster family are capable of synthesis of a broad spectrum of secondary metabolites that may aid in basal and induced defense response, as well as in competition against other plants (Bais et al., 2003; Benderson, 2003). Included in the list of secondary metabolites synthesized by *Centaurea spp.* are polyacetylenes and related thiophenes, flavonoids (flavones and flavonols and their derivatives in particular) and their glycosides, phenolics and lignans, coumarins, anthocyanins, cyanogenic glycosides (prunasin), mono-, sesqui-, di- and tri-terpenoids (with sesquiterpene lactones particularly diverse), and steroidal compounds (Benderson, 2003). Described below are several *Centaurea* unigenes which share sequence similarity with characterized genes involved in plant secondary metabolite biosynthesis. Further study of these candidate genes may aid in understanding the relative influence of AARS in *Centaurea* invasion.

Sesquiterpene lactones. *C. maculosa* is known to accumulate the sesquiterpene lactone, cnicin, at concentrations approaching 2% of dry weight on the leaves of the inflorescence stem (Locken and Kelsey, 1987). This compound is thought to act as a protectant against herbivory of generalist herbivores and acts as an oviposition stimulant for specialist herbivores (Landau et al., 1994) including *Agapeta zoegana*, a biological control agent introduced for the control of *C. maculosa* in the North America. Additionally, cnicin is phytotoxic to several plant species (Kelsey and Locken, 1987), appears to inhibit the rumen microbial activity of sheep (thereby reducing digestibility of

C. maculosa (Olson and Kelsey, 1997)), possesses broad spectrum anti-fungal activity (Panagouleas et al., 2003), and is being examined as a potential pesticide for control of formosan termite (Meepagala et al., 2006). Sesquiterpenes are synthesized through cyclization of farnesyl pyrophosphate (FPP) followed by further modification steps including oxidation, reduction, and glycosylation reactions. The genes responsible for the committed step that catalyzes the conversion of FPP to sesquiterpene hydrocarbons are well-characterized, including sesquiterpene synthases from aster family members such as *Artemisia annua*, *Solidago canadensis*, *Helianthus annuus*, *Ixeris dentata*, *Chicorium intybus*, and *Lactuca sativa*. In the *Centaurea* cDNA library, no unigenes were annotated as a sesquiterpene synthase. However, several unigenes revealed high sequence simlarity to genes involved in the synthesis of the iosprenoid pyrophosphates, metabolic precursors to the terpenoids. BLAST similarity searches revealed two unigenes which closely matched the *Antirrhinum majus* geranyl diphosphate synthase, which catalyzes the condensation of two isopenyl pyrophosphate units to form a ten-carbon precursor (geranyl pyrophosphate - GPP) of monoterpenoids. GPP can then be extended further to FPP, geranylgeranyl pyrophosphate (GGPP), and ultimately to dolichol phosphate, a polyprenoid involved in the formation of glycoproteins via the endomembrane system. Two unigenes were annotated as dehydrodolichol phosphate synthases by GO annotation.

Following formation of the ring structure, sesquiterpene skeletons can be modified through oxidation, reduction, and glycosylation reactions to form an enormous diversity of secondary products including cnicin, a sesquiterpene lactone found in *C. maculosa*. Sesquiterpene lactones commonly occur in the Asteraceae, but the

biosynthetic routes for individual metabolites are relatively poorly characterized. One of the most well-characterized of the biosynthetic pathways leading to sesquiterpene lactones is that for artemisinin, a compound of value as an anti-malarial drug (Towie, 2006) isolated from the aster *Artemisia annua*. Recently the oxidation steps which generate artemisinic acid, a precursor of artemisinin, were characterized and the entire pathway to artemisinic acid reconstructed in yeast (Ro et al., 2006). The reaction proceeds from FPP to the sesquiterpene, amorphadiene (catalyzed by amorphadiene synthase), and then to artemisinic acid, a reaction catalyzed by a single p450 enzyme which performs a three-step oxidation reaction to form a carboxylic acid. A unigene from the *Centaurea* library (CENT_UG_03500) demonstrates 93% amino acid sequence identity to this three-step oxidase and a nearly identical gene from the related *A. obtusifolia* (Figure 2.5), highly suggestive of a role in the biosynthesis of the cnicin or other sesquiterpene lactones of *C. maculosa*.

```
Arob            MALSLTTSIALATILLFVYKFATRSKSTKKSLPEPWRLPIIGHMHHLIGTTPHRGVRDLA 60
Aran            MALSLTTSIALATILLFVYKFATRSKSTKKSLPEPWRLPIIGHMHHLIGTTPHRGVRDLA 60
Cm03500         ---------------------------------RLPIIGHMHHLIGTMPHRGVMDLA 24
                : : :   : :  :::::  :          :    ************** ***** ***

Arob            RKYGSLMHLQLGEVPTIVVSSPKWAKEILTTYDITFANRPETLTGEIVLYHNTDVVLAPY 120
Aran            RKYGSLMHLQLGEVPTIVVSSPKWAKEILTTYDITFANRPETLTGEIVLYHNTDVVLAPY 120
Cm03500         RKYGSLMHLQLGEVSTIVVSSPKWAKEILTTYDITFANRPETLTGEIIAYHNTDIVLAPY 84
                **************.*******************************: *****:*****

Arob            GEYWRQLRKICTLELLSVKKVKSFQSLREEECWNLVQEIKASGSGRPVNLSENIFKLIAT 180
Aran            GEYWRQLRKICTLELLSVKKVKSFQSLREEECWNLVQEIKASGSGRPVNLSENIFKLIAT 180
Cm03500         GEYWRQLRKLCTLELLSVKKVKSFQSLREEECWNLVQEVKASGSGRPVDLSENIFKMIAT 144
                *********:****************************:*********:*******:***

Arob            ILSRAAFGKGIKDQKELTEIVKEILRQTGGFDVADIFPS-KKFLHHLSGKRARLTSLRKK 239
Aran            ILSRAAFGKGIKDQKELTEIVKEILRQTGGFDVADIFPS-KKFLHHLSGKRARLTSLRKK 239
Cm03500         ILSRAAFGKGIKDQKEFTEIVKEILRQTGGFDVADIFPS-KKFLHHLSGKRARLTSIHKK 203
                ****************:********************** ***************::**

Arob            IDNLIDNLVAEHTVNTSSKTNETLLDVLLRLKDSAEFPLTSDNIKAIILDMFGAGTDTSS 299
Aran            IDNLIDNLVAEHTVNTSSKTNETLLDVLLRLKDSAEFPLTSDNIKAIILDMFGAGTDTSS 299
Cm03500         LDNLINNIVAD-------------------------------------------------- 214
                :****:*:**:  :         :: ::: :     : :    :  ::: ::
```

Figure 2.5. Alignment of *Centaurea* unigene (CENT_UG_03500) and related sequences. Sesquiterpene lactone synthesis proteins from *Artemisia obtusifolia* and *A. annua* were aligned with *Centaurea* unigene 03500 using (Clustal W). Stars **(*)** indicate complete sequence conservation; **(:)** represents amino acids of a similar nature.

Acetylenes. The polyacetylelenes are secondary metabolites derived from fatty acids, and are characteristic of many aster genera. Acetylenes contain highly reduced carbon-carbon triple bonds. The biosynthetic route has been only recently characterized for a small group of acetylenes (Cahoon et al., 2003). Alignment of established acetylenases, Δ^{12} oleic acid desaturates, and *C. maculosa* unigenes annotated as desaturases reveals three *C. maculosa* ESTs which cluster closely to *H. annuus* acetylenase, but are distinct from *H. annuus* Δ^{12} oleic acid desaturates (Figure 2.6), suggesting that these genes may be involved in acetylene production in *C. maculosa.*

Flavonoids. The basic flavonoid pathway is the best-characterized metabolic pathway of plant secondary metabolism (Winkel-Shirley, 2001). More than 800 flavonoid structures have been characterized from the Asteraceae, in the Cardueae tribe, which contains *Centaurea spp.*, that are particularly rich in hydroxy-methylated flavonols and flavones (Emerenciano et al., 2001). The early steps of the pathway involve the generation of phenylpropanoid monomers which are condensed with three malonyl CoA units to form the chalcones, followed by isomerization, hydroxylation, methylation, glycosylation, and polymerizations steps (Winkel-Shirley, 2001). Many steps of the general flavonoid pathway are represented in the *Centaurea* cDNA library, with clones that show similarity to most characterized enzymatic functions (Table 2.2). However, no unigenes showed significant sequence similarity to flavonol synthase (FLS), the gene responsible for generation of the flavonols, or flavone synthase (FS), the gene product of which converts flavanones to flavones. Each of these genes are oxoglutarate-dependent dioxygenases, and several unigenes suggest this function, as revealed by GO annotation.

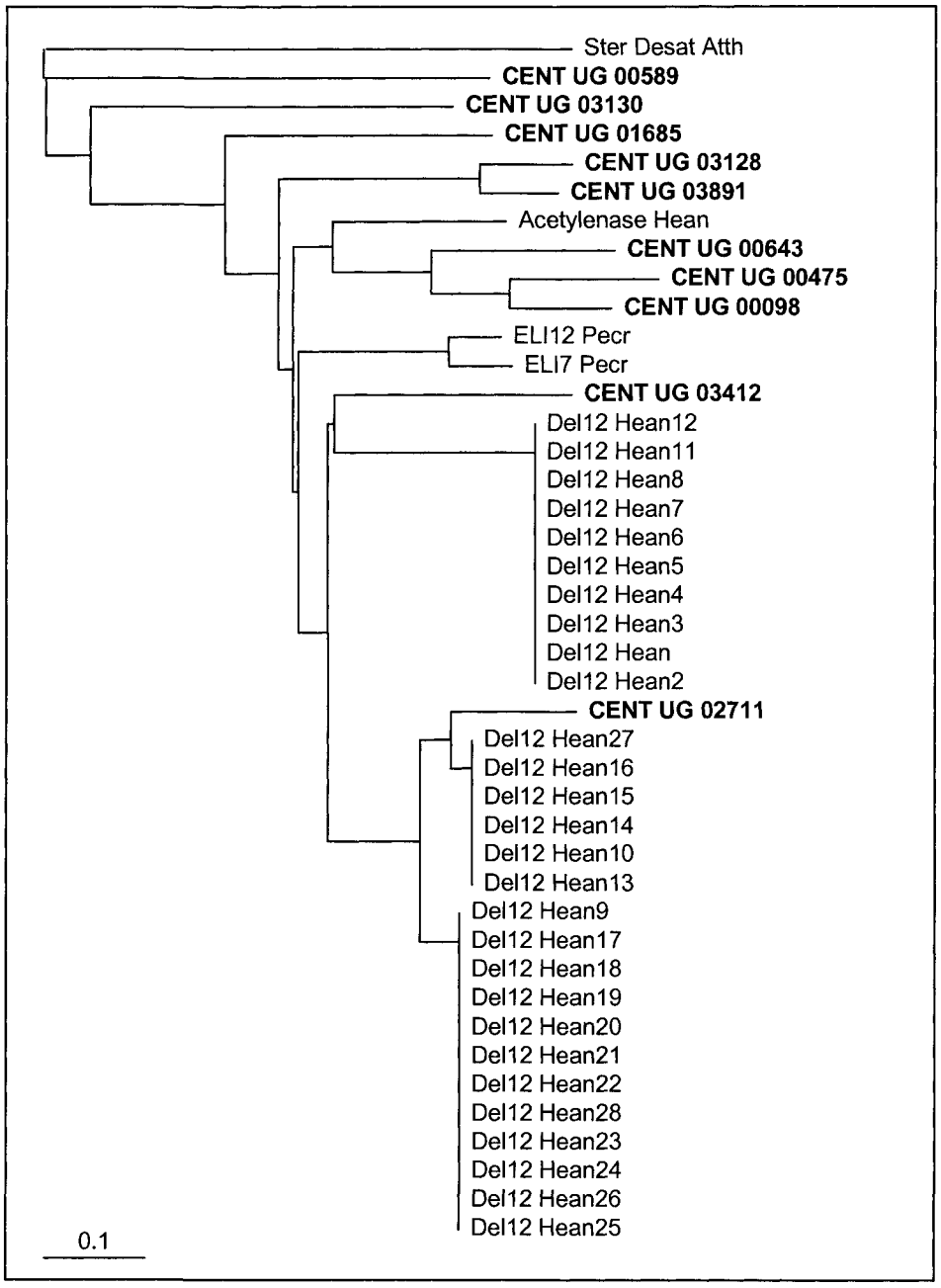

Figure 2.6. Phylogenetic analysis of acetylenease-related sequences from *Centaurea* cDNA library. *C. maculosa* EST sequences with similarity to fatty acid desaturase genes were clustered with previously characterized genes from *Helianthus annuus* (Hean), *Petroselinum crispum* (Pecr) fungal elicited desaturases (ELI), and with sterol desaturasase from *Arabidopsis thalania* as an outgroup. CENT_UG_00643, CENT_UG_00475, and CENT_UG_00098 cluster with the characterized acetylenase from *H. annuus*, distinct from the remaining Δ12 desaturases (Del12), suggesting potential for acetylenase activity.

Further, only two FS clones from Asteraceae members (*Gerbera hydrida* and *Callistephus chinensis*) are reported in GenBank, and only one Asteraceae sequence is annotated as an FLS gene (from *G. hybrida*), suggesting that one or more of the unannotated 2-oxoglutarate-dependent dioxygenases may be FLS or FS genes.

As *C. maculosa* has been reported to exude catechin, a phytotoxic secondary metabolite, from its root (Bais et al., 2002; Bais et al., 2003), the study of genes involved in secondary metabolism in *C. maculosa* may help reveal how this compound and other potential 'novel weapons' are synthesized. The AARS hypothesis suggests that plants may evolve to produce more of the effective 'weapon' compounds when in the invaded environment (Callaway and Ridenour, 2004), so it would also be interesting to test the activity of some of these proteins in native and invasive populations.

Table 2.2. Flavanoid Pathway- related sequences in *Centaurea* cDNA library. Proposed function (Func) of flavanoid pathway related sequences in the *Centaurea* cDNA library; PAL (phenylalanine ammonia lyase), C4H (cinnamate 4-hydroxylase), C4L (4-coumaryl-CoA ligase), CHS (chalcone synthase), CHI (chalcone isomerase), F3'H (flavanoid 3'-hydroxylase), GT (glycosyl transferase), OMT (O-methyltransferase). The number of unigenes and their identification numbers (PLAN database) are listed for each functional group.

Func	Unigenes (potenial genes)	Unigene IDs from PLAN database (CENT_UG_#####)
PAL	8 (3)	00151, 00435, 00996, 01487, 02666, 03772, 04127, 04157
C4H	1 (1)	00037
C4L	1 (1)	03187
CHS	5 (3)	00019, 00766, 01650, 02133, 02557
CHI	2 (1)	01866, 01424
F3'H	5 (3)	00279, 01543, 02213, 04214, 04285
GT	11 (7)	00763, 01670, 01900, 02397, 02287, 02550, 03048, 03142, 02000, 03287, 03943
OMT	15 (10)	00334, 00392, 00797, 01026, 01428, 01587, 02428, 02877, 02907, 03027, 03144, 03280, 03376, 03404, 04124

Defense-response genes

One of the main predictions of EICA is that plants in their new environment rapidly evolve to put more resources into growth/reproduction and less into defense, as they have escaped their co-evolved pathogens and predators (Blossey and Notzold, 1995). Thus, native plants should show higher levels of basal defense compounds and should out-perform invasives when both are exposed to pathogens from the native environment. The *Centaurea* cDNA library contains a variety of unigenes annotated as defense response genes (Table 2.3), as well as components of signaling pathways that may be involved in defense mechanisms. These include *Centaurea* sequences similar to three lipoxygenase (LOX) proteins, two phenylalanine ammonia lyase (PAL) proteins, and three calmodulin binding proteins. Also identified was a *Centaurea* sequence similar to the *Arabidopsis* activated disease resistance-like (ADR1-like) gene, which contains nucleotide binding site – leucine rich repeat (NBS-LRR) motifs characteristic of defense-response proteins (Chini and Loake, 2005). Four *Centaurea* unigenes show high similarity to another LRR containing *Arabidopsis* transcript (At3g20820) that is suspected to be involved in the defense response signal transduction pathway. The *Centaurea* library contains seven other unigenes, not annotated as "defense related," that contain LRR motifs or leucine zippers (Table 2.3). One *Centaurea* unigene shows similarity to the R gene-mediated disease resistance gene (EDS1) from *Arabidopsis*, which is required for SA accumulation and production of pathogenesis related (PR) proteins (Ochsenbein et al., 2006). Two unigenes show similarity to a PR-1 type protein from *Sambucus nigra*, similar to *Arabidopsis* PR-1-related transcript At4g33720.

Table 2.3. Defense-response-related sequences in *Centaurea* cDNA library. Defense-response-related sequences from the *Centaurea* cDNA library are represented by *Centaurea* unigene identification number (PLAN database). Accession number, organism, functional description, and E value of the top BLAST hit for each unigene is listed. Note; this table is continued on the following page.

Centaurea ID	Top BLAST hit ID	Organism	Function	E value
(A) Defense response-related sequences				
CENT_UG_01551	AT1G55020	*A. thaliana*	LOX1, lipoxygenase; response to wounding, jasmonic acid biosynthesis, response to abscisic and jasmonic acid stimulus, defense response to pathogenic bacteria, incompatible interaction, growth.	7e-90
CENT_UG_02121	AT1G17420.1		LOX3.	8e-11
CENT_UG_00731	AT1G72520.1		lipoxygenase, putative.	9e-90
CENT_UG_04157	AT2G37040.1	*A. thaliana*	Phenylalanine ammonia-lyase 1 (PAL1); defense response, response to wounding, phenylalanine ammonia-lyase activity.	2e-116
CENT_UG_01487				3e-116
CENT_UG_00996				2e-113
CENT_UG_02666				7e-108
CENT_UG_03772				4e-102
CENT_UG_04127				2e-24
CENT_UG_00435	AT3G53260.1		PAL2	2e-108
CENT_UG_00151				4e-81
CENT_UG_00144	AT4G02600.1	*A. thaliana*	Seven trans-membrane MLO family protein; calmodulin binding, defense response, cell death.	1e-99
CENT_UG_03728				2e-46
CENT_UG_00973	AT5G65970.1		Calmodulin binding, defense response, cell death.	2e-110
CENT_UG_03454			ATCNGC4 (DEFENSE,	4e-62
CENT_UG_01864	NP_851188 (AT5G54250)		NO DEATH 2, DND2); calmodulin binding, cation channel, cyclic nucleotide binding.	2e-76
CENT_UG_01096	AT3G20820.1	*A. thaliana*	Leucine-rich repeat, plant specific; protein binding, signal transduction, defense response.	4e-84
CENT_UG_00932				1e-78
CENT_UG_01174				5e-76
CENT_UG_03330				2e-74
CENT_UG_00611	AT3G48090.1	*A. thaliana*	Disease resistance protein (EDS1); signal transducer activity, triacylglycerol lipase activity, lipid metabolism, defense response.	4e-22
CENT_UG_00558	AT3G55230.1	*A. thaliana*	Disease resistance-responsive family protein; molecular function unknown, defense response.	8e-10
CENT_UG_03760	AT5G47910.1	*A. thaliana*	Respiratory burst oxidase protein D (RbohD);	2e-110
CENT_UG_02198				1e-65

CENT_UG_01724			oxygen and reactive oxygen species metabolism, defense response	2e-59
CENT_UG_00851 CENT_UG_04111	AT1G58170.1	*A. thaliana*	Disease resistance-responsive protein-related / dirigent protein-related; molecular function unknown, defense response, lignan biosynthesis.	5e-54 1e-53
CENT_UG_02248 CENT_UG_00531 CENT_UG_00565	AT5G04720.1	*A. thaliana*	Disease resistance protein (CC-NBS-LRR class), putative; ADR1-L2, ADR1-LIKE 2, defense response.	3e-65 4e-65 4e-65
CENT_UG_00867 CENT_UG_00804	CAA87071	*S. nigra*	Pathogenesis-related protein, PR-1 type	5e-57 5e-44
CENT_UG_02248	ABA99732	*O. sativa*	Disease resistance, putative	1e-71
CENT_UG_00565	Q9SZA7, AT4G33300	*A. thaliana*	Probable disease resistance protein	3e-65
(B) LRR and leucine zipper domain containing sequences				
CENT_UG_04303	AAL12626	*A. thaliana*	leucine-rich repeat receptor-like kinase F21M12.36	7e-71
CENT_UG_04152	AAM65656	*A. thaliana*	leucine rich repeat protein, putative	1e-88
CENT_UG_01096 CENT_UG_01174	CAE76632	*Cicer arietinum*	leucine rich repeat protein	3e-87 2e-76
CENT_UG_01558	NP_922826	*O. sativa*	putative leucine-rich repeat protein	2e-26
CENT_UG_02258	BAC22512	*Z. elegans*	homeobox leucine-zipper protein	9e-101
CENT_UG_04221	CAA11499	*S. oleracea*	basic leucine zipper protein	2e-20
CENT_UG_01937	CAA64221	*P. brachycarpa*	homeobox-leucine zipper protein	1e-14
(C) WRKY transcription factors				
CENT_UG_02392	AAW67002	*C. annuum*	WRKY transcription factor-c	1e-16
CENT_UG_02512	AAZ99027	*C. annuum*	WRKY-A1244	5e-11
CENT_UG_03094	BAB16432	*N. tabacum*	WRKY transcription factor NtEIG-D48	7e-07
CENT_UG_03349	BAE46417	*S. tuberosum*	Double WRKY type transfactor	1e-64
CENT_UG_02998	AAC49528	*P. crispum*	WRKY3	1e-05
CENT_UG_04064	AAC31956	*P. brachycarpa*	Zince finger protein WRKY1	7e-28
CENT_UG_03882 CENT_UG_00878	NP_564792	*A. thaliana*	WRKY6; transcription factor	3e-61 4e-56
CENT_UG_00803	NP_179913	*A. thanliana*	WRKY15; transcription factor	7e-08
CENT_UG_01139	NP_192034	*A. thaliana*	WRKY22; transcription factor	3e-42
CENT_UG_02403	NP_192845	*A. thaliana*	WRKY41; transcription factor	5e-26
CENT_UG_00385	NP_192354	*A. thalinaa*	WRKY42; transcription factor	6e-46

In addition, twelve of the unigenes annotated as having 'transcription factor activity' in the GO molecular function categorization are similar to WRKY transcription factors (Table 2.3) which may be involved in initial steps of the defense-response signaling pathway (Dong et al., 2003). With this *C. maculosa* sequence information it will be possible to test levels of basal defense responses at the level of gene expression in populations of native and invasive plants. Genes involved only in induced defense response may be under represented in the library, as the plants that were used in library creation did not undergo interspecific competition, pathogen stress, or herbivory and were not grown under field conditions. However, many of the unigenes identified have been implicated in induced defenses in other systems (PR-1 and EDS-like unigenes), and these may be good candidates for studying induced as well as basal defense response. Future experiments can be coupled with more traditional tests of morphology and biochemistry in order to supplement data concerning hypotheses of plant invasion.

2.5 CONCLUSIONS

This is 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 *C. maculosa* 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 *C. maculosa* 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 *C. maculosa* 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 *C. maculosa*, 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.

2.6 REFERENCES

Bais HP, Vepachedu R, Gilroy S, Callaway RM, Vivanco JM (2003) Allelopathy and exotic plant invasion: From molecules and genes to species interactions. Science **301:** 1377-1380

Bais HP, Walker TS, Stermitz FR, Hufbauer RA, Vivanco JM (2002) Enantiomeric-dependent phytotoxic and antimicrobial activity of (+/-)-catechin. A rhizosecreted racemic mixture from spotted knapweed. Plant Physiology **128:** 1173-1179

Basu C, Halfhill MD, Mueller TC, Stewart CN (2004) Weed genomics: new tools to understand weed biology. Trends in Plant Science **9:** 391-398

Benderson W (2003) Natural substances in the compositae: The Bohlmann Files at the BGBM (Freie Universitat Berlin, Botanischer Garten und Botanisches Museum) Berlin-Dahem. *In*, Vol 2007, Berlin

Bereczky Z, Wang HY, Schubert V, Ganal M, Bauer P (2003) Differential regulation of nramp and irt metal transporter genes in wild type and iron uptake mutants of tomato. Journal of Biological Chemistry **278:** 24697-24704

Blair AC, Hanson BD, Brunk GR, Marrs RA, Westra P, Nissen SJ, Hufbauer RA (2005) New techniques and findings in the study of a candidate allelochemical implicated in invasion success. Ecology Letters **8:** 1039-1047

Blossey B, Notzold R (1995) EVOLUTION OF INCREASED COMPETITIVE ABILITY IN INVASIVE NONINDIGENOUS PLANTS - A HYPOTHESIS. Journal of Ecology **83:** 887-889

Cahoon EB, Schnurr JA, Huffman EA, Minto RE (2003) Fungal responsive fatty acid acetylenases occur widely in evolutionarily distant plant families. Plant Journal **34:** 671-683

Callaway RM, Aschehoug ET (2000) Invasive plants versus their new and old neighbors: A mechanism for exotic invasion. Science **290:** 521-523

Callaway RM, DeLuca TH, Belliveau WM (1999) Biological-control herbivores may increase competitive ability of the noxious weed Centaurea maculosa. Ecology **80:** 1196-1201

Callaway RM, Ridenour WM (2004) Novel weapons: invasive success and the evolution of increased competitive ability. Frontiers in Ecology and the Environment **2:** 436-443

Chini A, Loake GJ (2005) Motifs specific for the ADR1 NBS-LRR protein family in Arabidopsis are conserved among NBS-LRR sequences from both dicotyledonous and monocotyledonous plants. Planta **221:** 597-601

Darwin C (1859) On the Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life. John Murray, London

Dong JX, Chen CH, Chen ZX (2003) Expression profiles of the Arabidopsis WRKY gene superfamily during plant defense response. Plant Molecular Biology **51:** 21-37

Ecker JR, Davis RW (1987) PLANT DEFENSE GENES ARE REGULATED BY ETHYLENE. Proceedings of the National Academy of Sciences of the United States of America **84:** 5202-5206

Elton C (1958) The Ecology of Invasions by Animals and Plants. Metheun, London

Emerenciano VP, Militao J, Campos CC, Romoff P, Kaplan MAC, Zambon M, Brant AJC (2001) Flavonoids as chemotaxonomic markers for Asteraceae. Biochemical Systematics and Ecology **29:** 947-957

Grime JP, Shacklock JML, Band SR (1985) NUCLEAR-DNA CONTENTS, SHOOT PHENOLOGY AND SPECIES CO-EXISTENCE IN A LIMESTONE GRASSLAND COMMUNITY. New Phytologist **100:** 435-445

He J, Dai XB, Zhao XC (2007) PLAN: a web platform for automating high-throughput BLAST searches and for managing and mining results. Bmc Bioinformatics **8**

Hierro JL, Maron JL, Callaway RM (2005) A biogeographical approach to plant invasions: the importance of studying exotics in their introduced and native range. Journal of Ecology **93:** 5-15

Kazazian HH (2004) Mobile elements: Drivers of genome evolution. Science **303:** 1626-1632

Kelsey RG, Locken LJ (1987) PHYTOTOXIC PROPERTIES OF CNICIN, A SESQUITERPENE LACTONE FROM CENTAUREA-MACULOSA (SPOTTED KNAPWEED). Journal of Chemical Ecology **13:** 19-33

Kimura Y, Tosa Y, Shimada S, Sogo R, Kusaba M, Sunaga T, Betsuyaku S, Eto Y, Nakayashiki H, Mayama S (2001) OARE-1, a Ty1-copia retrotransposon in oat activated by abiotic and biotic stresses. Plant and Cell Physiology **42:** 1345-1354

Kunkel BN, Brooks DM (2002) Cross talk between signaling pathways in pathogen defense. Current Opinion in Plant Biology **5:** 325-331

Kwon SJ, Park KC, Kim JH, Lee JK, Kim NS (2005) Rim 2/Hipa CACTA transposon display; A new genetic marker technique in Oryza species. Bmc Genetics **6**

Landau I, Mullerscharer H, Ward PI (1994) INFLUENCE OF CNICIN, A SESQUITERPENE LACTONE OF CENTAUREA-MACULOSA (ASTERACEAE), ON SPECIALIST AND GENERALIST INSECT HERBIVORES. Journal of Chemical Ecology **20:** 929-942

Lasat MM, Pence NS, Garvin DF, Ebbs SD, Kochian LV (2000) Molecular physiology of zinc transport in the Zn hyperaccumulator Thlaspi caerulescens. Journal of Experimental Botany **51:** 71-79

Liu YL, Burch-Smith T, Schiff M, Feng SH, Dinesh-Kumar SP (2004) Molecular chaperone Hsp90 associates with resistance protein n and its signaling proteins SGT1 and Rar1 to modulate an innate immune response in plants. Journal of Biological Chemistry **279:** 2101-2108

Locken LJ, Kelsey RG (1987) CNICIN CONCENTRATIONS IN CENTAUREA-MACULOSA, SPOTTED KNAPWEED. Biochemical Systematics and Ecology **15:** 313-320

Mauer T RM, Evans M (2001) Element Stewardship Abstract for Centaurea maculosa. *In*, Vol 2006. The Nature Conservancy, Arlington, VA

McClintock B (1984) THE SIGNIFICANCE OF RESPONSES OF THE GENOME TO CHALLENGE. Science **226:** 792-801

Meepagala KM, Osbrink W, Sturtz G, Lax A (2006) Plant-derived natural products exhibiting activity against Formosan subterranean termites (Coptotermes formosanus). Pest Management Science **62:** 565-570

Meerts P, Duchene P, Gruber W, Lefebvre C (2003) Metal accumulation and competitive ability in metallicolous and non-metallicolous Thlaspi caerulescens fed with different Zn salts. Plant and Soil **249:** 1-8

Mooney HA, Cleland EE (2001) The evolutionary impact of invasive species. Proceedings of the National Academy of Sciences of the United States of America **98:** 5446-5451

Morgante M (2006) Plant genome organisation and diversity: the year of the junk! Current Opinion in Biotechnology **17:** 168-173

Ochsenbein C, Przybyla D, Danon A, Landgraf F, Gobel C, Imboden A, Feussner I, Apel K (2006) The role of EDS1 (enhanced disease susceptibility) during singlet oxygen-mediated stress responses of Arabidopsis. Plant Journal **47:** 445-456

Ochsmann J (2001) On the taxonomy of spotted knapweed (Centaurea stoebe L.) *In* Proceedings from The First International Knapweed Symposium of the Twenty First Century, pp 33-41

Olson BE, Kelsey RG (1997) Effect of Centaurea maculosa on sheep rumen microbial activity and mass in vitro. Journal of Chemical Ecology **23:** 1131-1144

Panagouleas C, Skaltsa H, Lazari D, Skaltsounis AL, Sokovic M (2003) Antifungal activity of secondary metabolites of Centaurea raphanina ssp mixta, growing wild in Greece. Pharmaceutical Biology **41:** 266-270

Perry LG, Johnson C, Alford ER, Vivanco JM, Paschke MW (2005) Screening of grassland plants for restoration after spotted knapweed invasion. Restoration Ecology **13:** 725-735

Pimentel D, Lach L, Zuniga R, Morrison D (2000) Environmental and economic costs of nonindigenous species in the United States. Bioscience **50:** 53-65

Powell AM, Kyhos DW, Raven PH (1974) CHROMOSOME-NUMBERS IN COMPOSITAE X. American Journal of Botany **61:** 909-913

Queitsch C, Sangster TA, Lindquist S (2002) Hsp90 as a capacitor of phenotypic variation. Nature **417:** 618-624

Ro DK, Paradise EM, Ouellet M, Fisher KJ, Newman KL, Ndungu JM, Ho KA, Eachus RA, Ham TS, Kirby J, Chang MCY, Withers ST, Shiba Y, Sarpong R, Keasling JD (2006) Production of the antimalarial drug precursor artemisinic acid in engineered yeast. Nature **440:** 940-943

Shi CH, Dai Y, Xu XL, Xie YS, Xia BL, Liu QL (2001) UV/VIS spectra studies on the polyphenol oxidase II (PPO II) from tobacco. Spectroscopy Letters **34:** 675-683

Singh KB, Foley RC, Onate-Sanchez L (2002) Transcription factors in plant defense and stress responses. Current Opinion in Plant Biology **5:** 430-436

Takeda S, Sugimoto K, Otsuki H, Hirochika H (1998) Transcriptional activation of the tobacco retrotransposon Ttol by wounding and methyl jasmonate. Plant Molecular Biology **36:** 365-376

Towie N (2006) Malaria breakthrough raises spectre of drug resistance. Nature **440:** 852-853

Weir TL, Bais HP, Vivanco JM (2003) Intraspecific and interspecific interactions mediated by a phytotoxin, (-)-catechin, secreted by the roots of Centaurea maculosa (spotted knapweed). Journal of Chemical Ecology **29:** 2397-2412

Winkel-Shirley B (2001) Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiology **126:** 485-493

CHAPTER 3

Plant origin and ploidy influence gene expression and life cycle characteristics in the invasive plant spotted knapweed

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Plant origin and ploidy influence gene expression and life cycle characteristics in the invasive plant spotted knapweed

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AKB: designed and carried out tissue sampling, gene choice, gene expression experiment and data analysis, drafted manuscript
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All authors read and approved the final manuscript.

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3.1 ABSTRACT

Ecological, evolutionary and physiological studies have thus far provided an incomplete picture of why some plants become invasive; therefore we used genomic resources to complement and advance this field. In order to gain insight into the invasive mechanism of *Centaurea stoebe* we compared plants of three geo-cytotypes, native Eurasian diploids, native Eurasian tetraploids and introduced North American tetraploids, grown in a common greenhouse environment. We monitored plant performance characteristics and life cycle habits and characterized the expression of genes related to constitutive defense and genome stability using quantitative PCR. Plant origin and ploidy were found to have a significant effect on both life cycle characteristics and gene expression, highlighting the importance of comparing appropriate taxonomic groups in studies of native and introduced plant species. We found that introduced populations of *C. stoebe* 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. 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. A simulation of seed production over time predicts that introduced tetraploids have the highest fecundity of the three geo-cytotypes. Our results suggest that characterizing gene expression in an invasive species using populations from both its native and introduced range can provide insight into the biology of plant invasion that can complement traditional measurements of plant performance. In addition, these results highlight the importance of using appropriate taxonomic units in ecological genomics investigations.

3.2 INTRODUCTION

Plant invasion into new environments is an extremely costly problem, not only monetarily but also ecologically. Invasive plant infestations reduce biodiversity by displacing native species and can literally destroy some native ecosystems by altering important ecosystem characteristics (Pimental et al., 2002). However, the reasons why some plants remain at low abundance in their home range but become dominant in their new range is not well understood and remains one of the most perplexing questions in ecology. Multiple non-exclusive hypotheses have been proposed to explain plant invasion into new environments (Hierro et al., 2005).

A long standing idea in the field of invasion biology is that of enemy release (Elton, 1958). This hypothesis posits that introduced plants escape their native co-evolved specialist enemies, which allows them to rapidly increase their numbers (Elton, 1958). Blossey and Notzold (1995) proposed the evolution of increased competitive ability (EICA) hypothesis, which builds on the idea of enemy release and has generated much interest in recent years (Blossey and Notzold, 1995). The EICA hypothesis suggests that costly defense against specialists no longer enhances fitness of plants in the introduced range; therefore introduced plants will evolve to put fewer resources into defense allowing them to increase allocation of resources towards growth and reproduction (Blossey and Notzold, 1995). This hypothesis has been supported by experimental evidence, but only in part (Bossdorf et al., 2005). Multiple refinements to the EICA hypothesis have been proposed to account for altered selective pressures in the new environment including the presence of generalist enemies (van der Meijden, 1996;

Muller-Scharer et al., 2004; Joshi and Vrieling, 2005; Muller-Scharer and Schaffner, 2008) and changes in resource availability (Coley et al., 1985; Zhang and Jiang, 2006).

The majority of studies examining EICA and other hypotheses of plant invasion have focused on ecological, physiological and to some extent chemical plant characteristics (Bossdorf et al., 2005; Cipollini et al., 2005; Hierro et al., 2005; Inderjit et al., 2006). However, with the current revolution in genomics technology, the question arises as to whether ecological phenomena such as plant invasion can be better understood by studies of genetics or gene expression profiling. The development of genomics resources for non-model species of invasive weeds is increasingly becoming possible as new technologies become more available and affordable, as demonstrated by Broz et al. 2007 (spotted knapweed) and Anderson et al. 2007 (leafy spurge), aiding in the ability of researchers to investigate the biology of invasive weeds (Anderson et al., 2007; Broz et al., 2007). In regards to ecological hypotheses, it may be particularly useful to characterize expression of genes related to plant defense and competitive ability.

Recently, an EST (expressed sequence tag) library resource was developed for the problematic invasive plant, *Centaurea stoebe* L. (Gugler) Hayek (also known as *C. maculosa* Lam, *C. biebersteinii*, spotted knapweed) (Broz et al., 2007). *C. stoebe*, a native to Eurasia, is able to invade not only ruderal habitats, but also rangelands, pastures and prairies in North America, where it often establishes dense monocultures and excludes native plant species. *C. stoebe* first appeared on both coasts of North America around the late 1800s (Maddox, 1979; Mauer et al., 2001), and has since greatly expanded its range to all but three states in the continental US (United States Department of Agriculture, 2008).

Molecular marker studies revealed relatively large amounts of genetic diversity within and among populations in both the native and introduced ranges (Hufbauer and Sforza, 2008; Marrs et al., 2008), and suggest that this species has been introduced to North America multiple times. Thus, genetic drift resulting from bottle-necks or founder effects does not seem to have played an important role in the invasion success of this weed. Extensive field collections thus far conclude that the native range consists of morphologically indistinguishable diploid (2n = 2x = 18; *C. stoebe* ssp *stoebe*) and tetraploid (2n = 4x = 36; *C. stoebe* ssp *micranthos*) forms of the weed (Ochsmann, 2001) that occasionally occur in mixed stands (Treier et al., in press). In the introduced range, populations had been found to contain the tetraploid form exclusively (Ochsmann, 2001) until a recent extensive survey identified a single mixed stand of diploid and tetraploid plants in western Canada (Treier et al., in press). This suggests that both forms of the weed were introduced, but only the tetraploid has become an invasive problem (Treier et al., in press).

C. stoebe is able to tolerate a wide variety of soil types and precipitation amounts in both Eurasia and North America (Sheley et al., 1998; Ochsmann, 2001). Robust cross-continental comparisons have provided empirical evidence for a niche shift between native and introduced populations (Broennimann et al., 2007), and more recently between native and introduced tetraploid *C. stoebe*, with the invasive tetraploids occurring in drier and warmer climates (Treier et al., in press). Moreover, the range of the native tetraploid in Eurasia has expanded over the range of the native diploid within the past 100-150 years (Ochsmann, 2001), and introduced tetraploids appear to have a higher ecological tolerance, or niche breadth, than either of the native forms (Broennimann et al., 2007;

Treier et al., in press). Thus, the invasive success of *C. stoebe* appears to be partially due to pre-adaptation of the native tetraploid cytotype to drier climates, a trait which has been further selected for in the introduced range (Treier et al., in press). However, more studies are needed to rule out other alternatives related to the weeds invasive success.

Both diploid and tetraploid forms of *C. stoebe* are out-crossing, insect-pollinated asters, but the diploid tends to have a biennial monocarpic life cycle, whereas the tetraploid tends to be a polycarpic perennial, continuing to flower over multiple growing seasons (Muller, 1989; Ochsmann, 2001; Treier et al., in press). Compared to native populations, introduced tetraploids exhibit the highest proportion of polycarpic plants and have the greatest number of stems per plant (Treier et al., in press), which may increase their reproductive capacity. It is hypothesized that this perennial polycarpic life cycle is selected for, particularly in environments lacking natural enemies (Muller-Scharer et al., 2004), which may help explain why the tetraploid form became predominate in the introduced range.

Although there are a small number of studies that examine ploidy differences between native and introduced populations of plants, this factor is most often unaccounted for in ecological studies of invasive weeds (Bossdorf et al., 2005), including *C. stoebe*. Many of the worst weeds are polyploids, and changes in plant ploidy may lead to changes in life history traits, genetic diversity, gene expression or capacity for adaptation and evolution (Soltis and Soltis, 2000). Therefore, in a comparison of plants from both the native and introduced range, it is important to compare the same taxonomic unit (Bossdorf et al., 2005), and understand differences between taxonomic units.

As it appears that both ploidy pre-adaptation (European diploid vs. tetraploid) and selection (European vs. North American tetraploid) may be important factors in *C. stoebe* invasion, we were interested in characterizing the three distinct geo-cytotypes of *C. stoebe*: native diploids, native tetraploids and introduced tetraploids. We grew plants from multiple populations, representing each of the three geo-cytotypes in a common environment and monitored plant performance characteristics and life cycle habits. In addition, we identified gene sequences in the *C. stoebe* EST library that may be involved in constitutive basal plant defense or rapid evolution, as these traits may be important in the plants invasive success. Expression of these genes was characterized in each geo-cytotype using quantitative PCR.

Based on ideas of enemy release and rapid evolution of plants in the introduced range, and on trends in polyploidy, we developed hypotheses concerning plant performance and gene expression of the geo-cytotypes. First, we hypothesized that introduced tetraploids would exhibit reduced expression of constitutive defense and secondary metabolite related genes, but an increase in plant performance when compared to native tetraploids, due to a partial release from enemies. Second, we also expected that genes involved in genome stability would be expressed to a greater extent in introduced versus native tetraploids due to possible novel environmental stresses experienced in the introduced range. Although evolution is predominately thought to be due to random mutations, there is some evidence that expression of transposable elements and DNA repair enzymes influence genetic stability and stress-induced evolutionary strategies in organisms (Kazazian, 2004; Ponder et al., 2005; Morgante, 2006). Therefore, we also assessed transcript accumulation of two active transposable elements and a DNA repair

enzyme, which might facilitate rapid evolution in a new environment. Finally, we hypothesized that native tetraploids would exhibit increased levels of genes involved in secondary metabolite production compared to diploids, due to potential increases in the metabolic activities of polyploids (Dhawan and Lavania, 1996).

3.3 MATERIALS AND METHODS

CENTAUREA FIELD SAMPLING, GREENHOUSE EXPERIMENT AND TISSUE SAMPLE COLLECTION

Field Sampling

Populations of *C. stoebe* were sampled in Eurasia and North America during summer and fall of 2005 using a transect method ((Treier et al., in press) Table 3.1). One fifty-meter-long transect was chosen as the basic sampling unit for each population. Sixteen plants were sampled systematically every three meters (starting at 2.5 m and ending at 47.5 m) along each transect. At each sampling point, seeds were taken from the nearest fruiting plant. For each population, GPS coordinates were recorded. Seeds from each maternal plant were labeled and kept separate. Ploidy was determined for each population by growing four to sixteen seedlings from different parents and analyzing the nuclear DNA content using flow cytometry (Treier et al., in press). Although other populations were collected as part of this larger experiment, only populations that were sampled using the transect method and only those found to have exclusively diploid or tetraploid individuals (not mixed stands) were used in subsequent gene expression analyses. In total, plants of seven diploid and eight tetraploid populations from Eurasia,

and of eight tetraploid populations from North America were utilized; these are referred to as geo-cytotypes (populations listed in Table 3.1).

Table 3.1. Plant origin and ploidy of studied *C. stoebe* populations. Continents: North America (NA) or Eurasia (EU); Ploidy: 4x (tetraploid), 2x (diploid).

Continent	Ploidy	Country or State	Pop	Locality	Longitude	Latitude
NA	4x	Montana	MT 1	Missoula	-114.1008929	46.82048877
NA	4x	Montana	MT 2	Florence, Bitteroot Reserve	-114.1406713	46.58378483
NA	4x	Montana	MT 3	Ross Hole	-113.9748996	45.83464729
NA	4x	Montana	MT 10	Missoula, Blanchard Flat	-113.3832243	46.99937593
NA	4x	Montana	MT 11	Dixon, Moeise	-114.2997544	47.30836457
NA	4x	Oregon	OR 1	Portland, Rivergate	-122.7701958	45.61806134
NA	4x	Oregon	OR 3	Dee Flat	-121.6293944	45.5897611
NA	4x	Oregon	OR 11	Cougar Reservoir	-122.26225	44.15666
EU	4x	Hungary	H 2	Devecser, Zergeboglaros	17.44339689	47.11656667
EU	4x	Hungary	H 4	Barcs	17.49997063	45.96521169
EU	4x	Ukraine	UA 4	Khotyn	26.46580403	48.51591216
EU	4x	France	FRA 2	St-Clément-de-rivière	3.858896331	43.71806565
EU	4x	Germany	DE 3	Nürnberg	11.08564915	49.41683985
EU	4x	Germany	DE 4	Steinbach, Baggersee	10.63143809	49.99367438
EU	4x	Switzerland	CH 1	Grontenswill-Zetwill	8.15126773	47.28327703
EU	2x	Austria	AT 3	Hainburg	16.95549745	48.15341312
EU	2x	Switzerland	CH 4	Ausserberg	7.84454	46.31189
EU	2x	Germany	DE 1	Simbach am Inn	13.01505128	48.26064449
EU	2x	France	FRA D	St-Cirq Lapopie	1.679543126	44.46250283
EU	2x	Hungary	H 3	Tapolca	17.33497261	46.91410163
EU	2x	Hungary	H 6	Kiskunfelegyh aza	19.89586137	46.70589072
EU	2x	Ukraine	UA 2	Olesko	24.83581002	49.93014257

Greenhouse experiment

In May 2006, five seeds from each maternal plant were placed in multi-pot trays in a mixture of sand (20%) and compost (80%, made from yard waste at the Botanical Garden in Fribourg, Switzerland). The greenhouse was not heated but temperatures stayed above 0° C in winter. One plantlet per mother plant was re-potted at eight weeks in 1 L pots of sandy soils (20% sand, 80% compost) in a naturally lit greenhouse supplemented with artificial light. The greenhouse was located near the University of Fribourg, Switzerland. Plants were watered regularly, but were not given nutrient solution. Number of leaves and longest leaf length were measured three times (10^{th}-14^{th} July 2006, 7^{th}-11^{th} August 2006, 27^{th} April-3^{rd} May 2007) before plants started bolting. Number of leaves multiplied by the longest leaf size was used as a non-destructive proxy for plant biomass, and is referred to subsequently as "biomass index". When the first flower opened (6^{th} July-23^{rd} August), the date, number of stems, height of stems and number of buds larger than 5mm were recorded for each plant. Survival, number of capitula per flowering plant and number of newly formed rosettes were estimated once the stem had senesced at the beginning of October 2007. The percent of flowering plants and percent plant mortality was calculated for each population. Previous studies on *C. stoebe* have indicated that although environmental maternal effects on offspring are detectable, they are relatively weak compared to other factors such as plant genotype and environmental conditions (Weiner et al., 1997), therefore we do not expect maternal effects to confound the experimental results.

Tissue sampling

In November 2006 all plants remained in rosette form and had not bolted. One fully developed undamaged leaf was removed from each chosen plant using a razor blade. A few plants had minimal herbivore damage on the leaves, and these plants were avoided during tissue sampling. Four plants were sampled from each chosen population. Eight populations of North American tetraploids were sampled in addition to seven populations of Eurasian tetraploids and seven populations of Eurasian diploids (Table 3.1). Each leaf was immediately cut in half and the leaf tip was placed in a 5 mL vial containing RNAlater solution (Ambion, Austin TX). These samples were stored at -20° C for approximately four days, after which they were shipped on dry ice to Colorado State University. Upon arrival samples were placed at -20° C for storage.

Candidate gene choice

The *C. stoebe* EST library was found to contain a variety of unigenes that share sequence homology with known genes that are involved in plant secondary metabolism and defense response. Many of these unigenes are reported in Broz et al. 2007 (Broz et al., 2007). Although multiple candidate unigenes were selected for amplification in an initial analysis, only a small amount of primer sets resulted in reproducible amplification of a single product from *C. stoebe* cDNA (data not shown). Therefore only five candidate genes related to secondary metabolism or defense were quantified in the final analysis (Table 3.2).

Three distinct *C. stoebe* unigene homologs encoding phenylalanine ammonia lyase (PAL) were chosen to represent an important subset of secondary metabolism-

related genes (PAL1, PAL2a and PAL2b). One set of unigenes had top BLAST hits to PAL1 sequences from *Lactuca sativa* and *Arabidopsis thaliana* (AAL55242 and At2g37040, respectively), and the other two unigenes had top hits to PAL2 sequences from the same organisms (AAO13347 and At3g53260), but were distinct from each other upon sequence alignment. In addition, unigenes encoding a class II acidic chitinase (top BLAST hit *Helianthus annuus* chitinase AAB57694) and a beta-1,3-glucanase (top BLAST hit *A. thaliana* endo-glucanase At4g14080) were chosen to represent a subset of defense-related genes (Table 3.2).

The *C. stoebe* EST library was found to contain six transposable element homologs (Broz et al., 2007). Two unigenes encoding transposable elements were initially chosen to analyze the potential for active transposition, which could potentially facilitate rapid evolution. These had top BLAST hits to *Oryza sativa* japonica sequences ABB46630, a CACTA Enhancer Suppressor Mutator (En/Spm) subclass transposon and ABA99201, a mutator subclass transposon (Table 3.2). Both are type II transposons that move directly as DNA elements through a 'cut and paste' mechanism (Staginnus et al., 2001). Only the CACTA transposon gave reliable Q-PCR results, thus it is the only transposable element listed in the final expression analysis. Transcript accumulation of RAD, involved in homologous recombination and double strand break repair (Ivanov and Haber, 1997), was also analyzed. This sequence was identified by BLAST search and was not derived from the *C. stoebe* EST library. Three housekeeping genes; actin, ubiquitin, and cytochrome c oxidase were also analyzed as controls to normalize the expression of candidate genes (Table 3.2).

Table 3.2. Primer Information Table. The annotation of each *Centaurea stoebe* unigene(s) is given followed by Unigene ID numbers in parentheses (publicly accessible from the PLAN database, http://bioinfo.noble.org/plan/, project 30060). For each annotation, forward primer sequence is in the upper boxes and reverse primer sequence is in the lower boxes. The top BLAST hits (annotation, species, accession number) for each unigene are given in the column "homologs," and references describing information about the genes or gene families are given in the right column.

Name (Unigene ID)	Primer sequence (5'-3')	homologs	references
Secondary Metabolism			
PAL 1 phenylalanine ammonia lyase (03772, 01487, 04157, 00435, 00996)	GAAATGGACCCGTTGCAGAAGCC GCTTCGGCTGTTTTTCTTGCGGAAAT	PAL 1 *Arabidopsis* At2g37040 *Lactuca* AAL55242	Olsen et al. 2008 (Olsen et al., 2008) Rookes et al. 2003 (Rookes and Cahill, 2003) Winkel-Shirley 2001 (Winkel-Shirley, 2001) La Camera et al. 2004 (La Camera et al., 2004)
PAL 2a (00151)	AGCTCCACCCCTCGAGATTC GTCACCTTCTCACCGGTCAA	PAL 2 *Arabidopsis* At3g53260 *Lactuca* AAO13347	
PAL 2b (04127)	ATCGCGAGTACTTCTTCGCC GTCACCTTCTCACCGGTCAA	PAL 2 *Arabidopsis* At3g53260 *Lactuca* AAO13347	
Defense-related			
Chitinase (00271, 03889, 03038, 04202, 03133)	TGGCTCCATCGTTACTGCATCTG AGTTGTGGGATAGCTGGATAGGTC	Chitinase *Helianthus* AAB57694 Chitinase, class II *Arabidopsis* At4g01700	Kasprzewska 2003 (Kasprzewska, 2003) Jwa et al. 2006 (Jwa et al., 2006)
Glucanase (01113, 00896, 00032)	CGACCCGGTTAACATCAAGCTCG CGTCGAAAACTCCGTCGTCTTACC	Beta-1, 3-glucanase *Arabidopsis* At4g14080	Doxey et al. 2007 (Doxey et al., 2007)
Standards			
Actin (01058)	ACCAACATGAGAACAACCGATAC TCACACTGGTGTCATGGTCGGAAT	Actin *Gossypium hirsutum* AAP73454	
Cytochrome C oxidase (Weller et al. 2000)	CGTCGCATTCCAGATTATCCA CAACTACGGATATATAAGAGCCAAA ACTG		Weller et al. 2000 (Weller et al., 2000)
Ubiqutin	ACAACATCCAGAAGGAGTCC GCAACACAGCAAGCTTAACC		

GENE EXPRESSION ANALYSIS

RNA extraction and cDNA synthesis

Approximately 100 mg of each leaf sample (leaf tip) was removed from the RNAlater solution and quickly blotted on filter paper to remove excess liquid. Tissue was immediately frozen in liquid nitrogen and pulverized using a disposable pestle. RNA was isolated using Trizol reagent with its associated protocol (Invitrogen, Carlsbad CA). RNA pellets were resuspended in 30 μL RNase free water, and total RNA was quantified using a NanoDrop spectrophotometer (Wilmington DE). RNA samples were all diluted to the same concentration using RNase free water. RNA was treated with DNase to remove any genomic DNA contamination, and equal amounts of RNA from each sample were then individually translated into cDNA using reverse transcriptase, following a protocol from Invitrogen (Carlsbad CA). Samples were randomized in their preparation, such that RNA from plants from the same population (four plants tested per population) would not all be extracted on the same day.

Quantitative PCR

Candidate unigenes were chosen from the *C. stoebe* EST library based on a keyword search using the PLAN database (Table 3.2, (Broz et al., 2007; He et al., 2007)). Gene specific primers were designed to amplify a 200-600 basepair region of each candidate *C. stoebe* unigene sequence (Table 3.2). Initially, specific primer sets were designed for a wide array of genes potentially involved in constitutive defense or secondary metabolism. However, many resulted in either poor amplification or amplification of multiple *C. stoebe* cDNAs, so these were not used in the final Q-PCR

analysis. Successful primer sets included those for three distinct transcripts of phenylalanine ammonia lyase (PAL1, PAL2a and PAL2b), a chitinase, a glucanase, a transposable element and a DNA repair enzyme (Table 3.2). An additional transposable element was successfully amplified in preliminary experiments, but was expressed to a very low extent in the experimental plant samples.

When multiple unigenes had the same annotation, nucleotide sequences were aligned using the DNA alignment program in CLC Free Workbench (Cambridge MA) to determine similarities. Unigenes with over 90% similarity (after removing the terminal 100 bases in case of sequencing error) were grouped together under one annotation, and primers were designed to the alignments. When the ESTs were originally clustered to form unigenes, they had to have an overlap of at least 40 bp and at least 94% sequence identity to be clustered together. The reason some unigenes were grouped in this analysis, but not in the original clustering analysis, is likely due to sequencing errors at the terminal (3') ends of the ESTs, which exhibited the largest amount of variability. In this analysis the terminal 100 bp of sequence was removed, such that only the most reliable sequence information was included. In addition, a few single base changes within similar ESTs were identified and these may represent either sequencing errors or natural polymorphisms. In addition, three potential housekeeping genes were analyzed as controls: actin (*C. stoebe* unigene 01058, top BLAST hit AAP73454, *Gossypium hirsutum*) cytochrome c oxidase (originally designed for *Solanum tuberosum* cv Cara, (Weller et al., 2000)), and ubiquitin (originally designed for *Nicotiana*). All primer sets amplified a single product from *C. stoebe* cDNA.

All reactions were run and analyzed using the BioRad iCycler software (Hercules CA). A standard curve was created for each primer set using serial dilutions (concentrations of 5-625 ng/μL) of cDNA prepared from leaves of a greenhouse-grown *C. stoebe* plant (fresh tissue was frozen in liquid nitrogen, and RNA extraction and cDNA synthesis followed the protocol above), and negative controls using water instead of template were run for all reactions. The optimal annealing temperature for all primer sets was determined empirically, with all sets working well at an annealing temperature of 55° C. All PCR reactions had a final volume of 20 μL and contained 10μL of 2x Jumpstart cyber green reaction mix, 0.2 μL 1μM flourescein, 2.4 μL 25 mM $MgCl_2$, 0.2 μL 10μM forward primer, 0.2 μL 10 μM reverse primer, 2μL template (20 ng/μL) and 5 μL sterile H_2O. Reactions conditions for PCR were as follows: 95° C 30 seconds, 55° C 30 seconds, 72° C 30 seconds, for 40 cycles.

For each sample, total RNA (ng/μL) was estimated using the appropriate standard curves and normalized using the geometric mean of actin, cox, and ubiquitin, as suggested in Vandesompele et al. (2002) (Vandesompele et al., 2002). Any expression levels that fell below the standard curve for either the gene of interest or the three housekeeping gene standards were removed from the analysis.

STATISTICAL ANALYSES

In order to account for potential genetic variation within each geo-cytotype (native diploid, native tetraploids, and invasive tetraploid), three to four plants from a number of geographic populations (seven native diploid, seven native tetraploid, and eight invasive tetraploid respectively) were included in this study. We were interested in two *a priori* comparisons for all collected data; native tetraploid versus invasive

tetraploid, and native tetraploid versus native dipoid. Differences between geo-cytotypes for gene expression (log cDNA) and for plant characteristics were tested using the MIXED model procedure in SAS (vers 9.1) with geo-cytotype as a fixed variable and population as a random variable. When treating population as a fixed variable, no significant differences between populations within any of the three geo-cytotypes were detected at the $p < 0.1$ level in any of the analyses. Fisher's LSD was used for pair-wise comparisons of LSmeans to determine significant effects ($p < 0.05$) for the two pre-planned comparisons. For pair-wise comparisons, the degrees of freedom for all gene expression analysis was equal to 20, and for plant characteristics degrees of freedom are as follows; height = 18, flowering & biomass index = 19, mortality = 17, new rosettes = 15, capitula = 11. Using a Bonferroni multiple comparisons adjustment, the p-values for all reported comparisons remain significant at the $p < 0.05$ level, except for the chitinase gene expression when comparing native tetraploids to invasive tetraploids ($p=0.089$) and the number of new rosettes when comparing native diploids to native tetraploids ($p=0.061$). A similar mixed model was run for both gene expression data and plant performance data. All reported values are LSmeans and pooled standard errors.

SIMULATION OF SEED PRODUCTION

Total seed production over time was simulated for *C. stoebe* geo-cytotypes to understand possible differences in fecundity over multiple generations. Data was used from the plant performance analysis for each geo-cytotype (Table 3.1 and Table 3.3). The simulation followed a cohort of 1000 plants over fifteen generations (years) assuming that the number of flowering plants for each generation was 75.2, 82.1, and

44.3 % (invasive tetraploid, native tetraploid and native diploid, respectively) of the total population (Figure 3.1E); and each generation the number of flowering plants declined according to a mortality rate of 7.3, 23.6, and 62.3 % (invasive tetraploid, native tetraploid and native diploid, respectively) as shown in Figure 3.2F. For each flowering plant, the total number of seeds was estimated as the product of the number of new rosettes per plant (5.88, 5.75, and 2.8 for invasive tetraploid, native tetraploid and native diploid, respectively; Figure 3.1D), number of capitula per rosette (14.6, 18.6, and 15.7 for invasive tetraploid, native tetraploid and native diploid, respectively; Figure 3.1C), and 30 seeds per capitula (Mauer et al., 2001). See Table 3.3 for the LSmean values used.

Table 3.3. Measurements of plant performance and life-cycle traits for *C. stoebe* geo-cytotypes. *C. stoebe* plants were grown from seed in a common greenhouse environment. Plants were measured for leaf length and leaf number while in rosette form, and these values were multiplied to obtain an early indicator of biomass. After bolting, stem height of each bolting plant was measured the day the first flower opened and the number of capitula per plant were counted after the stems had senesced. The number of newly formed rosettes after flowering, the percent of flowering individuals, and the percent mortality after flowering were monitored. Legend; 2X EU, native Eurasian diploid populations; 4X EU, native Eurasian tetraploid populations; 4X US, invasive North American tetraploids. Significant differences in plant traits were determined for geo-cytotypes of interest (EU 2x versus EU 4x and EU 4x versus US 4x) using pair-wise comparisons of LSmeans. Reported values are LSmeans. Fisher's LSD and absolute t values are reported for each pair-wise comparison.

	EU 2x vs EU 4x		Measure of plant performance			EU 4x vs US 4x	
Trait	**t**	**p-value**	**EU 2x**	**EU 4x**	**US 4x**	**t**	**p-value**
Biomass index (cm)	0.72	0.479	95.47 [a]	86.07 [a]	72.01 [a]	1.12	0.278
Height (cm)	0.96	0.348	55.43 [a]	55.97 [a]	55.60 [a]	0.63	0.538
Capitula (#/rosette)	0.76	0.461	15.67 [a]	18.57 [a]	14.57 [a]	1.36	0.201
Rosettes (#/plant)	2.39	0.036	2.80 [a]	5.75 [b]	5.88 [a]	0.74	0.857
Flowering plants (%)	2.72	0.0137	44.34 [a]	82.14 [b]	75.15 [b]	0.52	0.608
Mortality rate (%)	2.78	0.0127	62.31 [a]	23.55 [b]	7.34 [b]	1.44	0.1685

3.4 RESULTS

PLANT PERFORMANCE AND LIFE CYCLE ANALYSIS

No significant differences in vegetative plant performance characteristics were found between *C. stoebe* geo-cytotypes (Figure 3.1, Table 3.3). Before bolting, the plant biomass index tended to be higher in diploid populations than in tetraploids, but the results were not significant (Figure 3.1A). Similarly, stem height was not different between the three geo-cytotypes (Figure 3.1B). However, differences in life cycle were noted between ploidy groups; a higher percentage of both native and invasive tetraploid plants flowered in the first year compared to the diploid plants (Figure 3.1E). Fewer than half of the diploid plants flowered in their first year of growth, and over 60% died after flowering (Figure 3.1F, Table 3.3). In comparison, over 75% of both native and introduced tetraploids flowered their first year and only 24% and 7% died after flowering, respectively (Figure 3.1E, F, Table 3.3). In addition, tetraploids produced more new rosettes after senescence of the parent plant than diploids (Figure 3.1D). Interestingly, the number of capitula per plant (Figure 3.1C) was not different between the three geo-cytotypes. The observed differences in life cycle characteristics reflect the moncarpic life cycle of the diploid and the polycarpic life cycle of the tetraploid (Ochsmann 2001), and are likely important in plant population fecundity over time, as illustrated by a simulation of seed production (Figure 3.2). Over a fifteen-year period, this simluation estimates production of 0.6, 8.8, and 16.4 million seeds for populations of the native diploid, native tetraploid, and introduced tetraploid, respectively (Figure 3.2).

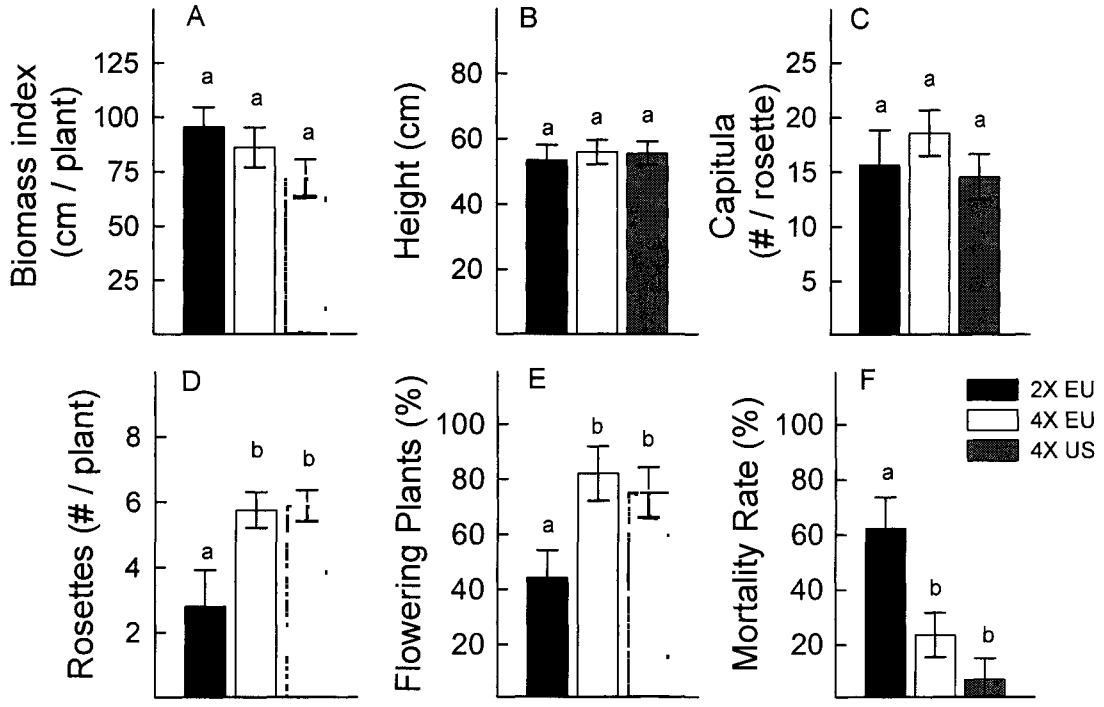

Figure 3.1. Plant performance and life-cycle traits of *C. stoebe* geo-cytotypes. *C. stoebe* plants were grown from seed in a common greenhouse environment. Plants were measured for leaf length and leaf number while in rosette form, and these values were multiplied to obtain an early indicator of biomass (A). After bolting, stem height (B) of each bolting plant was measured the day the first flower opened and the number of capitula per flowering plant (C) were counted after the stems had senesced. The number of newly formed rosettes after flowering (D), the percent of flowering individuals (E), and the percent mortality after flowering (F) were monitored. Legend; 2X EU, native Eurasian diploid populations; 4X EU, native Eurasian tetraploid populations; 4X US, invasive North American tetraploids. Significant differences in plant traits were determined for geo-cytotypes of interest (EU 2x versus EU 4x and EU 4x versus US 4x) using pair-wise comparisons of LSmeans. Bars represent LSmeans and standard errors. Fisher's LSD was used for pair-wise mean comparisons. Different letters above the columns indicate significant differences ($P < 0.05$) between pairs of geo-cytotypes.

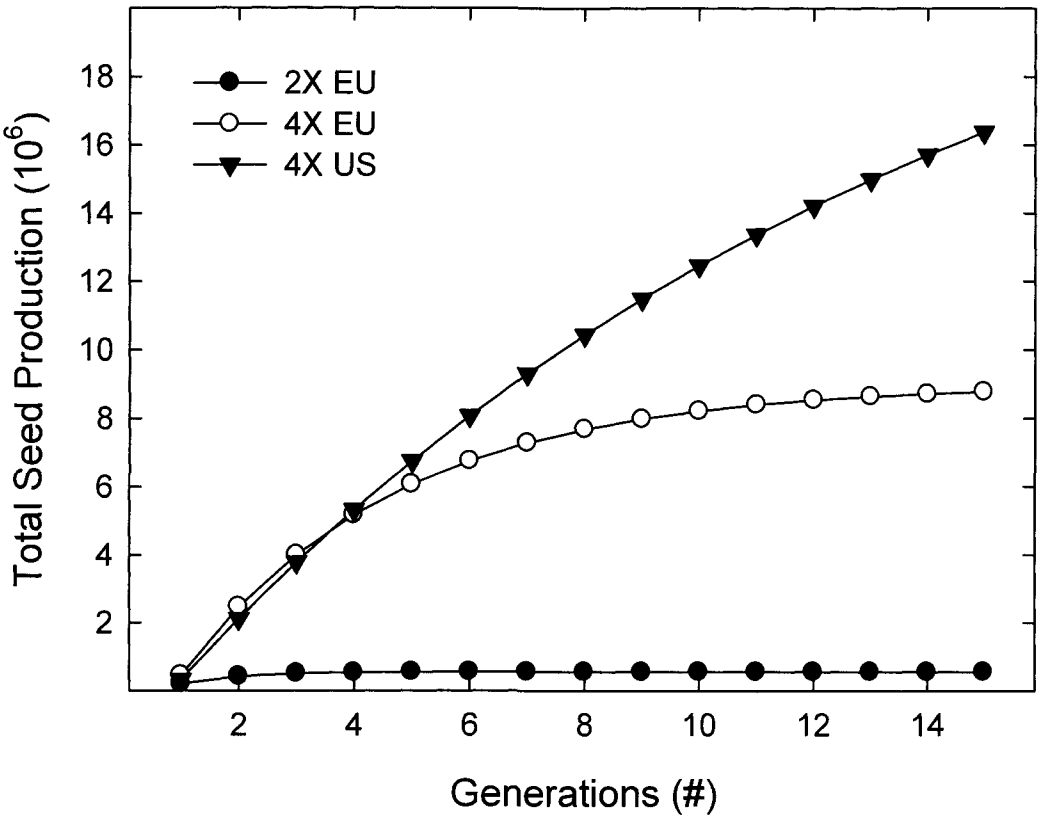

Figure 3.2. Simulation of total seed production over time. The simulation followed a cohort of 1000 plants over time assuming that the number of flowering plants for each generation was 75.2, 82.1, and 44.3 % (4X US, 4X EU, and 2X EU, respectively) of the total population; and each generation the number of flowering plants declined according to a mortality rate of 7.3, 23.6, and 62.3 % (4X US, 4X EU, and 2X EU, respectively). For each flowering plant, the total number of seeds was estimated as the product of the number of new rosettes per plant (5.88, 5.75, and 2.8 for the 4X US, 4X EU, and 2X EU, respectively), number of capitula per rosette (14.6, 18.6, and 15.7 for the 4X US, 4X EU, and 2X EU, respectively), and 30 seeds per capitula (Mauer et al., 2001). Legend; 2X EU, native Eurasian diploid populations; 4X EU, native Eurasian tetraploid populations; 4X US, invasive North American tetraploids. See Table 3.3 for mean values used in calculations.

GENE EXPRESSION ANALYSIS

Tetraploid plants from the introduced range had significantly lower rates of gene expression for all three PAL transcripts compared to tetraploid plants from the native range, providing evidence in favor of our hypothesis (Figure 3.3A). PAL1 transcript accumulation in introduced tetraploids was 2.4 times lower than the amount in native tetraploids, whereas PAL2a and PAL2b were 2.6 and 16.7 times lower, respectively (Table 3.4). PAL 1 expression was lower than expression for either form of PAL 2 in all geo-cytotypes (Figure 3.3A). Similarly, glucanase transcripts showed over a two-fold reduction in expression in introduced tetraploids than their native counterparts (Figure 3.3B, Table 3.4). Chitinase expression was 1.7 fold lower in introduced tetraploids than native tetraploids (Table 3.4). In general, expression of all tested secondary metabolism- and defense-related transcripts was lower in tetraploids from the introduced range compared to their native counterparts.

Contrary to our second hypothesis, introduced tetraploids showed over two-fold less expression of a transposable element (CACTA En/Spm subclass) transcript than native tetraploids (Figure 3.3C). The other transposable element (mutator subclass) showed extremely low levels of transcript accumulation in most samples, nearly all of which fell below the standard curve for that gene (data not shown). Of the usable values, the data suggested that introduced populations expressed this transposable element to a lower extent than native populations, but the sample size was very low and thus overall values may not accurately reflect expression in these populations. Expression of RAD was low in all plant types, but also showed the highest relative mean expression in native tetraploids, although this result was not significant (Figure 3.3D, Table 3.4).

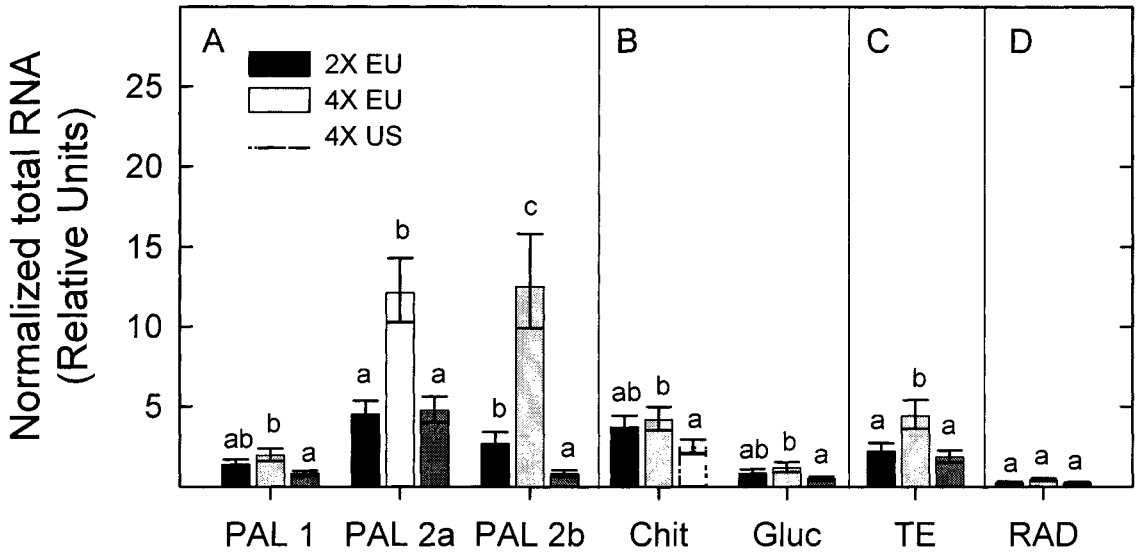

Figure 3.3. Gene expression profiles of *C. stoebe* geo-cytotypes. For each sample, total RNA (ng / ul) was estimated using the appropriate standard curve for each gene of interest and normalized using the geometric mean of the standards actin, cytochrome c oxidase, and ubiquitin as suggested in Vandersompele et al. 2002 (Vandesompele et al., 2002). Significant differences in gene expression (log cDNA) were determined for geo-cytotypes of interest (EU 2x versus EU 4x and EU 4x versus US 4x) using pair-wise comparisons of LSmeans. Bars represent back-transformed LSmeans and standard errors. Fisher's LSD was used for pair-wise mean comparisons, and values are reported in Table 3. Different letters above the columns indicate significant differences ($P < 0.05$) between pairs of geo-cytotypes. Legend; 2X EU, native Eurasian diploid populations; 4X EU, native Eurasian tetraploid populations; 4X US, invasive North American tetraploids. Panel A: Genes involved in secondary metabolism; PAL (Phenylalanine ammonia lyase) 1, 2a, 2b. Panel B: Genes involved in defense response; Chit (chitinase) and Gluc (glucanase); Panel C: Gene involved in transposition; TE (transposable element); Panel D: Gene involved in DNA repair and recombination, RAD.

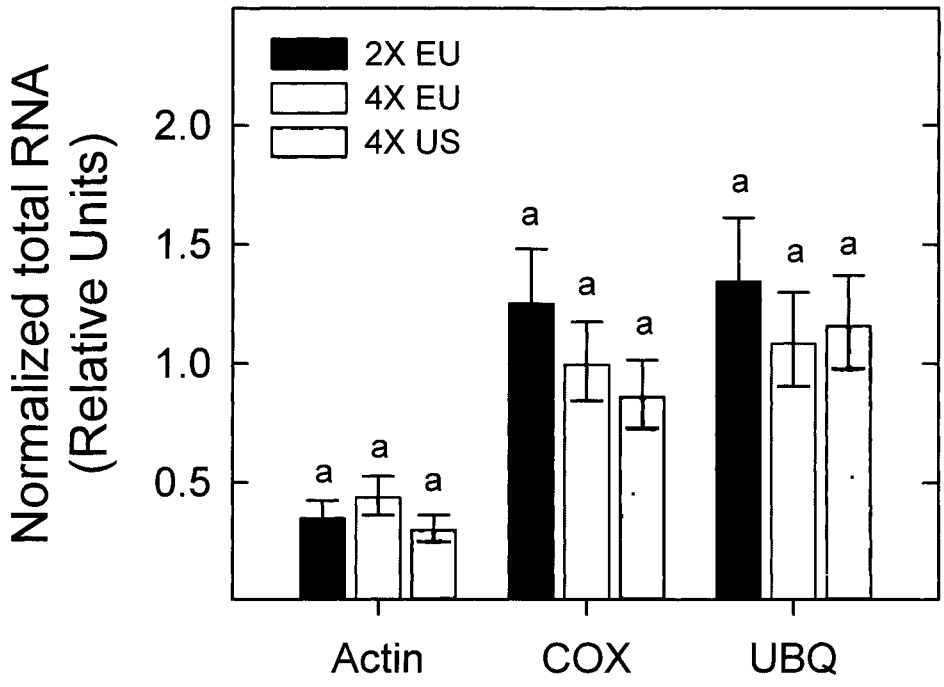

Figure 3.4. Housekeeping gene expression profiles of *C. stoebe* geo-cytotypes. Standards used to create normalization factors for analysis of genes of interest. For each sample, total RNA (ng / ul) was estimated using the appropriate standard curve and a normalization factor was calculated based on the geometric mean of all three standards, as suggested in Vandersompele *et al.* 2002. Significant differences in gene expression (log cDNA) were determined for geo-cytotypes of interest (EU 2x versus EU 4x and EU 4x versus US 4x) using pair-wise comparisons of LSmeans. Bars represent back-transformed LSmeans and standard errors. Fisher's LSD was used for pair-wise mean comparisons, and values are reported in Table 3. Different letters above the columns indicate significant differences ($P < 0.05$) between pairs of geo-cytotypes. Legend; 2X EU, native Eurasian diploid populations; 4X EU, native Eurasian tetraploid populations; 4X US, invasive North American tetraploids.

Table 3.4. Relative gene expression values of *C. stoebe* geo-cytotypes. For each sample, total RNA (ng / ul) was estimated using the appropriate standard curve for each gene of interest and normalized using the geometric mean of the three standards: actin, cytochrome c oxidase (COX) and ubiquitin (UBQ), as suggested in Vandersompele et al. 2002 (Vandesompele et al., 2002). Genes of interest included three isoforms of PAL (phenylalanine ammonia lyase) 1, 2a, 2b, involved in secondary metabolism; chitinase and glucanase, involved in defense response; and a transposable element (TE) and DNA repair/recombination gene (RAD), potentially involved in rapid evolution. Geo-cytotypes are 2X EU, native Eurasian diploid populations; 4X EU, native Eurasian tetraploid populations; 4X US, invasive North American tetraploids. Significant differences in gene expression (log cDNA) were determined for geo-cytotypes of interest (EU 2x versus EU 4x and EU 4x versus US 4x) using pair-wise comparisons of LSmeans. LSmeans were back-transformed and expression values are shown relative to native Eurasian tetraploid populations (4x EU). Fisher's LSD and absolute t values are reported for each pair-wise comparison.

	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

Diploid and tetraploid plants from the native range showed similar relative expression levels for seven out of ten genes tested; PAL1, glucanase, chitinase, RAD, and the three housekeeping genes (Figure 3.3A, B, D, Figure 3.4, Table 3.4). Expression of PAL2a and PAL2b was higher in native tetraploids compared to diploids (Figure 3.3A, Table 3.4) as hypothesized. Expression of CACTA transposable element was also higher in native tetraploids compared to diploids (Figure 3.3C, Table 3.4). Introduced tetraploids showed similar expression profiles when compared to diploids for nine of the ten genes tested (Figure 3.3). The expression of PAL2b was over three fold lower in introduced tetraploids compared to diploids (Table 3.4).

3.5 DISCUSSION

PLANT PERFORMANCE AND LIFE CYCLE ANALYSIS

Ridenour et al. (2008) recently reported that in a common garden in Montana, *C. stoebe* plants from North America exhibit greater biomass, tougher leaves and increased trichome density when compared to their Eurasian counterparts (Ridenour et al., 2008). Based on this finding and hypotheses such as EICA that suggest invasive plants may evolve to increase resource allocation to growth (Blossey and Notzold, 1995), we expected that introduced tetraploids would out-perform both native diploids and tetraploids. However, in our study, neither of the plant vegetative growth characteristics examined (biomass index and stem height, Figure 3.1A, B), showed a significant difference. Ridenour et al. (2008) performed the bulk of their experiments on populations with unknown ploidy; however, one experiment containing plants of known ploidy revealed greater rosette diameters of introduced tetraploids compared to native

tetraploids (Ridenour et al., 2008). Conversely, Müller et al. (1989) observed that Hungarian and German diploids had greater dry weights and shoot diameters than North American tetraploids when grown in a European soil, but sample sizes were relatively small (Muller, 1989). The observed differences may be due to the various populations chosen, the type and origin of soil used (ie; North American soil (Ridenour et al., 2008) versus European soil [(Muller, 1989; Treier et al., in press) present study], or other factors involved in each of the above studies. These inconsistencies may suggest that vegetative growth is not the best indicator of invasiveness.

As previously noted by Müller (1989), life cycle differences between *C. stoebe* geo-cytotypes may have greater relevance to fitness than single performance traits (Muller, 1989). In the first year of this study, flowering plants of all geo-cytotypes had a similar number of capitula (Figure 3.1C): however, fewer diploid plants flowered in the first year of growth than tetraploids, diploids formed fewer new rosettes, and diploids suffered greater mortalities after flowering (Figure 3.1D, E, F). In combination these measures suggest that the reproductive capacity of tetraploids is greater than that of diploids. Additionally, we expect introduced tetraploid populations to have a higher reproductive capacity when compared to the native tetraploids, as illustrated by a simulation of seed production (Figure 3.2). Ongoing experiments will provide more complete information about the life-cycle of these plants and seed production over their entire life span. Thus, although we did not detect any significant differences in vegetative traits between *C. stoebe* geo-cytotypes, there is some indication of a long-term difference in plant fecundity, with the invasive tetraploid showing highest performance of the three geo-cytotypes studied.

GENE EXPRESSION ANALYSIS

Secondary metabolism and defense

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 (Winkel-Shirley, 2001; La Camera et al., 2004; Treutter, 2005). Flavonoids are often stored in plant tissues as 'pre-formed' defense compounds and may act as pathogen and herbivore deterrents (Treutter, 2005). 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 (Winkel-Shirley, 2001; La Camera et al., 2004; Treutter, 2005).

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 (Kasprzewska, 2003; Jwa et al., 2006; Doxey et al., 2007). Different forms of chitinase are involved in both active and passive defense responses in plants (Kasprzewska, 2003). 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 (Doxey et al., 2007).

The fact that PAL, chitinase and glucanase transcripts were all reduced in introduced tetraploids compared to native tetraploids (Figure 3.3 A,B) 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. Some studies suggest that constitutive or basal levels of defense-related transcripts in plants, similar to those analyzed in this study, can be used to predict pathogen susceptibility and induced defense responses (Bowling et al., 1994; Vleeshouwers et al., 2000). Very subtle genetic mutations, such as those in the *Arabidopsis* cpr (constitutive expressers of pathogenesis related genes) mutant, have been shown to increase basal levels of systemic acquired resistance, which in turn increase levels of pathogen resistance (Bowling et al., 1994). In addition, the over-expression of PR proteins *in planta* typically results in a phenotype of enhanced disease resistance (Stintzi et al., 1993; Bowling et al., 1994; Maleck and Lawton, 1998). Plants with high constitutive defenses may, however, also have a lower degree of defense induction than those with low constitutive defenses (Coley et al., 1985; Cipollini et al., 2005).

Recent reports indicate that introduced *C. stoebe* 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.

It is important to note here that the release of *C. stoebe* from specialist enemies has been considered an important factor in the invasive success of the weed, and this has spurred the introduction of a number of biological control species to North America over the past thirty years (Maddox, 1979; Maddox, 1982; Smith and Story, 2003; Muller-

Scharer et al., 2004). Although many of these specialist herbivores have become established and widespread, *C. stoebe* densities have only been reduced in a few specific areas (e.g.(Story et al., 2006)), and the weed continues to expand its range at other sites (Sheley et al., 1998; Muller-Scharer et al., 2004). Interestingly, field observations in North America suggest that introduced *C. stoebe* experiences little pressure from generalist herbivores and pathogens (RM Callaway and WM Ridenour, personal communication), indicating that *C. stoebe* currently experiences a partial release from both specialist and generalist enemies in the introduced range.

In order to better understand defense responses in *C. stoebe*, 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 *C. stoebe*. In addition, it would be interesting to test the response of *C. stoebe* geo-cytotypes to a variety of generalist and specialist enemies at the level of gene expression.

Evolutionary capacity

The activity of transposable elements could facilitate evolution by reorganizing the genome, and may be one important aspect in this process (Kazazian, 2004; Morgante, 2006). Therefore, we hypothesized that introduced populations of *C. stoebe* would have the highest expression of the transposable elements analyzed, potentially due to novel stresses encountered in the introduced range. However, this was not the case. In fact, native tetraploid populations had the highest expression rate of one CATCA En/Spm

subclass transposable element (Figure 3.3C). The expression of RAD, which is involved in DNA recombination/repair (Ivanov and Haber, 1997), was also highest in native tetraploid populations, but was not significantly different from that of introduced populations (Figure 3.3D).

Although the expression of transposable elements could facilitate rapid evolution, transposition may not be adaptive and could cause deleterious genomic rearrangements as opposed to beneficial ones. In other studies, certain transposable elements have been detected in plants at specific growth stages or under conditions of biotic and abiotic stress (Takeda et al., 1998; Kimura et al., 2001); however, the biological role of active transposition currently remains unclear.

Plant ploidy

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 (Figure 3.3, Table 3.4). 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 (Guo et al., 1996; Albertin et al., 2005; Chen and Ni, 2006; Wang et al., 2006). This dosage effect results in gene or protein expression patterns in polyploids which 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 (Guo et al., 1996; Albertin et al., 2005; Chen and Ni, 2006), whereas evidence suggests that *C. stoebe* plants within the native range harbor different genetic backgrounds (Hufbauer and Sforza, 2008; Marrs et al., 2008). However, it appears that gene dosage compensation may be occurring to some extent in the native cytotypes of *C. stoebe*. 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 (Figure 3.3, Table 3.4), 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 highlights the importance of using appropriate plant types when examining a particular species in both the native and introduced range.

Alternative gene roles and regulation

Genes similar to those selected in the current study have been detected in response to a variety of cues and conditions that do not necessarily reflect their primary

annotation. For instance, many genes involved in defense response (Dangl and Jones, 2001), flavonoid biosynthesis (Winkel-Shirley, 2001) and active transposition (Takeda et al., 1998; Kimura et al., 2001) have been detected during particular points of plant growth and development. In this study we attempted to minimize any possible developmental differences in gene expression by sampling expanded, fully developed rosette leaves of similar age from all plants. All of the plants were grown in the same greenhouse environment and at the time of sampling remained in rosette form, none showing signs of bolting. If the genes tested here were expressed predominantly in response to developmental cues, it could be expected that expression of transcripts would be extremely similar across all geo-cytotypes, which is not what was observed.

Additionally, it is possible that the defense genes analyzed in this study are important for aspects other than plant defense against enemies. For instance, the production of certain flavonoids are thought to play important roles in photo-protection, frost hardiness and drought resistance (Treutter, 2005), which could influence expression of PAL genes. *C. stoebe* occupies areas in both the native and introduced range that are often subject to these types of abiotic stress (Ochsmann, 2001; Broennimann et al., 2007; Treier et al., in press). Thus, expression of PAL transcripts and resulting flavonoid accumulation may be important in both the biotic and abiotic stress response of the plant.

3.6 CONCLUSIONS

Although we sampled only a small subset of genes, we identified differences in gene expression between native and introduced populations of plants that may have ecological relevance. We found that introduced tetraploids exhibited lower expression of

constitutive defense genes than native tetraploids, as might be predicted based on general ideas of enemy release and rapid evolution. Plant origin and ploidy were found to have a significant effect on both life-cycle characteristics and gene expression. This highlights the importance of determining plant ploidy in ecological and genomics investigations, and suggests that *C. stoebe* invasion can be influenced by both plant ploidy and altered gene expression in the introduced range.

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.

3.7 REFERENCES

Albertin W, Brabant P, Catrice O, Eber F, Jenczewski E, Chevre AM, Thiellement H (2005) Autopolyploidy in cabbage (Brassica oleracea L.) does not alter significantly the proteomes of green tissues. Proteomics **5:** 2131-2139

Anderson JV, Horvath DP, Chao WS, Foley ME, Hernandez AG, Thimmapuram J, Liu L, Gong GL, Band M, Kim R, Mikel MA (2007) Characterization of an EST database for the perennial weed leafy spurge: An important resource for weed biology research. Weed Science **55:** 193-203

Blossey B, Notzold R (1995) EVOLUTION OF INCREASED COMPETITIVE ABILITY IN INVASIVE NONINDIGENOUS PLANTS - A HYPOTHESIS. Journal of Ecology **83:** 887-889

Bossdorf O, Auge H, Lafuma L, Rogers WE, Siemann E, Prati D (2005) Phenotypic and genetic differentiation between native and introduced plant populations. Oecologia **144:** 1-11

Bowling SA, Guo A, Cao H, Gordon AS, Klessig DF, Dong XI (1994) A MUTATION IN ARABIDOPSIS THAT LEADS TO CONSTITUTIVE EXPRESSION OF SYSTEMIC ACQUIRED-RESISTANCE. Plant Cell **6:** 1845-1857

Broennimann O, Treier UA, Muller-Scharer H, Thuiller W, Peterson AT, Guisan A (2007) Evidence of climatic niche shift during biological invasion. Ecology Letters **10:** 701-709

Broz AK, Broeckling CD, He JB, Dai X, Zhao PX, Vivanco JM (2007) A first step in understanding an invasive weed through its genes: an EST analysis of invasive Centaurea maculosa. Bmc Plant Biology **7**

Chen ZJ, Ni ZF (2006) Mechanisms of genomic rearrangements and gene expression changes in plant polyploids. Bioessays **28:** 240-252

Cipollini D, Mbagwu J, Barto K, Hillstrom C, Enright S (2005) Expression of constitutive and inducible chemical defenses in native and invasive populations of Alliaria petiolata. Journal of Chemical Ecology **31:** 1255-1267

Coley PD, Bryant JP, Chapin FS (1985) RESOURCE AVAILABILITY AND PLANT ANTIHERBIVORE DEFENSE. Science **230:** 895-899

Dangl JL, Jones JDG (2001) Plant pathogens and integrated defence responses to infection. Nature **411:** 826-833

Dhawan OP, Lavania UC (1996) Enhancing the productivity of secondary metabolites via induced polyploidy: A review. Euphytica **87:** 81-89

Doxey AC, Yaish MWF, Moffatt BA, Griffith M, McConkey BJ (2007) Functional divergence in the Arabidopsis beta-1,3-glucanase gene family inferred by phylogenetic reconstruction of expression states. Molecular Biology and Evolution **24:** 1045-1055

Elton C, ed (1958) The ecology of invasions by animals and plants. The University of Chicago Press, Chicago

Guo M, Davis D, Birchler JA (1996) Dosage effects on gene expression in a maize ploidy series. Genetics **142:** 1349-1355

He J, Dai XB, Zhao XC (2007) PLAN: a web platform for automating high-throughput BLAST searches and for managing and mining results. Bmc Bioinformatics **8**

Hierro JL, Maron JL, Callaway RM (2005) A biogeographical approach to plant invasions: the importance of studying exotics in their introduced and native range. Journal of Ecology **93:** 5-15

Hufbauer RA, Sforza R (2008) Multiple introductions of two invasive Centaurea taxa inferred from cpDNA haplotypes. Diversity and Distributions **14:** 252-261

Inderjit, Callaway RM, Vivanco JM (2006) Can plant biochemistry contribute to understanding of invasion ecology? Trends in Plant Science **11:** 574-580

Ivanov EL, Haber JE (1997) DNA repair: RAD alert. Current Biology **7:** R492-R495

Joshi J, Vrieling K (2005) The enemy release and EICA hypothesis revisited: incorporating the fundamental difference between specialist and generalist herbivores. Ecology Letters **8:** 704-714

Jwa NS, Agrawal GK, Tamogami S, Yonekura M, Han O, Iwahashi H, Rakwal R (2006) Role of defense/stress-related marker genes, proteins and secondary metabolites in defining rice self-defense mechanisms. Plant Physiology and Biochemistry **44:** 261-273

Kasprzewska A (2003) Plant chitinases - Regulation and function. Cellular & Molecular Biology Letters **8:** 809-824

Kazazian HH (2004) Mobile elements: Drivers of genome evolution. Science **303:** 1626-1632

Kimura Y, Tosa Y, Shimada S, Sogo R, Kusaba M, Sunaga T, Betsuyaku S, Eto Y, Nakayashiki H, Mayama S (2001) OARE-1, a Ty1-copia retrotransposon in oat activated by abiotic and biotic stresses. Plant and Cell Physiology **42:** 1345-1354

La Camera S, Gouzerh G, Dhondt S, Hoffmann L, Fritig B, Legrand M, Heitz T (2004) Metabolic reprogramming in plant innate immunity: the contributions of phenylpropanoid and oxylipin pathways. Immunological Reviews **198:** 267-284

Maddox DM (1979) The knapweeds: their economics and biological control in the western states. USA Rangelands **1:** 139-141

Maddox DM (1982) BIOLOGICAL-CONTROL OF DIFFUSE KNAPWEED (CENTAUREA-DIFFUSA) AND SPOTTED KNAPWEED (C MACULOSA). Weed Science **30:** 76-82

Maleck K, Lawton K (1998) Plant strategies for resistance to pathogens. Current Opinion in Biotechnology **9:** 208-213

Marrs RA, Sforza R, Hufbauer RA (2008) When invasion increases population genetic structure: a study with Centaurea diffusa. Biological Invasions **10:** 561-572

Mauer T, Russo MJ, Evans M (2001) Element Stewardship Abstract for Centaurea maculosa. *In*. The Nature Conservancy Arlington, Virginia, p tncweeds.ucdavis.edu/esadocs/documnts/centmac.html

Morgante M (2006) Plant genome organisation and diversity: the year of the junk! Current Opinion in Biotechnology **17:** 168-173

Muller-Scharer H, Schaffner U (2008) Classical biological control: exploiting enemy escape to manage plant invasions. Biological Invasions **10:** 859-874

Muller-Scharer H, Schaffner U, Steinger T (2004) Evolution in invasive plants: implications for biological control. Trends in Ecology & Evolution **19:** 417-422

Muller H (1989) GROWTH-PATTERN OF DIPLOID AND TETRAPLOID SPOTTED KNAPWEED, CENTAUREA-MACULOSA LAM (COMPOSITAE), AND EFFECTS OF THE ROOT-MINING MOTH AGAPETA-ZOEGANA (L) (LEP, COCHYLIDAE). Weed Research **29:** 103-111

Ochsmann J (2001) On the taxonomy of spotted knapweed (Centaurea stoebe L.) *In* Proceedings from The First International Knapweed Symposium of the Twenty First Century, pp 33-41

Olsen KM, Lea US, Slimestad R, Verheul M, Lillo C (2008) Differential expression of four Arabidopsis PAL genes; PAL1 and PAL2 have functional specialization in abiotic environmental-triggered flavonoid synthesis. Journal of Plant Physiology **165:** 1491-1499

Pimental D, Lach L, Zuniga R, Morrison D (2002) Environmental and economic costs associated with nonindigenous species in the United States. Biological Invasions**:** 285-303

Ponder RG, Fonville NC, Rosenberg SM (2005) A switch from high-fidelity to error-prone DNA double-strand break repair underlies stress-induced mutation. Molecular Cell **19:** 791-804

Ridenour WM, Vivanco JM, Feng YL, Horiuchi J, Callaway RM (2008) No evidence for trade-offs: Centaurea plants from America are better competitors and defenders. Ecological Monographs **78:** 369-386

Rookes JE, Cahill DM (2003) A PAL1 gene promoter-green fluorescent protein reporter system to analyse defence responses in live cells of Arabidopsis thaliana. European Journal of Plant Pathology **109:** 83-94

Sheley RL, Jacobs JS, Carpinelli MF (1998) Distribution, biology, and management of diffuse knapweed (Centaurea diffusa) and spotted knapweed (Centaurea maculosa). Weed Technology **12:** 353-362

Smith L, Story JM (2003) Plant size preference of Agapeta zoegana L. (Lepidoptera : Tortricidae), a root-feeding biological control agent of spotted knapweed. Biological Control **26:** 270-278

Soltis PS, Soltis DE (2000) The role of genetic and genomic attributes in the success of polyploids. Proceedings of the National Academy of Sciences of the United States of America **97:** 7051-7057

Staginnus C, Huettel B, Desel C, Schmidt T, Kahl G (2001) A PCR-based assay to detect En/Spm-like transposon sequences in plants. Chromosome Research **9:** 591-605

Stintzi A, Heitz T, Prasad V, Wiedemannmerdinoglu S, Kauffmann S, Geoffroy P, Legrand M, Fritig B (1993) PLANT PATHOGENESIS-RELATED PROTEINS AND THEIR ROLE IN DEFENSE AGAINST PATHOGENS. Biochimie **75:** 687-706

Story JM, Callan NW, Corn JG, White LJ (2006) Decline of spotted knapweed density at two sites in western Montana with large populations of the introduced root weevil, Cyphocleonus achates (Fahraeus). Biological Control **38:** 227-232

Takeda S, Sugimoto K, Otsuki H, Hirochika H (1998) Transcriptional activation of the tobacco retrotransposon Tto1 by wounding and methyl jasmonate. Plant Molecular Biology **36:** 365-376

Treier UA, Broennimann O, Normand S, Guisan A, Schaffner U, Steinger T, Muller-Scharer H (in press, 2009) Shift in cytotype frequency and niche space in the invasive plant Centaurea maculosa. Ecology **90**: 1366-1377

Treutter D (2005) Significance of flavonoids in plant resistance and enhancement of their biosynthesis. Plant Biology **7:** 581-591

United States Department of Agriculture NRCS (2008) Plants Database. *In*, p http://plants.usda.gov

van der Meijden E (1996) Plant defence, an evolutionary dilemma: contrasting effects of (specialist and generalist) herbivores and natural enemies. Entomologia Experimentalis et Applicata **80:** 307-310

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology **3:** research0034.0031 - research0034.0011

Vleeshouwers V, Van Dooijeweert W, Govers F, Kamoun S, Colon LT (2000) Does basal PR gene expression in Solanum species contribute to non-specific resistance to Phytophthora infestans? Physiological and Molecular Plant Pathology **57:** 35-42

Wang JL, Tian L, Lee HS, Wei NE, Jiang HM, Watson B, Madlung A, Osborn TC, Doerge RW, Comai L, Chen ZJ (2006) Genomewide nonadditive gene regulation in Arabidopsis allotetraploids. Genetics **172:** 507-517

Weiner J, Martinez S, MullerScharer H, Stoll P, Schmid B (1997) How important are environmental maternal effects in plants? A study with Centaurea maculosa. Journal of Ecology **85:** 133-142

Weller SA, Elphinstone JG, Smith NC, Boonham N, Stead DE (2000) Detection of Ralstonia solanacearum strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) assay. Applied and Environmental Microbiology **66:** 2853-2858

Winkel-Shirley B (2001) Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiology **126:** 485-493

Zhang DY, Jiang XH (2006) Interactive effects of habitat productivity and herbivore pressure on the evolution of anti-herbivore defense in invasive plant populations. Journal of Theoretical Biology **242:** 935-940

CHAPTER 4

A molecular approach to understanding plant–plant interactions in the context of invasion biology

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A molecular approach to understanding plant–plant interactions in the context of invasion biology.

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AKB: designed and performed research, analyzed data, wrote manuscript
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RMC, MWP, JMV: designed research, edited manuscript

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4.1 ABSTRACT

Competition is a major determinant of plant community structure, and can influence the size and reproductive fitness of a species. Therefore, competitive responses may arise from alterations in gene expression and plant function when an individual is confronted with new competitors. This study explored competition at the level of gene expression by hybridising transcripts from *Centaurea maculosa* Lam., one of North America's most invasive exotic plant species, to an *Arabidopsis thaliana* L. (Heynh) microarray chip. *Centaurea* was grown in competition with *Festuca idahoensis* Elmer, a native species that generally has weak competitive effects against *Centaurea*; *Gaillardia aristata* Pursh, a native species that tends to be a much stronger competitor against *Centaurea*; and alone (control). Some transcripts were induced or repressed to a similar extent regardless of the plant neighbour grown with *Centaurea*. Other transcripts showed differential expression that was specific to the competitor species, possibly indicating a species-specific aspect of the competitive response of *Centaurea*. These results are the first to identify genes in an invasive plant that are induced or repressed by plant neighbours and provide a new avenue of insight into the molecular aspects of plant competitive ability.

4.2 INTRODUCTION

Competition has long been considered one of the most important forces structuring communities (Griggs, 1940; Watt, 1947; Harper, 1977). Griggs (1940) argued that 'the explanation of rarity must, therefore, lie in an evaluation of the competitive competency of species'. Invasive species would appear to have substantial 'competitive competency' under some conditions because they reduce the abundance of native species, in some cases to the point of forming monocultures. Due to this competitive ability, invasive species are responsible for over 100 billion dollars of economic losses each year in the USA (Pimentel et al., 2000) and are regarded as major threats to biodiversity and human health. There are many non-exclusive hypotheses for explaining plant invasion in new habitats (Hierro et al., 2005), but competitive ability is ultimately the outcome of all of these. Competitive interactions between invasive plants and their neighbours have been studied experimentally and results tend to support the idea that the competitive effects of invaders on natives are generally stronger than the competitive effects of natives on invaders (Vila and Weiner, 2004). Competition is often resource based; however, allelopathic interactions with other plants (Bertin et al., 2003; Hierro and Callaway, 2003), flexibility in mycorrhizal partners (Bonnardeaux et al., 2007) and modification of soil microbial communities (Marler et al., 1999; Zabinski et al., 2002; Callaway et al., 2004; Carey et al., 2004; Broz et al., 2007b) may give invasive plants an additional competitive advantage. The way in which competition structures communities depends to some extent on competitive interactions that are species-specific, rather than broadly deterministic, or related to simple functional traits such as plant size. This study explores the specificity of plant competitive interactions by quantifying the

molecular response of a highly aggressive invasive species to two different native species, one shown to be a weak competitor and the other a strong competitor.

Competitive interactions among plants have been studied to a large extent at the ecological, physiological, and biochemical levels. However, there have been very few studies that consider gene expression profiles of plants subjected to competition with different neighbours. For exotics, this problem is exacerbated by the lack of molecular tools available for invasive plants and other weedy species (Basu et al., 2004). In contrast, molecular tools for model plant species such as *Arabidopsis thaliana* L. (Heynh) allow monitoring of genome-wide changes in gene expression under a variety of experimental conditions that have varying ecological relevance (Initiative, 2000; Baldwin et al., 2001; Mitchell-Olds, 2001). Because many genes are well conserved across plant species, a microarray chip representing genes from one plant can potentially be used to understand gene expression changes in another plant (Basu et al., 2004; Travers et al., 2007). Cross-species hybridisation to microarray is not yet a common technique, but has been utilised successfully in the cross-hybridisation of leafy spurge (the North American exotic, *Euphorbia esula* L.) and wild oat (*Avena fatua* L. to an *Arabidopsis thaliana* L. (Heynh) microarray (Horvath and Anderson, 2002; Horvath et al., 2003a; Horvath et al., 2003b), in hybridisation of *Andropogon gerardii* Vitman to a *Zea mays* L. array, and others (Horvath et al., 2007; Travers et al., 2007). In this context, this study attempted to apply molecular tools to an important ecological problem; exotic plant invasion.

Centaurea maculosa Lam. (*Centaurea stoebe* L. spp. *micranthos* (Gugler) Hayek, spotted knapweed) is a problematic invasive weed in the north-west USA. Although ecological and biochemical work has been done concerning the invasion process of this

noxious weed, limited genetic information is available. In this study, cross-species hybridisation of *Centaurea* cDNAs to an *Arabidopsis thaliana* L. (Heynh) microarray chip was used to help understand gene expression changes in *Centaurea* grown with a strong native North American competitor (*Gaillardia aristata* Pursh, Indian blanket flower) or a weak native competitor (*Festuca idahoensis* Elmer, Idaho fescue) (Ridenour and Callaway, 2003; Callaway et al., 2004; Callaway et al., 2005; Perry et al., 2005; Weir et al., 2006). This study attempted to validate microarray results by quantitative PCR (Q-PCR) using information from two recent *C. maculosa* expressed sequence tag (EST) sequencing projects (Broz et al., 2007a; Michelmore, 2008).

4.3 MATERIALS AND METHODS

PLANT GROWTH

Experiment 1. *Centaurea maculosa* Lam. plants were grown from seed collected near Missoula (MT, USA), plants were grown in the greenhouse for approximately six months. Seeds were germinated in 8-cm diameter pots of soil gathered from Larimer County (CO, USA), and soils were maintained near saturation with watering. These plants were used in an initial experiment in order to determine the viability of the study system and to get an idea of genes represented in the Centaurea transcriptome.

Experiment 2. In April 2005, 1.5 L of sterile 20/30 grit sand (Lane Mountain, Inc., Valley, WA, USA) was added to the bottom of 2.4-L pots and 1 L of weed-free sifted soil collected from native grasslands near Mansion Heights, MT, USA (Latitude 46.8234°, Longitude –113.996°) was placed on top of the sand. The pots were placed in a naturally

lit greenhouse supplemented with artificial light and each pot was seeded with five seeds of *Gaillardia aristata* Pursh (strong competitor) or *Festuca idahoensis* Elmer (weak competitor), species which are both native to North American rangelands invaded by Centaurea. Once seedlings were established they were thinned to one plant per pot. After the natives had grown for 30 days, a single Centaurea seed collected from an invasive population in Missoula, was added to each pot containing the native species and allowed to establish (n = 10). Single Centaurea plants were also grown in pots without natives (n = 13) as controls. The design outlined above was part of a larger experiment to investigate the competitive effects of Centaurea with conspecific and heterospecific plant neighbours. Plants were fertilised every three weeks with 100 mL of Miracle Gro (The Scotts Miracle Gro Company, Marysville, OH, USA) solution per pot (0.34 g of Miracle Gro per litre water containing 15% total nitrogen, 2% available phosphate, 20% soluble potash), watered to near saturation, and rotated on the bench every 10 days. Plants were harvested in September of 2005, when native plants were ~153 days old and Centaurea plants were 123 days old. At this time, root and shoot biomass was separated, dried at 60° C and weighed.

RNA EXTRACTION

Experiment 1. Leaves or roots were removed from one *Centaurea* plant, placed into separate tubes, and immediately frozen in liquid nitrogen. Each tissue type was ground with a mortar and pestle, and RNA was separately extracted from one 200-mg sample of roots and one 200-mg sample of leaves using the RNeasy plant mini-kit (Qiagen, Valencia, CA, USA). Total RNA was quantified using the NanoDrop spectrophotometer

(Thermo Scientific, Wilmington, DE, USA), and 0.5–1 μg of sample was run on a 1.5% formaldehyde gel to ensure samples were free of genomic DNA.

Experiment 2. Root samples of ~400 mg were harvested from at least three *Centaurea* individuals per treatment and immediately placed in RNAlater reagent (Ambion, Austin, TX, USA) in 1.6-mL tubes. Samples were stored at –80°C until processed. Each individual root sample was removed from RNAlater solution, quickly blotted on filter paper to remove excess liquid, frozen in liquid nitrogen, and pulverised. RNA was extracted using the RNeasy plant mini-kit (Qiagen). Root samples from each individual plant were kept separate during RNA isolation and subsequent microarray analyses in order to allow for biological replication. RNA samples were quantified and checked for purity as described above.

MICROARRAY ANALYSIS

Experiment 1. The *Arabidopsis* OAR27K gene chip was hybridised with labelled cDNA probes produced from samples of *Centaurea* RNA at the W. M. Keck Foundation Biotechnology Resource Laboratory, Yale University, USA. The OAR27K gene chip consists of 70-mer oligos representing ~27 000 genes from *Arabidopsis*. One leaf sample and one root sample were reverse-transcribed into cDNA and each was labelled with a fluorescent dye (Cy3 and Cy5, respectively) using the Genisphere Array 900 kit (Cat No.W500180) (Genisphere, Hatfield, PA, USA). Labelled cDNAs were hybridised to the

OAR27Kchip using the Advalytix ArrayBooster DNA Microarray Incubator (Advalytix, Boston, MA, USA). Approximately 10 μg of each sample was used for hybridisation.

Experiment 2. Hybridisation and labelling followed the procedure above, except the experimental control was *Centaurea* plants grown alone, and the test situations were *Centaurea* grown with *Gaillardia* or *Festuca* neighbours. In each experiment, RNA from *Centaurea* grown alone was used as the control (labelled with Cy3) and RNA from *Centaurea* grown with a neighbour was used as the test (labelled with Cy5). Two biological replicates were performed for each test situation. Tissue samples were limited so the second repetition of the *Centaurea-Festuca* experiment used the control of *Centaurea* grown with another *Centaurea* plant.

MICROARRAY DATA ANALYSIS

Gene Pix Pro 5.0 analysis software (Axon Instruments Inc., Foster City, CA, USA) was used to analyse the gene chips. Gene Pix Pro (gpr) and image files for each experiment were uploaded to a secure site through the Yale University server where they could be accessed for analysis (http://ymd.med.yale.edu/ymd_prod/cgi-bin/gz_login.cgi, accessed January 2007). Features were flagged, as described in the Gene Pix manual (page 21), if they were not found or if they were overlapping adjacent features. In the preliminary experiment, all of the genes that were not flagged were considered in the analysis without using fold cut-offs for up-regulation or down-regulation between tissues, to get a general overview of hybridisation. For each chip, flagged genes were removed

from the analysis. The average background was calculated for each dye channel (wavelength 635 and 532 nanometers), and only the genes that had pixel counts (median minus background) over two times the average background, in at least one channel, were considered. In Experiment 2, each chip was initially analysed separately. Flagged values and all channel values that were less than two standard deviations above the mean background were removed from the analysis. Ratios ((F635-B635)/(F532-B532)) of the remaining values were averaged per chip, and correction factors were calculated for each chip, in order to set the average of all ratios equal to one. Ratios were multiplied by the appropriate correction factor to globally normalise the data. Oligos (array spots) that were present in all four chips were chosen for further analysis, which amounted to 547 oligos. Although this lowered the number of genes in the final analysis, it increased confidence in the data, and allowed comparison of the two experimental conditions. Replicates (two per experimental condition) were combined and the average ratios and respective standard deviations were calculated for each oligo. A one sample *t*-test was performed to identify oligos in which the test condition (*Centaurea-Gaillardia* or *Centaurea-Festuca*) was significantly different from the control (*Centaurea* alone). A two sample *t*-test was performed to identify oligos that were differentially regulated between experiments (*Centaurea-Gaillardia* and *Centaurea-Festuca*). Only *P*-values of 0.05 or less were considered significant for both the one and two sample *t*-tests, and only changes in expression exceeding 2-fold were considered differentially expressed. Because the gene list was relatively short after filtering, and because both fold-change and *P*-value ($P < 0.05$) were used to determine genes that were differentially expressed, the values were not further adjusted to account for multiple testing issues. This method

was appropriate because, as stated by the MicroArray Quality Control (MAQC) Consortium (Shi et al., 2006), 'a straightforward approach of fold change ranking plus a non-stringent *P* cut-off can be successful in identifying reproducible gene lists, whereas ranking and selecting differentially expressed genes based solely by the *t*-test statistic predestine a poor concordance in results, in particular for shorter gene lists, due to the relatively unstable nature of the variance (noise) estimate in the *t*-statistic measure'. Accession numbers for each of the 547 genes present on all four chips were used to query the TAIR (www.arabidopsis.org, January 2007) database to determine function, and genes were then grouped into functional categories based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) Ontology System (www.genome.jp/kegg/brite.html, February 2007). All microarray data was uploaded to the NCBI Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/, August 2008).

QUANTITATIVE PCR ANALYSIS OF GENE EXPRESSION

Remaining RNA from the initial extractions (Experiment 2) was treated with DNase and translated to cDNA using reverse transcriptase, following a protocol from Invitrogen (Carlsbad, CA, USA). This cDNA was used to analyse specific expression profiles for a subset of genes identified in the microarray analysis. Primers were designed (Table 4.1) based on sequence information from two recent *Centaurea maculosa* EST sequencing projects: (Broz et al., 2007a), (http://bioinfo.noble.org/plan/, project 30060, accessed November 2007) and a collection of ESTs in the NCBI database from The Compositae Genome Project at the University of California, Davis

(Michelmore, 2008). Full-length *Arabidopsis* gene sequences corresponding to eight oligos on the microarray were used as queries in a tBLASTx homology search of *Centaurea* EST sequences in the NCBI database.

Table 4.1. Primer information table for quantitative PCR using *Centaurea* cDNA.

Gene annotation	Arabidopsis Accession	Centaurea Accession	Primer sequence (5'-->3')
ABC transporter, GCN subfamily	At5g60790	EH727505	ATTCTCGTGGTTGGCTTATCGGCA
			ACACCCATATCTCGTGAGCCACTT
RAD 16 homolog, DNA repair	At1g05120	EH744349	TGCACGAGATAAAGCGGTGAAGGA
			CATCACATGGCAAGCAGCAACCAT
GTPase, ADP ribosylation	At1g70490	EH747103	AGATGCAGTGTTGCTTGTGTTCGC
			TGGAAAGCCAATCGAGTCCCTCAT
Adenine nucleotide translocator	At3g08580	EH723883	ATGCCAAGGCTGCAAAGAAAGGAG
			ATCCAGCAATACCGTCTGAAGCCA
COR13, cystine and ethylene synthesis	At4g23600	EH715585	GCTGCACTCAAGCCATACAAACCA
			CTGCAAGAGCTTCAACCGCATCAA
Unknown	At1g22750	EH749036	TGGTGTACTAGGCTGCTTGATGCT
			TCCATACCGCTAAAGCCACAACGA
Cyclin dependant kinase	At1g76540	EH727616	AGAGATGCAGCATGGGAACATCGT
			ACTCGGGACAAGAGTCCATGTGTT
Spermidine synthase (Polyamines)	At5g53120	EH726127	GCATGTGGCTTCACACCCATCTTA
			AATCAACATCTGGTCCCTCCGTTG
Actin	NA	EL933268	ACCAACATGAGAACAACCGATAC
			TCACACTGGTGTCATGGTCGGAAT
Cytochrome C Oxidase[a]	NA	NA	CGTCGCATTCCAGATTATCCA
			CAACTACGGATATATAAGAGCCAAAA CTG
Ubiquitin[b]	NA	NA	ACAACATCCAGAAGGAGTCC
			GCAACACAGCAAGCTTAACC
Elongation factor 1[b]	NA	NA	CTCCAAGGCTAGGTATGATG
			TTCGTGGTGCATCTCAACAG

[a]Weller *et al.*, 2000, [b]designed using tobacco

Candidate *Centaurea* ESTs were identified and nucleotide sequences were aligned with the *Arabidopsis* 70-mer oligo from the OAR27K chip and the full-length *Arabidopsis* gene sequence from which the oligo was derived using CLC free workbench software (CLC Bio, Cambridge, MA, USA). Primers were designed to be specific to the *Centaurea* EST sequence that showed the highest homology to the oligo and/or full-length *Arabidopsis* sequence. The first and last 100 base pairs of each candidate EST sequence were not utilised for primer design to avoid issues with possible EST sequencing errors.

All reactions were run in duplicate and analysed using the BioRad iCycler machine and software (Hercules, CA, USA). A standard curve was created for each primer set using serial dilutions (concentrations of 5–625 ng μL^{-1}) of cDNA prepared from roots of a greenhouse-grown *Centaurea* plant (fresh tissue was frozen in liquid nitrogen, and RNA extraction and cDNA synthesis followed the protocol above), and negative controls using water instead of template were run for all reactions. The optimal annealing temperature for all primer sets was found to be 55°C. All PCR reactions had a final volume of 20 μL and contained 10μL of 2x Jumpstart SYBR green reaction mix (Sigma Aldrich, Saint Louis, MO, USA), 0.2 μL of 1μM flourescein, 2.4 μL of 25 mM $MgCl_2$, 0.2 μL of 10μM forward primer, 0.2 μL of 10 μM reverse primer, 2μL of template (118 ng μL^{-1}) and 5 μL of sterile H_2O. Reactions conditions for PCR were as follows: 95°C for 30 s, 55°C for 30 s and 72°C for 30 s, for 40 cycles. Amplified gene products ranged from 90–200 base pairs.

For each sample, total RNA (ng μL^{-1}) was estimated using the appropriate standard curves and normalised using the geometric mean of several housekeeping genes

(actin, cytochrome *c* oxidase, elongation factor 1, and ubiquitin) as suggested in Vandesompele et al. (2002) (Vandesompele et al., 2002). A ratio was then calculated to determine differential gene expression between the two competitive systems verses *Centaurea* grown alone.

4.4 RESULTS

INITIAL HYBRIDISATION: PARTIAL REPRESENTATION OF CENTAUREA TRANSCRIPTOME

In an initial experiment, 1254 *Arabidopsis* oligos (4.6% of oligos on the microarray) reliably hybridised with *Centaurea* root and leaf samples. Over 10% of the transcripts hybridising in the initial experiment were functionally categorised as transcription-related factors (Figure 4.1). Multiple transcription factor families were represented including members of WRKY, myb domain, scarecrow-like, MADS box, bZIP, and hormone responsive families. Putative components of signal transduction pathways were identified, including G-proteins, calmodulin-binding proteins, kinases, phosphatases, and sequences containing the Leucine-Rich-Repeat (LRR) and F-box protein interaction domains. A variety of stress response genes were found in the analysis including cytochrome P450, cytochrome *c*, dehydrins, pathogen responsive genes, and heat shock family proteins. Families of transport proteins, such as ABC-transporters, AAA-type ATPases, and nutrient/ion/organic-acid transporters were represented in the analysis. Over 23% of the transcripts were annotated as metabolic enzymes, representing groups such as carbohydrate, lipid, amino acid, and energy

metabolism (Figure 4.1). Approximately 37% of the hybridised transcripts fell into the unknown category.

Functional annotation of genes in preliminary microarray experiment

Functional Category	#	%
Cellular Processes and Signaling	102	7.3
Environmental Information Processing	111	8.0
Genetic Information Processing	336	24.2
Metabolism	323	23.3
Unknown	517	37.2

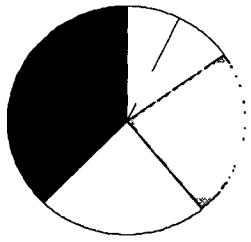

Figure 4.1. Functional categorization of genes identified in preliminary cross-species hybridization to microarray. In a preliminary experiment, *Centaurea maculosa* root and leaf cDNAs were used to probe the *Arabidopsis* OAR27K microarray chip. Accession numbers for each gene identified in the microarray analysis were used to query the *Arabidopsis* gene database to determine function, and genes were then grouped into functional categories based on the Kyoto Encyclopedia of Genes and Genomics (KEGG) Orthology System. The number and percentage of annotations is given for each functional category and is represented as a pie chart.

Despite the overall low level of hybridisation, some expected patterns of gene expression between roots and leaves were observed. Approximately 6% of the identified transcripts were preferentially expressed over 2-fold in roots verses leaves (data table available as online supplement for this publication at CSIRO publishing website; http://www.publish.csiro.au/?act=view_file&file_id=FP08155_AC.pdf, pages 1-99). Although the majority of these transcripts have not been annotated as root-specific proteins, a variety were identified that may be of greater importance in roots than in leaves. For instance, two transcripts involved in drought resistance (*At3g57600* and *At5g45340*), a metal transporter (*At2g35730*), an organic cation transporter (*At1g16390*), and a nodulin related transcript (*At4g19450*) were all preferentially expressed in roots.

Additionally, a multi-copper oxidase (*At1g23010*), primarily involved in root growth and phosphate sensing (Svistoonoff et al., 2007), was highly expressed in roots. Twenty transcripts involved in photosynthesis were identified in the analysis, nineteen of which showed preferential expression in leaves compared with roots (see online supplement, green highlights). These included plastocyanins, chlorophyll binding proteins involved in the light harvesting complex, and components of photosystems I and II.

COMPETITION EXPERIMENTS: PHENOTYPE AND BIOMASS MEASUREMENTS

There were notable phenotypic differences in *Centaurea* plants growing with the strong competitor (*Gaillardia aristata*) compared with plants grown with the weak competitor (*Festuca idahoensis*) (Figure 4.2). *Centaurea* plants grown with the strong competitor *Gaillardia* showed a 75% reduction in mean dry weight when compared with *Centaurea* grown alone, whereas *Centaurea* grown with the weak competitor *Festuca* showed a ~30% reduction in biomass. This trend was similar for measurements of below and above ground biomass.

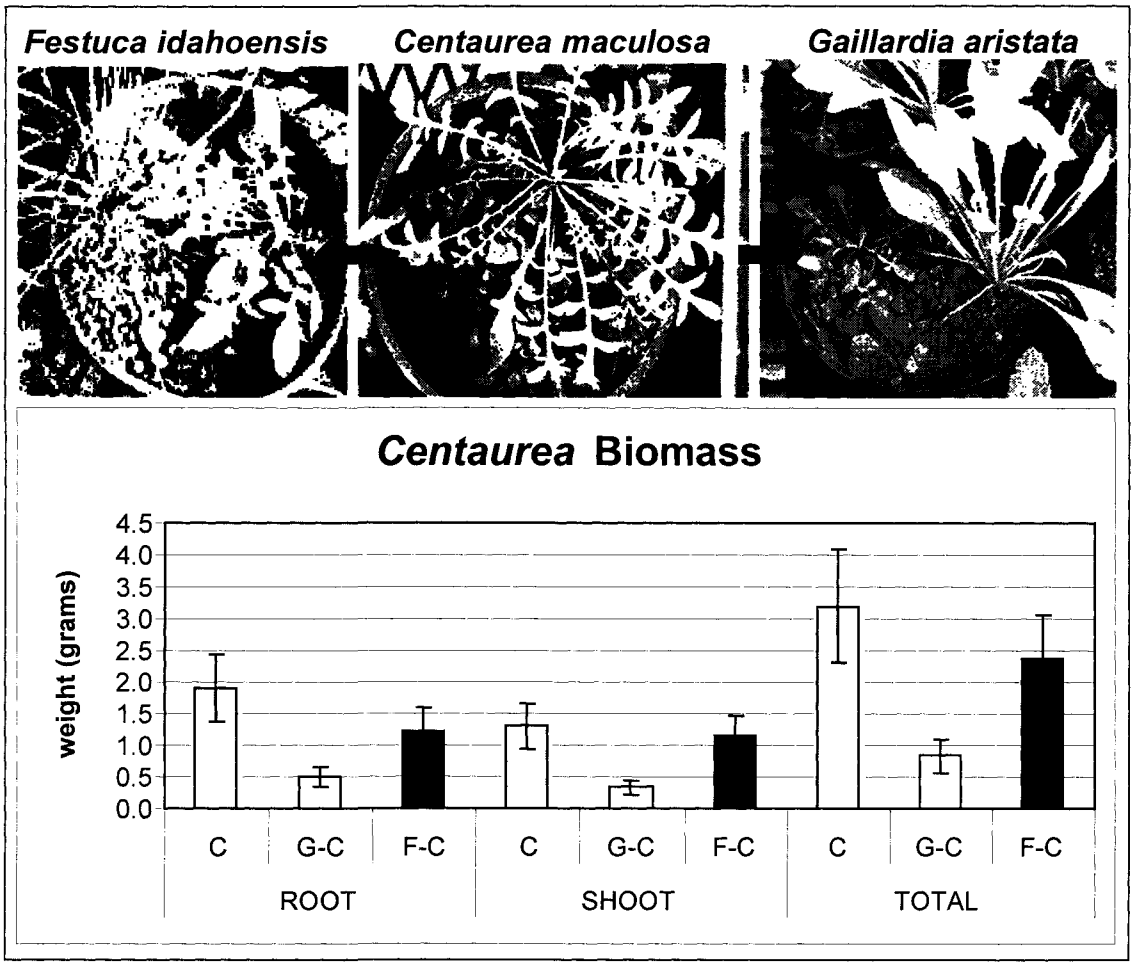

Figure 4.2. Biomass of *Centaurea* plants grown alone and with neighbors. *Centaurea maculosa* plants were grown either alone (C), with *Gaillardia aristata* (strong competitor) (G-C), or with *Festuca idahoensis* (weak competitor) (F-C) for approximately four months. At this time, *Centaurea* roots and shoots were separated, dried and weighed for biomass measurements. Error bars represent one standard error of the mean.

COMPETITION EXPERIMENTS: SIMILAR REGULATION OF TRANSCRIPTS BETWEEN EXPERIMENTS

In order to compare the two competitive situations, only transcripts that met filtering criteria (see section 4.3 Materials and Methods) and that were present in all four

arrays were included in the final analysis, resulting in a total of 547 transcripts. Of these, 454 transcripts were not differentially regulated to our threshold of a 2-fold increase or decrease, and were not significantly different between competitive situations using a 2-sample *t*-test ($P < 0.05$). This group included multiple genes associated with nutrient transport and abiotic stress response (data table available as online supplement for this publication at CSIRO publishing website; http://www.publish.csiro.au/?act=view_file&file_id=FP08155_AC.pdf, pages 100-128). In addition, genes involved in photosynthesis were not differentially expressed between conditions.

In the microarray analysis, six transcripts were downregulated over 2-fold when *Centaurea* was grown with either plant neighbour, relative to *Centaurea* grown alone. Gene accession numbers and functional annotations of these transcripts are listed in Table 4.2 under the section 'Down in Both'. Only one transcript was shown to be upregulated in both experimental conditions (Table 4.2, 'Up in Both').

COMPETITION EXPERIMENTS: DIFFERENTIAL REGULATION OF TRANSCRIPTS BETWEEN EXPERIMENTS

Ninety-three transcripts were differentially regulated between experiments; their regulation depended on the plant neighbour grown with *Centaurea*. Of these, only 36 were significantly upregulated or downregulated over 2-fold in at least one experimental condition ($P < 0.05$) (Table 4.2).

Table 4.2. Differentially expressed genes identified in microarray analysis of competition experiments. *Centaurea maculosa* Lam. plants were grown alone (control) or with a strong competitor (*Gaillardia aristata* Pursh) or a weak competitor (*Festuca idahoensis* Elmer). Roots were collected from *Centaurea* plants grown in each of these conditions and resulting cDNAs were used to probe the *Arabidopsis thaliana* L. (Heynh) OAR27K microarray chip. Duplicate microarrays were performed for each test condition, and only genes up or downregulated over two times were further analysed. Accession numbers for each gene identified in the microarray analysis were used to query the *Arabidopsis* gene database to determine function, and genes were then grouped into functional categories based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) Ontology System. Number and percent representation of genes up or downregulated over 2-fold in at least one test condition (in comparison to the control) are listed by functional category. There were 36 genes and 39 functional annotations. Table legend information: ID, accession number; Function, functional annotation; FunCat, KEGG functional category (CPS, Cell processing and signalling; GIP, Genetic information processing; EIP, Environmental information processing; M, Metabolism; U, Unknown), Average C-G (average ratio of *Centaurea-Gaillardia* to *Centaurea* alone), Average C-F (average ratio of *Centaurea-Festuca* to *Centaurea* alone), *P*-values in parentheses refer to 1 sample *t*-test (comparison of test condition to control), final column represents *P* values from 2 sample *t*-test (comparison between two test conditions). Note; this table is continued on the following three pages.

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

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	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

Of the 36 transcripts, nine were significantly downregulated when *Centaurea* was grown with *Gaillardia*, but showed no change when *Centaurea* was grown with *Festuca* in comparison to the control, *Centaurea* grown alone (Table 4.2, 'Down in Gaillardia, no change in Festuca'). One transcript of unknown function showed no change when *Centaurea* was grown with *Gaillardia*, but was significantly downregulated when *Centaurea* was grown with *Festuca* in comparison to the control (Table 4.2, 'No change in Gaillardia, down in Festuca'). Twenty-five transcripts showed no change when *Centaurea* was grown with *Gaillardia*, but were significantly upregulated when *Centaurea* was grown with *Festuca* (Table 4.2 'No change in Gaillardia, up in Festuca').

QUANTITATIVE PCR

In order to assess the validity of the microarray results using another technique, eight transcripts were chosen for analysis by Q-PCR, which is often regarded as the 'gold standard' assay for measuring gene expression (Qin et al., 2006). Remaining cDNA from the microarray analyses was used to represent each competitive situation. Primers were designed to be specific to the *Centaurea* EST sequence in NCBI that showed the highest homology to the oligo and full-length *Arabidopsis* sequence upon BLAST search. The general trends of gene expression observed using the microarray were validated for half of the transcripts tested (Table 4.3). However, validation of expression trends was not based solely on transcript or on competitive situation. For instance, results for only two of the eight transcripts were validated for both competitive situations (ABC transporter and cyclin dependant kinase, Table 4.3). Three results were validated for *Centaurea*

grown with *Festuca* and five for *Centaurea* grown with *Gaillardia*, verses *Centaurea* grown alone.

Table 4.3. Q-PCR validation of microarray. Eight genes were selected for quantitative PCR analysis. Gene annotations and accessions for both the *Arabidopsis* oligo and the *Centaurea* EST sequence are listed in the table above followed by values generated from microarray and RT-PCR. For each sample, total RNA (ng/μL) was estimated using the appropriate standard curves and normalized using the geometric mean of actin, cytochrome c oxidase, elongation factor 1, and ubiquitin, as suggested in Vandersompele *et al.*, (2002). A ratio was then calculated to determine differential gene expression between the two competitive systems versus the control. Symbols denote values of the ratio of each competitive condition (*Gaillardia* or *Festuca*) relative to the control (*Centaurea* alone). Transcripts that showed similar expression trends using both microarray and Q-PCR are boxed. Expression ratio notation: <0.2 (---); 0.21-0.5 (--); 0.51-0.9 (-); 1.1-2 (+); 2.1-5 (++); >5.1 (+++).

Gene annotation	Arabidopsis Accession	Centaurea Accession	Competitor	Array	Q-PCR
ABC transporter, GCN subfamily	At5g60790	EH727505	*Gaillardia*	---	-
			Festuca	---	--
RAD 16 homolog, DNA repair	At1g05120	EH744349	*Gaillardia*	--	+
			Festuca	--	+
GTPase, ADP ribosylation	At1g70490	EH747103	*Gaillardia*	+++	--
			Festuca	+++	+
Adenine nucleotide translocator	At3g08580	EH723883	*Gaillardia*	--	-
			Festuca	++	-
COR13, cystine and ethylene synthesis	At4g23600	EH715585	*Gaillardia*	--	-
			Festuca	+	--
Unknown	At1g22750	EH749036	*Gaillardia*	-	++
			Festuca	---	++
Cyclin dependant kinase, cell cycle	At1g76540	EH727616	*Gaillardia*	-	-
			Festuca	++	+
Spermidine synthase (Polyamines)	At5g53120	EH726127	*Gaillardia*	--	--
			Festuca	++	-

4.5 DISCUSSION

Cross-species hybridisation to microarray chips may represent a valuable resource for understanding gene expression changes in plants that have not been genetically or molecularly well characterised (Basu et al., 2004; Travers et al., 2007), such as *Centaurea*. In the preliminary hybridisation experiment using root and leaf tissue, 4.6% of the *Arabidopsis* microarray oligos hybridised with *Centaurea* cDNAs. This value is low in comparison with other cross-species hybridisations, which report between 23 and 47% hybridisation (Horvath and Anderson, 2002; Horvath et al., 2003a; Horvath et al., 2003b; Horvath et al., 2007). It is possible that the genomes of *Centaurea* (asteraceae) and *Arabidopsis* (brassicasceae) are highly divergent resulting in poor hybridisation; however, Horvath *et al.* (2003a) successfully hybridised the distantly related wild oat (*Avena fatua* L., poaceae) cDNAs to an *Arabidopsis* microarray. Alternatively, the low amount of hybridisation could be due to the fact that the *Arabidopsis* array used in the current study contained relatively short oligos as opposed to longer, full-length cDNAs that were contained on spotted arrays used in other studies. In addition, the spotted arrays used in Horvath *et al.* (2003a; 2003b) contained ~32% redundant cDNAs (Schaffer et al., 2001), which may help to explain the higher rates of hybridisation observed in those experiments. Regardless, in the current study, a wide variety of functional categories were represented by the hybridised transcripts making the discovery of important regulatory changes in competition experiments possible. In addition, transcripts involved in photosynthesis were found to be preferentially expressed in *Centaurea* leaves, whereas a gene primarily involved in root growth was preferentially

expressed in the roots. This expected result increased our confidence that large changes between competition systems would be detectable using cross-species hybridisation.

In the competition experiments, two biological replications were performed for each of the two experimental conditions (*Centaurea* grown with *Gaillardia* or *Centaurea* grown with *Festuca*) and stringent filtering criteria were used to analyse the microarray data. This resulted in only 6 transcripts that were induced or repressed similarly in *Centaurea* regardless of the plant neighbour (in comparison to *Centaurea* grown alone), and 36 transcripts showing differential expression dependant on the identity of the plant neighbour.

In the initial hybridisation experiment, no attempts were made to validate the microarray results using an alternative technique. However, independent validation of microarray remains an important consideration for gene expression data (Dallas et al., 2005; Morey et al., 2006; Qin et al., 2006). Therefore, the results from the competition microarray experiments were verified with a Q-PCR based approach using recently deposited *Centaurea* sequence information in the NCBI database (Broz et al., 2007a; Michelmore, 2008). When both competition systems were considered, only two of the eight genes examined by Q-PCR validated the gene expression trends identified by microarray (Table 4.3). More generally, eight transcriptional profiles were validated for at least one competition system, but the other eight showed the opposite trend. Although only 50% of the expression trends found on the array were validated using Q-PCR, genes were identified that were either differentially or similarly expressed based on plant neighbour identity.

The discrepancies between the microarray and Q-PCR data could be the result of many factors. One potential reason for this discrepancy could be related to genome size, and its relationship to hybridisation. The tetraploid form of *C. maculosa* examined in this experiment has an approximate genome size of 3600 Mbp (diploid, $2n = 18$, 2C ~3.6 pg, (Grime et al., 1985), almost 30 times larger than that of *Arabidopsis* (The Arabidopsis Genome Initiative 2000, (Initiative, 2000)). Because *Centaurea* occur as tetraploids, they are expected to have at least two copies of every gene, although all copies may not be functional or expressed. There is not a complete genome sequence for *Centaurea* and the EST libraries available contain only a limited amount of sequence information; thus, it is highly possible that multiple *Centaurea* cDNAs hybridised with oligos on the *Arabidopsis* microarray. As only one candidate *Centaurea* EST was examined by Q-PCR per microarry spot, it ma y not be reasonable to expect that the same trends would be found using both techniques. If multiple *Centaurea* cDNAs are hybridising to a single spot on the *Arabidopsis* microarray, this constitutes a problem for any validation technique. Correlating the expression data from one microarray spot to expression profiles for multiple potentially hybridising genes would be extremely challenging using methods of Q-PCR or other generally accepted validation techniques such as Northern hybridisations. The observed lack of correlation between the techniques does not necessarily invalidate either dataset. The Q-PCR approach is gene specific, whereas the microarray approach may represent either hybridisation of multiple transcripts or transcripts that were not investigated using Q-PCR due to a lack of *Centaurea* sequence information.

In a general sense, there is currently no standard protocol for validation of microarray results (Morey et al., 2006). Differences between array platforms, validation techniques, normalisation practices and analysis methods often result in different gene expression profiles, even when the original data are obtained from the same sample or raw dataset (Morey et al., 2006; Qin et al., 2006). Oligonucleotide microarray and Q-PCR results are often in disagreement, even in model organisms with sequenced genomes (Dallas et al., 2005; Morey et al., 2006; Qin et al., 2006). For instance, in a study of mouse (*Mus* sp.) brain and blood samples, Morey et al. (2006) found that microarray expression trends were not validated by Q-PCR for 27% of 277 genes examined. Similarly, in a clinical setting, Dallas et al. (2005) observed poor correlations between normalised array and Q-PCR data for up to 16% of 48 genes examined from human (*Homo sapien*) brain tumour and leukemia specimens. As this study was working with a non-model organism and used cross-species hybridisation to microarray, it was expected that there might be a greater percentage of inconsistencies between the two techniques than have been found in studies of model organisms.

Microarray analysis is often used as a starting point to identify candidate genes that exhibit modified expression in response to a particular experimental condition; however, it is important to confirm microarray data using a complimentary technique such as Q-PCR to ensure the validity of the results (Dallas et al., 2005; Morey et al., 2006; Qin et al., 2006). Because there were no specific *a priori* assumptions concerning gene expression changes related to plant neighbour identity, a cross-species hybridisation microarray was used here in order to identify candidate genes that may be important in plant-neighbour interactions. Although a variety of candidate genes were identified,

interesting genes may have been missed due to the overall low levels of hybridisation. This approach was an appropriate starting point; however, these results demonstrate the necessity of gene-specific approaches such as Q-PCR, particularly when microarray data is generated from cross-species hybridisations.

Q-PCR is generally considered a more robust technique than microarray, and because of the multiple issues involved in validating genes from cross-species hybridisations, Q-PCR data was considered to be more reliable than the data generated from the microarray. In addition, dye-swaps were not performed on the samples in the microarray experiments and thus, different levels of dye may have been incorporated into samples, biasing the results. Therefore, the remaining discussion focuses predominately on gene expression results gained using the Q-PCR approach, as the microarray data may contain a large amount of false positives. Although the results for specific genes often differed using the two different techniques, the general result, that the *Centaurea* transcriptome responds distinctly to different neighbour plants, remained the same using either technique.

Gene expression profiles of *Centaurea* varied depending on whether the species was competing with *Gaillardia*, competing with *Festuca*, or experiencing no competition. These results suggest that different plant competitors can induce or suppress the expression of certain genes in neighbouring plants, and suggest that competition can be highly neighbour-species specific.

Currently, little is known about specific genes involved in the competitive ability of plants, particularly those involved in non-resource based competition. This makes it difficult to mechanistically link identified upregulation and downregulation of genes to

specific aspects of plant competition. Some competitive processes appear to be regulated by specific networks of transcription factors (Pabo and Sauer, 1992) or signalling pathways that trigger enhanced metabolic activity or defence responses (Schenk et al., 2000; Kunkel and Brooks, 2002; Cipollini, 2004). Other studies suggest that shade avoidance and the adjustment of nutrient allocation are important aspects of plant competition (Rajcan et al., 2004).

Interestingly, in this microarray analysis, many genes involved in the transport of specific nutrients and abiotic stress response were not significantly induced or repressed, and their expression did not differ between competitive situations. The observed similarity in expression of these genes may indicate that plants were not competing intensively for resources, and were affected to a greater extent by non-resource based competition. In addition, expression of genes involved in photosynthesis did not differ between competitive situations. However, because root tissue was used in this experiment it cannot be concluded from the expression analysis alone that the plants were not competing for light.

Centaurea has been hypothesised to gain a competitive advantage over susceptible plant competitors through allelopathy (Ridenour and Callaway, 2001), by exuding the flavonoid (±)–catechin from its roots (Bais et al., 2002). However, this study was only able to identify one gene potentially involved in flavonoid biosynthesis (*Flavanol Synthase 1*, *FLS1*) in the microarray analysis and it was not differentially regulated (data table available as online supplement at CSIRO publishing website; http://www.publish.csiro.au/?act=view_file&file_id=FP08155_AC.pdf, pages 100-128). As catechin exudation appears to be sporadic under field conditions (Perry et al., 2007),

one would not necessarily expect to see induction of these types of genes under greenhouse conditions at the single time point sampled.

Using Q-PCR, this study identified six transcripts that were similarly induced or repressed when *Centaurea* was competing with either plant neighbour (Table 4.3). Four of these transcripts were downregulated in both competition systems. Three of the downregulated transcripts are potentially involved in cell growth and signalling, including an adenine nucleotide translocator, *COR13* which is involved in cystine and ethylene synthesis, and a spermidine synthase which produces secondary metabolites that are important regulators of cell growth (Hanzawa et al., 2002). An ABC transporter was also downregulated in both competition systems. ABC transporters are directly involved in the active transport of molecules (Schulz and Kolukisaglu, 2006), and were also found to be downregulated in corn (*Zea mays* L.) due to competition with velvetleaf (*Abutilon theophrasti* L.) (Horvath et al., 2006). Interestingly, *RAD16* a transcript involved in DNA repair and chromatin remodelling (Lindsey-Boltz et al., 2001) was upregulated in both competition systems, which may indicate a response to competition at the level of DNA modification.

The fact that six transcripts were induced or repressed to similar extents in both the *Centaurea-Festuca* and *Centaurea-Gaillardia* competition systems suggests that these genes may act as general 'plant-neighbour interaction' genes, the expression of which is modulated merely by the presence of a heterospecific plant neighbour. The genes identified in this experiment are expected to be part of a sustained response, as *Centaurea* plants had been growing with neighbours for roughly four months, and this

may help explain the variety of transcripts that were found to be similarly regulated between test conditions.

Genes that were differentially regulated between the competitive situations may be indicative of a species-specific plant neighbour response. When *Centaurea* was grown with the weak competitor *Festuca* Q-PCR results showed a significant upregulation of two genes that are potentially involved in growth and cell cycle regulation (Table 4.3): a cyclin dependant kinase which regulates the cell cycle and a GTPase/ADP-ribosylation factor which is involved in genetic information processing and plays an indirect role in transport through signalling cascades (Kahn and Gilman, 1984; Lee et al., 2002). These transcripts were both downregulated when *Centaurea* was grown with *Gaillardia*, which may reflect the reduced size of the *Centaurea* plant when it is grown with the stronger competitor.

It seems somewhat paradoxical that *Centaurea* grown with *Festuca* would show induction of more growth genes than *Centaurea* grown alone considering that the total *Centaurea* biomass was not significantly different between the two conditions. However, final biomass is not necessarily related to the rate of plant growth at the time of sampling. In addition, several studies suggest that under some conditions *Festuca* can actually increase the growth of *Centaurea* though interactions with arbuscular mycorrhizal (AM) fungi (Marler et al., 1999; Zabinski et al., 2002; Callaway et al., 2004; Carey et al., 2004). Underground connections through microorganisms can cause a relocation of nutrients between plants (van der Heijden et al., 2003; Wolfe and Klironomos, 2005), and *Centaurea* may benefit from these associations, undergoing increased cell division when it is grown with a *Festuca* neighbour as opposed to growing alone. Most fungal

connections can form only after the development of significant root biomass which could lead to a relative increase in *Centaurea* growth rates and upregulation of growth related genes later in its life-cycle. As this experiment was conducted using native Montana soils, activity of microorganisms could influence the final competitive advantage of one plant over another.

An alternative explanation for the increase in growth related genes in *Centaurea* due to *Festuca* competition may be related to differences in the shade avoidance response of *Centaurea* when it is grown with different competitors. *Centaurea* plants develop as low growing rosettes, and in this study *Centaurea* was partially shaded by both neighbouring plant species (Figure 4.2). Therefore, one might expect that *Centaurea* plants would exhibit a strong shade avoidance response in both competitive situations compared with the control. One hallmark of the shade avoidance response is a decrease in the root : shoot ratio resulting from a considerable increase in shoot growth (Rajcan et al., 2004). The average root : shoot ratio of *Centaurea* growing alone (1.45) did not differ greatly from the root : shoot ratio of *Centaurea* growing with the strong competitor *Gaillardia* (1.52), even though their mean weights differed over 3-fold (Figure 4.2). However, the root : shoot ratio of *Centaurea* growing with *Festuca* was substantially reduced (1.07) compared with *Centaurea* alone, indicating that the *Festuca* neighbour may induce a shade avoidance response in *Centaurea*.

As mentioned previously, two genes potentially involved in growth, a cyclin dependant kinase and an ADP ribosylation factor, were preferentially expressed in the roots of *Centaurea* growing with *Festuca* (Table 4.3) which may be related to the shade

avoidance response. However, other genes potentially involved in growth and signalling processes (*COR13*, spermidine synthase, and an adenine nucleotide translocator) were downregulated; a response which was also found in *Centaurea* growing with *Gaillardia* (Table 4.3).

In a study of velvetleaf-corn interactions, Horvath *et al.* (2007) found preferential expression of cell growth and ethylene responsive genes in velvetleaf plants that were shaded by corn, a potential result of the shade avoidance response. A complimentary study found decreased expression of these types of genes in corn due to velvetleaf competition (Horvath et al., 2006). In this experiment corn overtopped velvetleaf throughout the growing period and the sampled tissue had not experienced shading (Horvath et al., 2006). In *Arabidopsis*, simulated shading resulted in preferential expression of a substantial number of growth related genes including a spermine synthase gene (*At5g119530*) (Devlin et al., 2003), the opposite of what was found for the spermidine synthase expression in both competition systems of the present study (Table 4.3). One difficulty in comparing the results of previous studies to the current study is that different plant tissues were used. Other studies investigated transcriptional changes in leaf tissue (Devlin et al., 2003; Horvath et al., 2006; Horvath et al., 2007), whereas the present study investigated root tissue. It is difficult to know if the transcriptional changes that take place in leaves due to competition or shading would be similar to the changes that occur in root tissue. Studies investigating gene expression changes in *Centaurea* leaves due to competition with neighbouring plants may help clarify this issue.

4.6 CONCLUSIONS

The data presented here provide new information about gene regulation in plant competition; however, they are limited in scope. The data describe interactions between *Centaurea* and specific neighbours 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 analyse transcript profiles of *Centaurea* grown in various environmental conditions, including a sterile environment. It would also be beneficial to analyse 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 neighbours 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 neighbours 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 hybridise reliably with *Centaurea* cDNAs, greatly reducing the amount of genes that could be analysed. Q-PCR techniques revealed neighbour 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 neighbour identity are having an effect on *Centaurea* gene expression.

This is the first report of using cross-species hybridisation 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 characterising competitive systems, it may be possible to identify molecular factors that increase plant competitive ability and facilitate invasion by exotics.

4.7 REFERENCES

Bais HP, Walker TS, Stermitz FR, Hufbauer RA, Vivanco JM (2002) Enantiomeric-dependent phytotoxic and antimicrobial activity of (+/-)-catechin. A rhizosecreted racemic mixture from spotted knapweed. Plant Physiology **128:** 1173-1179

Baldwin IT, Halitschke R, Kessler A, Schittko U (2001) Merging molecular and ecological approaches in plant-insect interactions. Current Opinion in Plant Biology **4:** 351-358

Basu C, Halfhill MD, Mueller TC, Stewart CN (2004) Weed genomics: new tools to understand weed biology. Trends in Plant Science **9:** 391-398

Bertin C, Yang XH, Weston LA (2003) The role of root exudates and allelochemicals in the rhizosphere. Plant and Soil **256:** 67-83

Bonnardeaux Y, Brundrett M, Batty A, Dixon K, Koch J, Sivasithamparam K (2007) Diversity of mycorrhizal fungi of terrestrial orchids: compatibility webs, brief encounters, lasting relationships and alien invasions. Mycological Research **111:** 51-61

Broz AK, Broeckling CD, He JB, Dai X, Zhao PX, Vivanco JM (2007a) A first step in understanding an invasive weed through its genes: an EST analysis of invasive Centaurea maculosa. Bmc Plant Biology **7**

Broz AK, Manter DK, Vivanco JM (2007b) Soil fungal abundance and diversity: another victim of the invasive plant Centaurea maculosa. Isme Journal **1:** 763-765

Callaway RM, Ridenour WM, Laboski T, Weir T, Vivanco JM (2005) Natural selection for resistance to the allelopathic effects of invasive plants. Journal of Ecology **93:** 576-583

Callaway RM, Thelen GC, Barth S, Ramsey PW, Gannon JE (2004) Soil fungi alter interactions between the invader Centaurea maculosa and North American natives. Ecology **85:** 1062-1071

Carey EV, Marler MJ, Callaway RM (2004) Mycorrhizae transfer carbon from a native grass to an invasive weed: evidence from stable isotopes and physiology. Plant Ecology **172:** 133-141

Cipollini D (2004) Stretching the limits of plasticity: Can a plant defend against both competitors and herbivores? Ecology **85:** 28-37

Dallas PB, Gottardo NG, Firth MJ, Beesley AH, Hoffmann K, Terry PA, Freitas JR, Boag JM, Cummings AJ, Kees UR (2005) Gene expression levels assessed by oligonucleotide microarray analysis and quantitative real-time RT-PCR - how well do they correlate? Bmc Genomics **6**

Devlin PF, Yanovsky MJ, Kay SA (2003) A genomic analysis of the shade avoidance response in Arabidopsis. Plant Physiology **133:** 1617-1629

Griggs R (1940) The ecology of rare plants. Bulletin of the Torrey Botanical Club **67:** 575–594

Grime JP, Shacklock JML, Band SR (1985) NUCLEAR-DNA CONTENTS, SHOOT PHENOLOGY AND SPECIES CO-EXISTENCE IN A LIMESTONE GRASSLAND COMMUNITY. New Phytologist **100:** 435-445

Hanzawa Y, Imai A, Michael AJ, Komeda Y, Takahashi T (2002) Characterization of the spermidine synthase-related gene family in Arabidopsis thaliana. Febs Letters **527:** 176-180

Harper J (1977) The population biology of plants. Academic Press, London, UK

Hierro JL, Callaway RM (2003) Allelopathy and exotic plant invasion. Plant and Soil **256:** 29-39

Hierro JL, Maron JL, Callaway RM (2005) A biogeographical approach to plant invasions: the importance of studying exotics in their introduced and native range. Journal of Ecology **93:** 5-15

Horvath DP, Anderson JV (2002) A molecular approach to understanding root bud dormancy in leafy spurge. Weed Science **50:** 227-231

Horvath DP, Gulden R, Clay SA (2006) Microarray analysis of late-season velvetleaf (Abutilon theophrasti) effect on corn. Weed Science **54:** 983-994

Horvath DP, Llewellyn D, Clay SA (2007) Heterologous hybridization of cotton microarrays with velvetleaf (Abutilon theophrasti) reveals physiological responses due to corn competition. Weed Science **55:** 546-557

Horvath DP, Schaffer R, West M, Wisman E (2003a) Arabidopsis microarrays identify conserved and differentially expressed genes involved in shoot growth and development from distantly related plant species. Plant Journal **34:** 125-134

Horvath DP, Schaffer R, Wisman E (2003b) Identification of genes induced in emerging tillers of wild oat (Avena fatua) using Arabidopsis microarrays. Weed Science **51:** 503-508

Initiative TAG (2000) Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. Nature **408:** 796-815

Kahn RA, Gilman AG (1984) PURIFICATION OF A PROTEIN COFACTOR REQUIRED FOR ADP-RIBOSYLATION OF THE STIMULATORY REGULATORY COMPONENT OF ADENYLATE-CYCLASE BY CHOLERA-TOXIN. Journal of Biological Chemistry **259:** 6228-6234

Kunkel BN, Brooks DM (2002) Cross talk between signaling pathways in pathogen defense. Current Opinion in Plant Biology **5:** 325-331

Lee MH, Min MK, Lee YJ, Jin JB, Shin DH, Kim DH, Lee KH, Hwang I (2002) ADP-ribosylation factor 1 of arabidopsis plays a critical role in intracellular trafficking and maintenance of endoplasmic reticulum morphology in Arabidopsis. Plant Physiology **129:** 1507-1520

Lindsey-Boltz LA, Bermudez VP, Hurwitz J, Sancar A (2001) Purification and characterization of human DNA damage checkpoint Rad complexes. Proceedings of the National Academy of Sciences of the United States of America **98:** 11236-11241

Marler MJ, Zabinski CA, Callaway RM (1999) Mycorrhizae indirectly enhance competitive effects of an invasive forb on a native bunchgrass. Ecology **80:** 1180-1186

Michelmore R (2008) The Compositae Genome Project at the University of California, Davis. *In* MLaUDG Center, ed, Vol 2007, Davis, CA

Mitchell-Olds T (2001) Arabidopsis thaliana and its wild relatives: a model system for ecology and evolution. Trends in Ecology & Evolution **16:** 693-700

Morey JS, Ryan JC, Van Dolah FM (2006) Microarray validation: factors influencing correlation between oligonucleotide microarrays and real-time PCR. Biological Procedures Online**:** 175-193

Pabo CO, Sauer RT (1992) TRANSCRIPTION FACTORS - STRUCTURAL FAMILIES AND PRINCIPLES OF DNA RECOGNITION. Annual Review of Biochemistry **61:** 1053-1095

Perry LG, Johnson C, Alford ER, Vivanco JM, Paschke MW (2005) Screening of grassland plants for restoration after spotted knapweed invasion. Restoration Ecology **13:** 725-735

Perry LG, Thelen GC, Ridenour WM, Callaway RM, Paschke MW, Vivanco JM (2007) Concentrations of the allelochemical (+/-)-catechin in Centaurea maculosa soils. Journal of Chemical Ecology **33:** 2337-2344

Pimentel D, Lach L, Zuniga R, Morrison D (2000) Environmental and economic costs of nonindigenous species in the United States. Bioscience **50:** 53-65

Qin LX, Beyer RP, Hudson FN, Linford NJ, Morris DE, Kerr KF (2006) Evaluation of methods for oligonucleotide array data via quantitative real-time PCR. Bmc Bioinformatics **7**

Rajcan I, Chandler KJ, Swanton CJ (2004) Red-far-red ratio of reflected light: a hypothesis of why early-season weed control is important in corn. Weed Science **52:** 774-778

Ridenour WL, Callaway RM (2003) Root herbivores, pathogenic fungi, and competition between Centaurea maculosa and Festuca idahoensis. Plant Ecology **169:** 161-170

Ridenour WM, Callaway RM (2001) The relative importance of allelopathy in interference: the effects of an invasive weed on a native bunchgrass. Oecologia **126:** 444-450

Schaffer R, Landgraf J, Accerbi M, Simon V, Larson M, Wisman E (2001) Microarray analysis of diurnal and circadian-regulated genes in Arabidopsis. Plant Cell **13:** 113-123

Schenk PM, Kazan K, Wilson I, Anderson JP, Richmond T, Somerville SC, Manners JM (2000) Coordinated plant defense responses in Arabidopsis revealed by microarray analysis. Proceedings of the National Academy of Sciences of the United States of America **97:** 11655-11660

Schulz B, Kolukisaglu HU (2006) Genomics of plant ABC transporters: The alphabet of photosynthetic life forms or just holes in membranes? Febs Letters **580:** 1010-1016

Shi LM, Reid LH, Jones WD, Shippy R, Warrington JA, Baker SC, Collins PJ, de Longueville F, Kawasaki ES, Lee KY, Luo YL, Sun YMA, Willey JC, Setterquist RA, Fischer GM, Tong WD, Dragan YP, Dix DJ, Frueh FW, Goodsaid FM, Herman D, Jensen RV, Johnson CD, Lobenhofer EK, Puri RK, Scherf U, Thierry-Mieg J, Wang C, Wilson M, Wolber PK, Zhang L, Amur S, Bao WJ, Barbacioru CC, Lucas AB, Bertholet V, Boysen C, Bromley B, Brown D, Brunner A, Canales R, Cao XXM, Cebula TA, Chen JJ, Cheng J, Chu TM, Chudin E, Corson J, Corton JC, Croner LJ, Davies C, Davison TS, Delenstarr G, Deng XT, Dorris D, Eklund AC, Fan XH, Fang H, Fulmer-Smentek S, Fuscoe JC, Gallagher K, Ge WG, Guo L, Guo X, Hager J, Haje PK, Han J, Han T, Harbottle HC, Harris SC, Hatchwell E, Hauser CA, Hester S, Hong HX, Hurban P, Jackson SA, Ji HL, Knight CR, Kuo WP, LeClerc JE, Levy S, Li QZ, Liu CM, Liu Y, Lombardi MJ, Ma YQ, Magnuson SR, Maqsodi B, McDaniel T, Mei N, Myklebost O, Ning BT, Novoradovskaya N, Orr MS, Osborn TW, Papallo A, Patterson TA, Perkins RG, Peters EH, Peterson R, Philips KL, Pine PS, Pusztai L, Qian F, Ren HZ, Rosen M, Rosenzweig BA, Samaha RR, Schena M, Schroth GP, Shchegrova S, Smith DD, Staedtler F, Su ZQ, Sun HM, Szallasi Z, Tezak Z, Thierry-Mieg D, Thompson KL, Tikhonova I, Turpaz Y, Vallanat B, Van C, Walker SJ, Wang SJ, Wang YH, Wolfinger R, Wong A, Wu J, Xiao CL, Xie Q, Xu J, Yang W, Zhong S, Zong YP, Slikker W (2006) The MicroArray Quality Control (MAQC) project shows inter- and intraplatform reproducibility of gene expression measurements. Nature Biotechnology **24:** 1151-1161

Svistoonoff S, Creff A, Reymond M, Sigoillot-Claude C, Ricaud L, Blanchet A, Nussaume L, Desnos T (2007) Root tip contact with low-phosphate media reprograms plant root architecture. Nature Genetics **39:** 792-796

Travers SE, Smith MD, Bai JF, Hulbert SH, Leach JE, Schnable PS, Knapp AK, Milliken GA, Fay PA, Saleh A, Garrett KA (2007) Ecological genomics: making the leap from model systems in the lab to native populations in the field. Frontiers in Ecology and the Environment **5:** 19-24

van der Heijden MGA, Wiemken A, Sanders IR (2003) Different arbuscular mycorrhizal fungi alter coexistence and resource distribution between co-occurring plant. New Phytologist **157:** 569-578

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology **3**

Vila M, Weiner J (2004) Are invasive plant species better competitors than native plant species? evidence from pair-wise experiments. Oikos **105:** 229-238

Watt AS (1947) PATTERN AND PROCESS IN THE PLANT COMMUNITY. Journal of Ecology **35:** 1-22

Weir TL, Bais HP, Stull VJ, Callaway RM, Thelen GC, Ridenour WM, Bhamidi S, Stermitz FR, Vivanco JM (2006) Oxalate contributes to the resistance of Gaillardia grandiflora and Lupinus sericeas to a phytotoxin produced by Centaurea maculosa. Planta **223:** 785-795

Wolfe BE, Klironomos JN (2005) Breaking new ground: Soil communities and exotic plant invasion. Bioscience **55:** 477-487

Zabinski CA, Quinn L, Callaway RM (2002) Phosphorus uptake, not carbon transfer, explains arbuscular mycorrhizal enhancement of Centaurea maculosa in the presence of native grassland species. Functional Ecology **16:** 758-765

CHAPTER 5

Plant neighbor identity influences individual plant biochemistry and physiology

Parts of this work have been submitted for publication and are under review.

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Plant neighbor identity influences individual plant biochemistry and physiology

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Authors' contributions

AKB: conceived, designed, performed and analyzed data for the greenhouse experiment and wrote manuscript
CDB: conceived, designed and performed field collections and metabolomic analysis and wrote manuscript
RMC and EG: assisted with field collections
LWS and ML: assisted with metabolomic work and data analyis
CD: ran GC-MS samples for field metabolomics experiment.
JMV: conceived experiments and edited manuscript

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5.1 ABSTRACT

A non-targeted metabolomics analysis demonstrated that individuals of the invasive weed, *Centaurea maculosa* Lam., accumulate high levels of defense related secondary metabolites and low levels of primary metabolites when growing in conspecific versus heterospecific stands in the field. Here, in a greenhouse experiment designed to further investigate these results, I found that *C. maculosa* 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 paralleled the metabolomics results found for field samples of *C. maculosa*. Similarly, the North American native grass *Festuca idahoensis* accumulated greater amounts of total phenolics in the presence of a conspecific versus a heterospecific plant neighbor after elicitation. Conspecific plant neighbors resulted in increased accumulation of defense related secondary metabolites for both plant types grown in two different nutrient conditions. However, the two species responded differently in terms of biomass accumulation to the parameters tested. These results indicate that individual *C. maculosa* and *F. idahoensis* individuals can differentially modify their metabolic response based on the composition of the plant community in which they grow and further suggest that plant neighbor identity is an important factor influencing individual plant biochemistry and physiology in both species.

5.2 INTRODUCTION

Competition between organisms is thought to be largely responsible for structuring ecological communities and may help to explain observed spatial patterns of species density and diversity. The spatial distribution of plants in a community impacts the way in which plants interact with each other, mutualists (Tirado and Pugnaire, 2003), consumers (Janzen, 1970) and other aspects of the environment. Due to the sessile nature of plants, spatial distribution greatly influences the amount of intra- and inter–specific competition an individual plant experiences. Competition between conspecifics can be strikingly different than competition between heterospecific plant neighbors (Fonteyn and Mahall, 1978), and can alter plant growth habits, metabolism (Cipollini and Bergelson, 2002) and gene expression (Broz et al., 2008).

Aggregation of conspecifics is widely observed in plant communities. This is often due to plant reproductive features, such as limited seed dispersal distance and clonal reproduction, or to heterogeneous environments that may favor the growth of one particular species over others. Although the life history and environmental characteristics that contribute to plant aggregation are relatively straight forward, the impact of conspecific aggregation on individual plant biochemistry and physiology is not well understood. In experimental communities, aggregation of conspecifics affects parameters such as growth, fecundity (Stoll and Prati, 2001) and the production of defense compounds (Lankau and Strauss, 2007; Wentzell and Kliebenstein, 2008). However, the impact of conspecific aggregation on species performance is expected to differ depending on the species and its competitive competency (Stoll and Prati, 2001).

Exotic invasive plants would appear to have substantial competitive competency, as they are often observed to reduce the abundance of native species and are typically considered a major threat to biodiversity in native ecosystems (Pimentel et al., 2000). Invasive plants often establish very high population densities, resulting in a near monoculture of conspecific neighbors. However, they also exist at much lower relative densities within a matrix of heterospecific neighbors. Because of this wide variation in relative density, invasive plants represent useful ecological models to examine the effect of plant community composition on plant biochemistry, under both natural field conditions and in greenhouse experiments. Biochemical characteristics of plants may play a role in invasive success (Callaway and Ridenour, 2004; Cappuccino and Arnason, 2006), and thus detailed understanding of plant biochemistry is likely to provide insight into the mechanisms of invasion (Inderjit et al., 2006).

Centaurea maculosa Lam. (*Centaurea stoebe* L. spp. *micranthos* (Gugler) Hayek, *C. biebersteinii,* spotted knapweed) is a particularly problematic invasive weed in the north western United States that tends to form dense stands, excluding native species. A metabolomics-based analyses of field collected *C. maculosa* plants from conspecific stands revealed reduced accumulation of many small primary metabolites in comparison to plants from heterospecific stands (Broeckling, 2008). In contrast, multiple secondary metabolites demonstrated increased accumulation in conspecific stands including chlorogenic acid, quinic acid and the sesquiterpene lactone cnicin (Broeckling, 2008). Multivariate analyses of these data demonstrated that samples from conspecific and heterospecific stands group separately in a partial least square-discriminant analysis (PLS-DA), suggesting that plant neighbor identity could be affecting individual plant

metabolism in the field (Broeckling, 2008). However, other factors, particularly resource availability and herbivory are known to impact plant primary and secondary metabolism, and could have contributed to the metabolomics results. Analysis of soil characteristics revealed no consistent differences between the conspecific and heterospecific *C. maculosa* field stands sampled, and leaf herbivory damage data taken on site did not improve ANOVA model fit (Broeckling, 2008). However, a separate field study found that specialist root and flower herbivores occurred more frequently in conspecific versus heterospecific stands of *C. maculosa* (RM Callaway and E Greene, unpublished data), providing a possible explanation for the metabolomics results.

To more closely address the potential influences of resource availability, herbivory and plant neighbor identity on *C. maculosa* biochemistry and physiology, I manipulated these factors in a greenhouse experiment. Individual *C. maculosa* plants were paired with either a conspecific (*C. maculosa*) neighbor or a heterospecific (*Festuca idahoensis*, a native North American grass species) neighbor and grown in the greenhouse under high or low nutrient conditions. *Festuca idahoensis* plants were also paired with a conspecific (*F. idahoensis*) neighbor to investigate the impact of the experimental factors on a North American native plant that is generally considered to be a weak competitor against *C. maculosa* (Ridenour and Callaway, 2003; Callaway et al., 2004; Perry et al., 2005). Half of these plant pairs were elicited with jasmonic acid (JA) to simulate herbivory prior to harvest. This experimental design helped tease apart factors that affect biomass accumulation and secondary metabolism in both *C. maculosa* and *F. idahoensis*.

5.3 MATERIALS AND METHODS

GREENHOUSE EXPERIMENT

Centaurea maculosa seeds were collected from field populations near Missoula, MT, USA by the laboratory of R.M. Callaway, and *F. idahoensis* Elmer seeds were obtained from Wind River Seed Company. In late April 2008, a piece of sterile glass wool was placed into the bottom of 96 conetainers (volume 164 cm^3 each, Stuewe and Sons, Tangent OR, USA) to keep the soil substrate from leaking out. The conetainers were then filled with a mix of 2 parts sand (Play Sand obtained from US Mix, Denver CO) and one part soil clay conditioner (100% arcillite obtained from Schultz, Bridgeton MO). Conetainers were placed into racks in the greenhouse, flooded with water to settle soil media, seeded and covered with plastic wrap to maintain a humid environment. The factors in the experimental design were plant-neighbor identity (3; *C. maculosa-C. maculosa, C. maculosa-F. idahoensis,* or *F. idahoensis-F. idahoensis*) nutrient level (2; high nutrient condition or low nutrient condition) and elicitation (2; elicited with jasmonic acid or not elicited), and for each combination there were 8 repetitions giving a total of 96 experimental units. Three seeds of each competitor were added to the appropriate containers, resulting in six seeds per conetainer. Once seedlings were established (approximately 2 weeks) the plants were thinned to two total plants per pot. Pots were continuously monitored in case of late seedling emergence and thinned as needed. On May 12, 2008 the plants were randomized in a complete block design, with nutrient level as the blocking factor.

Hoagland's solution was used as a nutrient solution for the experiment. Separate 1M stock solutions were made for $Ca(NO_3)_2$-$4H_2O$ (236.1 g/L), KNO_3 (101.1 g/L),

KH_2PO_4 (136.1 g/L), and $MgSO_4$-$7H_2O$ (246.5 g/L). Trace elements were made in a separate stock solution with H_2BO_3 (2.8 g/L), $MnCl_2$-$4H_2O$ (1.8 g/L), $ZnSO_4$-$7H_2O$ (0.2 g/L), $CuSO_4$-$5H_2O$ (0.1 g/L), and $NaMoO_4$ (0.025 g/L). A final stock solution was made containing an EDTA Iron (III) Sodium Salt ($C_{10}H_{12}FeN_2NaO_8$) (9.71 g/L). All stock solutions were stored at 4° C when not in use. To make 1 L of full strength Hoagland's solution from these stocks, 7 mL of 1M $Ca(NO_3)_2$-$4H_2O$, 5 mL of 1M KNO_3, 2mL of 1M KH_2PO_4, 2mL of 1M $MgSO_4$-$7H_2O$, 1 mL of trace element stock and 1 mL of iron stock were combined and brought to 1 L with water. For the high nutrient condition the full strength Hoagland's solution was diluted 1:1 with water, resulting in a ½ strength solution. For the low nutrient condition a portion of the ½ strength Hoagland's solution was further diluted with water to give a 1/20 strength solution.

Plants were given one of two nutrient treatments: high nutrient condition (plants were watered with ½ strength Hoagland's solution twice per week) or low nutrient condition (plants were watered with 1/20 strength Hoagland's solution twice per week). Approximately 20 mL of nutrient solution was put into each conetainer on Mondays and Fridays, and plants were watered thoroughly on Wednesdays using a misting wand over the course of the experiment.

On July 18th, the pots were un-randomized and re-randomized based on two blocking factors: nutrient level and elicitation. On this day, two weeks before harvest, half the pots were elicited by spraying both plants in the pot with a 0.5 mM jasmonic acid (JA) solution until they were thoroughly soaked. The jasmonic acid solution was made by adding 57.3 µL of methyl jasmonate 95% (Sigma #392707, mw 224.3, Saint Louis, Missouri) to 25 mL of methanol to create a 10 mM solution. Fifteen mL of the 10 mM

solution was brought up to 300 mL with water to make a 0.5mM solution. For a control, 15 mL of methanol was added to 285 mL of water. Plants were again treated with 0.5 mM JA solution again on July 25th (one week before harvest) and July 31st (one day before harvest). The control plants were sprayed with a water-methanol solution. During JA treatments racks (blocks) of conetainers were separated by at least three feet and a temporary barrier was set up to ensure that control plants did not come into contact with the JA solution.

On August 1st all plants were removed from conetainers. Roots of competitors were placed in water to rinse away soil media and separate the two plants. After the two plants per pot were separated, the roots and shoots of plants were cut apart with a razor blade. Total root samples from each plant were placed into individual envelopes and dried at 60° C for two days. Dried roots were weighed to the nearest mg. In the conetainers containing two *F. idahoensis* plants it was extremely difficult to separate the roots of the two competitors; thus only one root sample was collected per conetainer. The weights of these dry root samples were divided by two to determine total root biomass per *F. idahoensis* competitor for analysis. Shoot samples from each plant were placed in individual Falcon tubes and placed at -80° C for at least four hours. Shoot samples were then freeze dried under a vacuum at -75° C for two days, after which they were stored at 4° C. All freeze dried shoot samples were weighed to the nearest mg. Shoot samples of *C. maculosa* were then ground with a coffee grinder to a fine powder and stored in falcon tubes at 4° C. Shoot samples from *F. idahoensis* did not grind well in the coffee grinder and were instead placed in liquid nitrogen and pulverized using a mortar and pestle.

TOTAL PHENOLICS ASSAY

Total phenolics accumulation was determined for field collected *C. maculosa* leaf tissues (for collection sites and methods see Broeckling 2008) and plant tissues (*C. maculosa* and *F. idahoensis*) collected from the greenhouse experiment described above. For each plant sample, one mL of 80% acetone was added to 20 mg of ground plant tissue, vortexed 30 seconds, rotated at 250 rpm in the dark for 15 minutes, vortexed briefly, and centrifuged at 10,000 rpm at 4° C for 15 min. A 100-µL aliquot of supernatant was removed and stored at -20° C overnight. The remaining supernatant was dried under a continuous flow of air. The 100-µL aliquot was brought to a total volume of 1 mL with distilled water, vortexed, and used in a colorimetric microplate assay for phenolics determination.

The Folin-Ciocalteu assay (Folin and Ciocalteu, 1927) was used to determine the amount of total phenolics in all samples, using gallic acid to create a standard curve. Briefly, 35 µL of each sample was mixed with 150 µL of 0.2 M Folin-Ciocalteu reagent and incubated at room temperature for 5 minutes, after which 115 µL of saturated (7.5% w/v) sodium bicarbonate was added to the reaction. The reaction was mixed briefly, incubated at 45° C for 30 minutes, and then allowed to cool to room temperature for 60 minutes. Absorbance at wavelength 765 nm was read in a SPECTRA max plus 384 microplate reader (Molecular devices, Sunnyvale CA). All samples were run in triplicate. Total phenolics were calculated as gallic acid equivalents (GAE) per gram of sample dry weight.

STATISTICAL ANALYSES

Analyses of total biomass and phenolics data were performed using the mixed procedure in the SAS 9.1 program (Cary NC, USA). Total biomass data were not normally distributed, and were log transformed to normalize distribution. However, significance of interactions and pairwise comparisons did not change with log transformation of the data. Phenolics data were normally distributed. An overall ANOVA of *Centaurea* total biomass data suggested that there were significant interactions between nutrient condition and neighbour identity ($p=0.0013$) and elicitation and neighbour identity ($p=0.0059$). The interaction between elicitation and nutrient condition was not significant ($p=0.5692$) but the three way interaction between all factors was near significant at a level of $\alpha=0.05$ ($p=0.0899$). In an overall ANOVA of *Centaurea* total phenolics data there were significant interactions between nutrient condition and elicitation ($p<0.0001$) and elicitation and neighbour identity ($p<0.0001$). The interaction between nutrient level and neighbour identity was not significant ($p=0.2951$) and the three way interaction between all factors was near significant at a level of $\alpha=0.05$ ($p=0.0556$). In an overall ANOVA of *Festuca* total biomass data, a significant interaction was identified between nutrient condition and neighbour identity ($p<0.0001$). Other interactions were not significant, including the three way interaction ($p=0.3382$). In an overall ANOVA of *Festuca* total phenolics data there was a significant interaction between neighbour identity and elicitation ($p<0.0001$), and near significant (at $\alpha=0.05$) interactions between elicitation and nutrient condition ($p=0.0551$) and neighbour identity and nutrient condition ($p=0.0773$). The three way interaction between all factors was also near significant at a level of $\alpha=0.05$ ($p=0.0704$).

Because of large differences in total biomass between nutrient conditions, I chose to analyze each nutrient condition with separate ANOVAs for each plant. Within each nutrient condition, the interaction between elicitation and neighbour identity were significant for total *Centaurea* biomass (Table 5.2; high nutrient, $p=0.0257$; low nutrient $p=0.0182$) and *Centaurea* total phenolics (Table 5.2; high nutrient, $p=0.0069$; low nutrient, $p<0.0001$). Within each nutrient condition the interaction between elicitation and neighbour identity were significant for *Festuca* total phenolics (Table 5.5; low nutrient, $p<0.0001$; high nutrient $p=0.0007$). This interaction was significant for *Festuca* biomass in the low nutrient condition only (Table 5.5; low nutrient, $p=0.0346$, high nutrient $p=0.8321$). Pair-wise comparisons between means were made to determine significant differences between conditions of interest using Fischers LSD.

5.4 RESULTS

TOTAL PHENOLICS IN FIELD COLLECTED PLANTS

An analysis of total phenolics confirmed the overall increased accumulation of phenolic compounds in *C. maculosa* plants from conspecific versus heterospecific field stands (Figure 5.1).

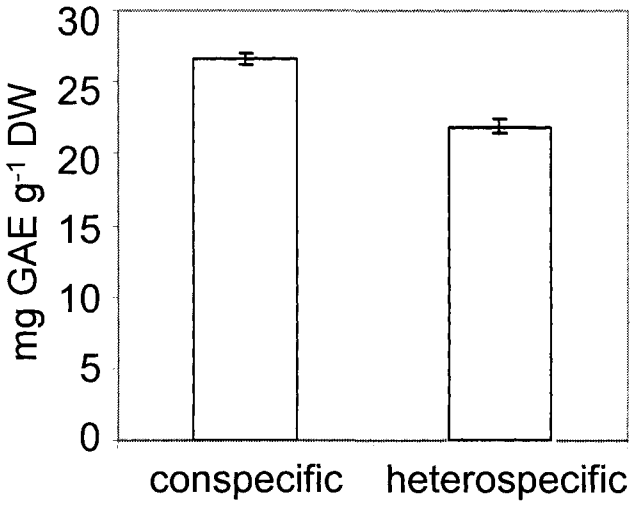

Figure 5.1. Total phenolics accumulation of *Centaurea maculosa* plants collected from conspecific and hetetrospecific field stands. Accumulation of total phenolics was analyzed in freeze dried, field collected *C. maculosa* leaf tissues collected by Broeckling (2008) using the Folin-Ciocalteu method. Values are expressed as mg gallic acid equivalents (GAE) per gram dry weight of plant tissue. Means and standard errors are shown. Samples collected from conspecific stands are represented by the white bar, and those from heterospecific stands are represented by the gray bar. Stand type significantly affected total phenolics accumulation ($t=6.94$, $p<0.0001$).

TOTAL PHENOLICS AND BIOMASS IN CENTAUREA MACULOSA

The extent of phenolics and biomass accumulation in *C. maculosa* individuals was influenced by a combination of the three factors tested; plant neighbor identity, nutrient level and whether or not the plants were elicited with JA. As expected, higher nutrient levels produced larger plants, and additionally resulted in plants with lower amounts of total phenolics (Table 5.1, Figure 5.2). When data for the two nutrient levels were analyzed in separate ANOVAs, a significant interaction ($p<0.03$) was always identified between plant neighbor identity and JA elicitation (Table 5.2). Pair-wise comparisons of this interaction revealed an interesting trend that was consistent for both nutrient levels (Table 5.3). In non-elicited conditions, I found no significant differences between *C. maculosa* total phenolic concentrations or biomass due to plant neighbor identity; however, when the plants were elicited with JA, *C. maculosa* individuals accumulated a significantly greater amount of total phenolics and exhibited reduced biomass when growing with a conspecific versus a heterospecific neighbor (Figure 5.2, Table 5.3).

Although a conspecific neighbor was consistently correlated with significant increases in total phenolics and significant reductions in biomass accumulation under JA elicited conditions, the extent of these changes was influenced by the level of available nutrients. For instance, when plants were elicited with JA under low nutrient conditions, the relative increase in total phenolics accumulation due to a conspecific versus a heterospecific neighbor was 17% (total increase ~5 mg gallic acid equivalents (GAE) per gram dry weight), whereas in high nutrient conditions this increase was on the order of 10% (total ~2 mg GAE per gram dry weight) (Figure 5.2, Table 5.1). Biomass data

indicated a 28% relative decrease in dry weight (total ~65 mg) under low nutrient conditions versus a 39% decrease (total ~488 mg) under high nutrient conditions due to the presence of a conspecific versus heterospecific neighbor when plants were elicited (Figure 5.2, Table 5.1). The opposing trends identified for total phenolics and biomass accumulation suggest that under elicited conditions *C. maculosa* individuals are experiencing a trade-off between growth and defense. The directional trends associated with this trade-off are influenced predominately by the interaction between plant neighbor identity and elicitation, as opposed to resource levels.

Table 5.1. *Centaurea maculosa* mean values for biomass and total phenolics. Means and standard errors for total biomass (mg dry weight) and total phenolics (mg gallic acid equivalents (GAE) per gram dry tissue) of *C. maculosa* grown in one of two nutrient conditions (low or high), with one of two neighbor plants (C. maculosa; C or *F. idahoensis*; F), and either elicited (+) or not elicited (-) with jasmonic acid (JA). For each reported mean, n=8.

Nutrient level	Competitor Identity	JA	Biomass (mg dry weight)		Total Phenolics (mg GAE g^{-1} dry weight)	
			mean	se	mean	se
Low	Centaurea	-	214.3	15.66	24.12	0.71
	Festuca	-	186.7	22.14	26.05	1.00
	Centaurea	+	167.6	14.76	34.18	0.67
	Festuca	+	233.0	22.14	29.16	1.00
High	Centaurea	-	903.6	61.96	8.80	0.2085
	Festuca	-	1,010.7	95.49	9.65	0.2949
	Centaurea	+	760.8	72.19	10.21	0.2085
	Festuca	+	1,249.1	95.49	9.24	0.3152

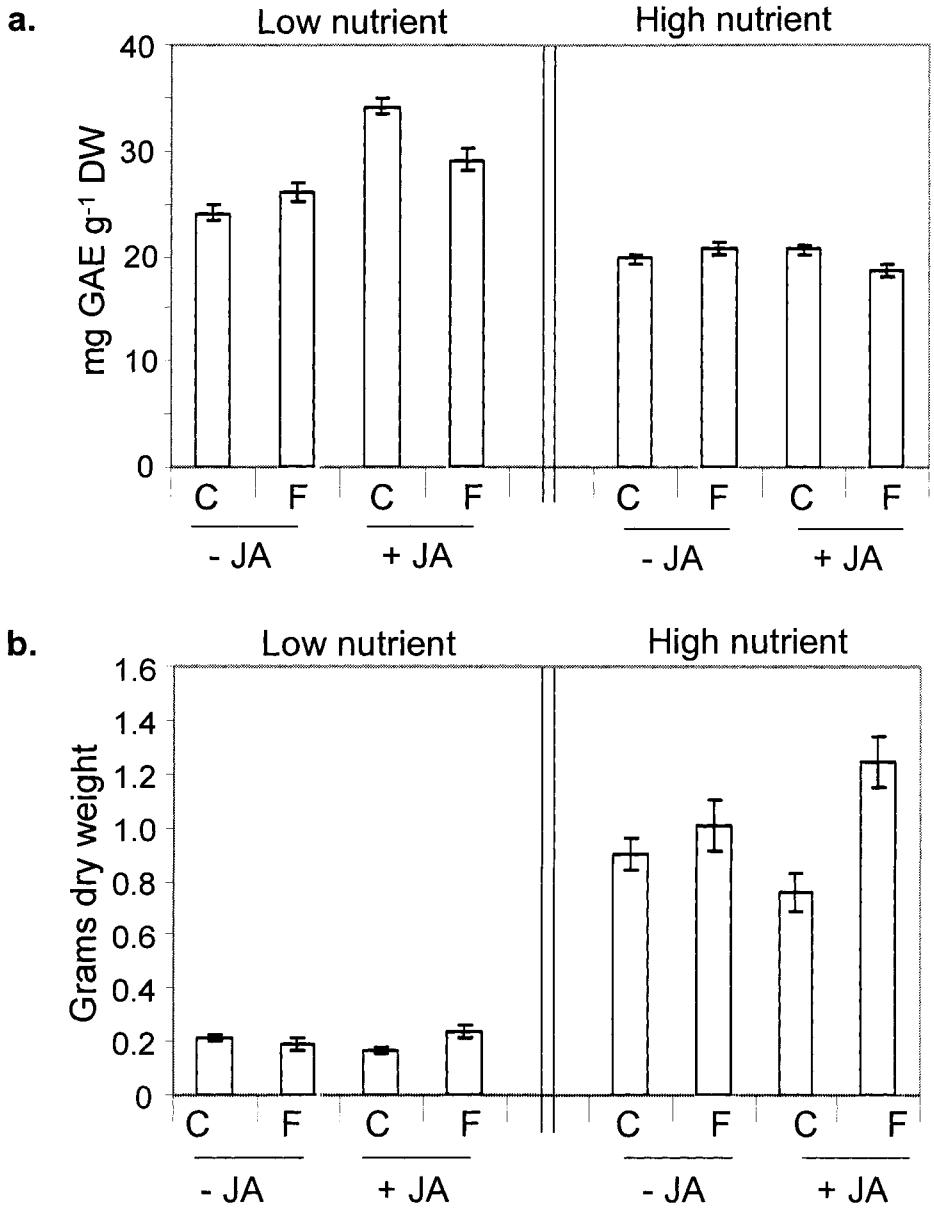

Figure 5.2. *Centaurea maculosa* total phenolics biomass accumulation. *Centaurea maculosa* plants were paired with either a conspecific (*C. maculosa*; C) or heterospecific (*Festuca idahoensis*; F) neighbor and grown in high or low nutrient conditions in the greenhouse. Half of the plant pairs in each nutrient condition were elicited with jasmonic acid (+JA) to simulate herbivory. Accumulation of total phenolics was analyzed in *C. maculosa* leaf tissues using the Folin-Ciocalteu method, and values are expressed as mg gallic acid equivalents (GAE) per gram dry weight of plant tissue (a). Total dry weight of leaves and roots was also measured for all plants (b). Bars represent mean values with standard errors. Refer to pair-wise comparisons (Table 1) for t and p values for comparisons of interest.

Table 5.2 *Centaurea maculosa* total phenolics and total biomass ANOVA F and p values

Total phenolics				
Plant	*Nutrient level*	*Effect*	*F*	*Pr > F*
Centaurea	low	Neighbor	3.20	0.0755
		Elicitation	58.44	<0.0001
		N*E	16.28	<0.0001
Centaurea	high	Neighbor	0.72	0.3964
		Elicitation	1.64	0.2029
		N*E	7.52	0.0069
Total biomass				
Plant	*Nutrient level*	*Effect*	*F*	*Pr > F*
Centaurea	low	Neighbor	0.99	0.3242
		Elicitation	0.00	0.9917
		N*E	6.00	0.0182
Centaurea	high	Neighbor	12.99	0.0008
		Elicitation	0.34	0.5656
		N*E	5.32	0.0257

Table 5.3. *Centaurea maculosa* pairwise comparisons. Test statistics and p values for pairwise comparisons of the interaction between plant neighbor identity and elicitation with jasmonic acid (JA) in both high and low nutrient conditions.

Nutrient level	**Competitor Identity**	**JA**	**Competitor Identity**	**JA**	**Biomass**		**Total Phenolics**	
					t	**p**	**t**	**p**
Low	Centaurea	-	Centaurea	+	2.17	0.0350	10.31	<0.0001
	Festuca	-	Festuca	+	1.48	0.1457	2.19	0.0301
	Centaurea	-	*Festuca*	-	*1.02*	*0.3140*	*1.57*	*0.1180*
	Centaurea	+	*Festcua*	+	*2.46*	*0.0177*	*4.16*	*<0.0001*
High	Centaurea	-	Centaurea	+	1.50	0.1404	1.28	0.2025
	Festuca	-	Festuca	+	1.77	0.0843	2.45	0.0155
	Centaurea	-	*Festuca*	-	*0.94*	*0.3517*	*1.34*	*0.1808*
	Centaurea	+	*Festcua*	+	*4.08*	*0.0002*	*2.45*	*0.0125*

TOTAL PHENOLICS AND BIOMASS IN FESTUCA IDAHOENSIS

The extent of phenolics and biomass accumulation in *F. idahoensis* individuals was also influenced by a combination plant neighbor identity, nutrient level and whether or not the plants were elicited with JA. Again, higher nutrient levels produced larger plants with generally lower amounts of total phenolics (Figure 5.3, Table 5.4). In ANOVAs for each nutrient condition I identified a significant interaction between elicitation and neighbor identity for the total phenolics data, and for the total biomass data in the low nutrient condition (Table 5.5). The *F. idahoensis* total phenolics data showed a similar result to that identified for *C. maculosa*: in elicited conditions plants with conspecific neighbors accumulated more total phenolics than those with heterospecific neighbors (Figure 5.3, Table 5.6). In the absence of elicitation, *F. idahoensis* total phenolics accumulation was not significantly different due to plant neighbor identity under low nutrient conditions, similar to the results found for *C. maculosa*. However, in high nutrient conditions *F. idahoensis* plants with conspecific neighbors exhibited reduced accumulation of total phenolics (Figure 5.3, Table 5.6).

As found for *C. maculosa*, the extent of the change in total phenolics due to plant neighbor and elicitation differed between the two nutrient levels. In low nutrient elicited conditions, growth with a conspecific neighbor resulted in a relative increase of 22% (2.5 mg GAE per gram dry weight) in *F. idahoensis* total phenolics (Figure5.3, Table 5.4). In high nutrient elicited conditions this relative increase was ~9.5% (0.97 mg GAE per gram dry weight) (Table 5.4). However, in high nutrient, non-elicited conditions, growth with a conspecific neighbor resulted in a relative decrease of ~9.6% (0.85 mg GAE per gram dry weight) in *F. idahoensis* total phenolics (Table 5.4).

Total biomass trends for *F. idahoensis* did not parallel those found for *C. maculosa*. In the high nutrient condition, *F. idahoensis* biomass accumulation was significantly inhibited by the presence of the heterospecific *C. maculosa* neighbor, regardless of whether or not the plants were elicited. Total biomass of *F. idahoensis* was reduced over 70% (>500 mg) due to the presence of *C. maculosa* versus a conspecific neighbor in high nutrient conditions (Table 5.4). In the low nutrient condition there was not a significant difference in biomass accumulation due to plant neighbor when plants were elicited; however, without elicitation *F. idahoensis* accumulated significantly more biomass when grown with *C. maculosa* (Figure 5.3). Here, total biomass of *F. idahoensis* was increased nearly 30% (137 mg) in the presence of *C. maculosa* versus a conspecific neighbor. In one instance (high nutrient non-elicited condition) larger plants contained lower amounts of total phenolics; however in the high nutrient elicited condition larger plants exhibited higher amounts of total phenolics.

Table 5.4. *Festuca idahoensis* mean values for biomass and total phenolics. Means and standard errors for total biomass (mg dry weight) and total phenolics (mg gallic acid equivalents (GAE) per gram dry tissue) of *F. idahoensis* grown in one of two nutrient conditions (low or high), with one of two neighbor plants (*Festuca idahoensis* or *Centaurea maculosa*), and either elicited (+) or not elicited (-) with jasmonic acid (JA). For each reported mean, n=8 individual plants.

Nutrient level	Competitor Identity	JA	Biomass (mg dry weight)		Total Phenolics (mg GAE g^{-1} dry weight)	
			mean	se	mean	se
Low	Festuca	-	326.2	33.36	10.02	0.28
	Centaurea	-	463.2	47.18	10.89	0.39
	Festuca	+	420.5	33.36	11.38	0.28
	Centaurea	+	379.3	47.18	8.87	0.39
High	Festuca	-	1,376.0	88.97	8.80	0.21
	Centaurea	-	796.8	125.80	9.65	0.29
	Festuca	+	1,212.9	88.97	10.21	0.21
	Centaurea	+	681.3	134.50	9.24	0.32

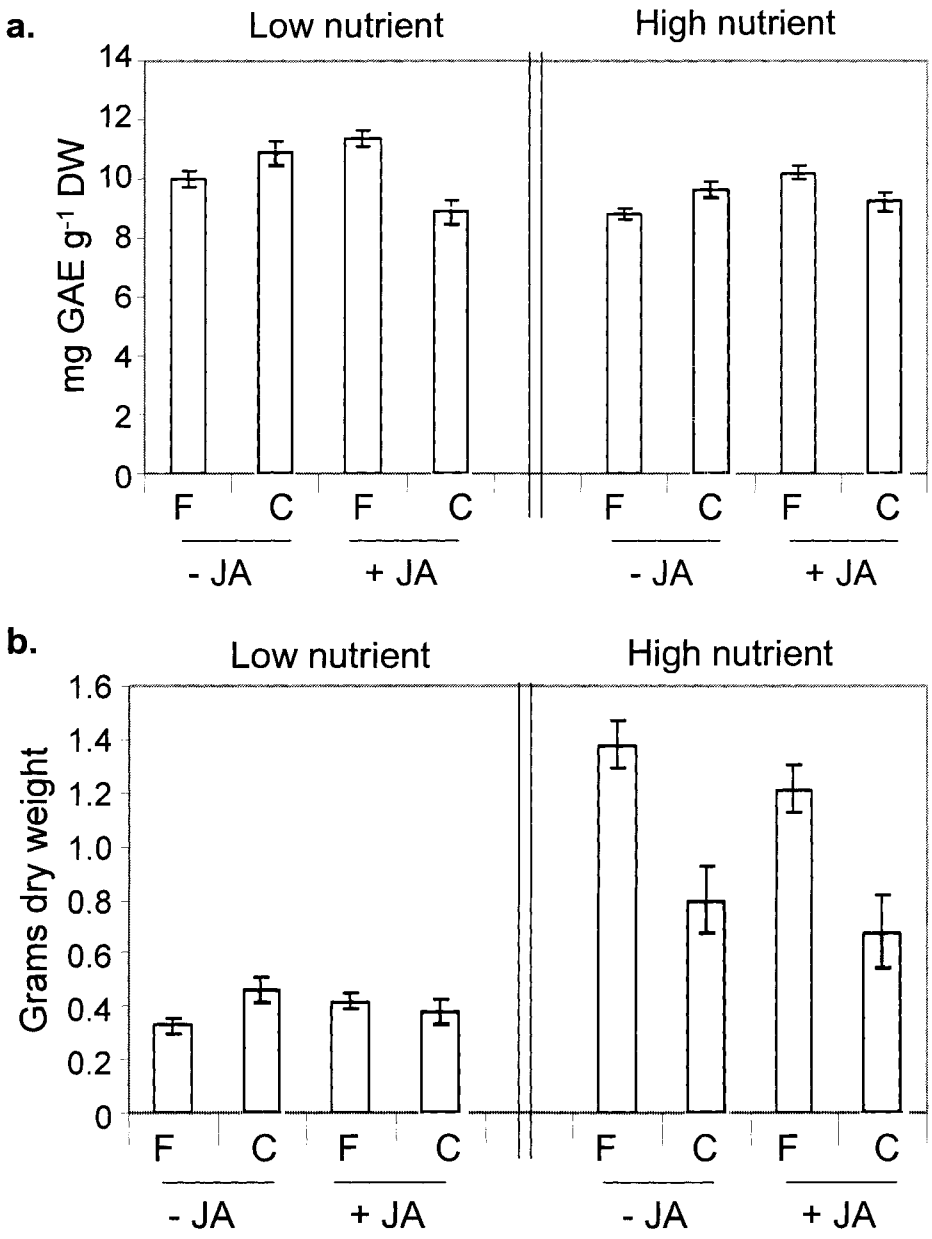

Figure 5.3. *Festuca idahoensis* total phenolics biomass accumulation. *Festuca idahoensis* plants were paired with either a conspecific (*F. idahoensis*; F) or heterospecific (*Centaurea maculosa*; C) neighbor and grown in high or low nutrient conditions in the greenhouse. Half of the plant pairs in each nutrient condition were elicited with jasmonic acid (+JA) to simulate herbivory. Accumulation of total phenolics was analyzed in *F. idahoensis* leaf tissues using the Folin-Ciocalteu method, and values are expressed as mg gallic acid equivalents (GAE) per gram dry weight of plant tissue (a). Total dry weight of leaves and roots was also measured for all plants (b). Bars represent mean values with standard errors. Refer to pair-wise comparisons (Table 5.6) for t and p values for comparisons of interest.

Table 5.5 Festuca idahoensis total phenolics and total biomass ANOVA F and p values

Total phenolics				
Plant	*Nutrient level*	*Effect*	*F*	*Pr > F*
Festuca	low	Neighbor	5.88	0.0166
		Elicitation	0.93	0.3377
		N*E	24.85	<0.0001
Festuca	high	Neighbor	0.05	0.8213
		Elicitation	3.69	0.0567
		N*E	12.06	0.0007
Total biomass				
Plant	*Nutrient level*	*Effect*	*F*	*Pr > F*
Festuca	low	Neighbor	1.37	0.2474
		Elicitation	0.02	0.8982
		N*E	4.76	0.0346
Festuca	high	Neighbor	24.79	<0.0001
		Elicitation	1.56	0.2184
		N*E	0.05	0.8321

Table 5.6. *Festuca idahoensis* pairwise comparisons. Test statistics and p values for pairwise comparisons of the interaction between plant neighbor identity and elicitation with jasmonic acid (JA) in both high and low nutrient conditions.

Nutrient level	**Competitor Identity**	**JA**	**Competitor Identity**	**JA**	**Biomass**		**Total Phenolics**	
					t	**p**	**t**	**p**
Low	Centaurea	-	Centaurea	+	1.26	0.2155	3.64	0.0004
	Festuca	-	Festuca	+	2.00	0.0517	3.48	0.0007
	Centaurea	-	Festuca	-	2.37	0.0222	1.81	0.0724
	Centaurea	+	Festcua	+	0.71	0.4796	5.24	<0.0001
High	Centaurea	-	Centaurea	+	0.63	0.5339	0.94	0.3493
	Festuca	-	Festuca	+	1.30	0.2018	4.78	<0.0001
	Centaurea	-	Festuca	-	3.76	0.0005	2.35	0.0202
	Centaurea	+	Festcua	+	3.30	0.0020	2.56	0.0116

5.5 DISCUSSION

CENTAUREA MACULOSA

Broeckling (2008) found that *C. maculosa* plants growing in conspecific stands accumulated more secondary metabolites than those growing in heterospecific stands. In particular, the phenolic compound, chlorogenic acid showed over a two fold increase in conspecific versus heterospecific stands. Phenolic compounds are often implicated in plant anti-herbivore defense mechanisms (Elliger et al., 1981; Appel, 1993; Dudt and Shure, 1994; Maher et al., 1994; Matsuki, 1996; Stamp and Yang, 1996), and have been considered important factors in multiple hypotheses of plant defense against herbivores (Feeny, 1976; Coley et al., 1985). Here, I demonstrate that the tissues collected by Broeckling (2008) from conspecific stands contain more total phenolics than those from heterospecific stands. This result is consistent with my expectations based on the metabolomics data. Further, data for both biomass and total phenolics accumulation measured in the current experiment parallel the *C. maculosa* field metabolomics results of Broeckling (2008), and provide additional information on the relative influences of herbivory, plant neighbor and resource availability on secondary metabolite accumulation.

I found that, in the absence of elicitation, plant neighbor identity had no effect on *C. maculosa* biomass accumulation or total phenolics. However, when plants were elicited with JA to simulate herbivory, *C. maculosa* plants accumulated more total phenolics than when they were grown with a conspecific versus a heterospecific neighbor. This trend was robust over the two nutrient levels examined, suggesting that resource availability was not the main driver of this effect. However, resource availability did impact the extent of the effect. Similarly, Broeckling (2008) found no consistent

differences between field soils collected from conspecific and heterospecific stands of *C. maculosa* and concluded that resource availability could not fully explain the metabolomics results.

Broeckling (2008) measured rates of leaf herbivory in conspecific stands at the time of sampling. Integrating this data into an ANOVA model of the metabolomics results did not improve the model, suggesting that leaf herbivory was not the major factor impacting *C. maculosa* metabolic profiles between stand types. However, multiple specialist root herbivores have been introduced to North America in an attempt to control populations of *Centaurea*; thus, it was possible that unseen herbivory was influencing individual plant metabolism in the field. A recent field study indicated that high rates of specialist root and seed head herbivory could be positively correlated with increased densities of *C. maculosa* (RM Callaway and E Greene, unpublished data). As herbivory is expected to increase secondary metabolite production, specialist herbivory provided a possible explanation for the field metabolomics results. Here, I found that elicitation with JA (simulated herbivory) and plant neighbor identity were both major factors affecting *C. maculosa* total phenolic production. Thus, a combination of these two factors, stand type and relative rates of herbivory are most likely the major drivers of the metabolomics field results.

In *C. maculosa* individuals from heterospecific stands, Broeckling (2008) found increased levels of primary metabolites, which are crucial to plant growth. Here, I demonstrate that under elicited conditions, *C. maculosa* plants with heterospecific neighbors exhibit greater total biomass than those with conspecific neighbors. Combined, these results suggest that *C. maculosa* plants growing with heterospecific neighbors

produce larger amounts of primary metabolites which are allocated towards growth, as opposed to producing secondary compounds, such as total phenolics, for defense. Alternatively, *C. maculosa* plants growing with conspecific neighbors produce larger amounts of defense related secondary metabolites, but by doing so may sacrifice biomass accumulation. These trends are suggestive of a trade-off of between growth and defense in *C. maculosa* plants that are exposed to elicitors.

It seems plausible that a modification of a defense strategy based on plant neighbor identity would confer evolutionary advantages that allow for the growth, maintenance and spread of *C. maculosa*. For instance, high conspecific plant density often correlates with higher attack rates by consumers, and this density-dependent function is fundamental to theory for the maintenance of community diversity (Janzen, 1970; Connell, 1971). Individual *C. maculosa* plants growing in conspecific stands are more likely to be subject to specialist herbivore attack than those growing in diverse plant communities (RM Callaway, E Greene, unpublished results). Thus, in a conspecific stand, a defensive strategy involving the accumulation of chemical herbivore deterrents, such as phenolic compounds, is likely to be more effective than a strategy based on growth. In heterospecific stands where the probability of herbivore attack is lower, factors such as plant competition for light or nutrients could have larger impacts on individual success. Thus, investing resources in growth rather than defense may be more effective in a diverse plant community.

FESTUCA IDAHOENSIS

Biomass trends for *F. idahoensis* differed greatly from those found in *C. maculosa*. Although *F. idahoensis* is generally considered to be a weak compeitor against *C. maculosa*, my results suggest that this may only be true in conditions of high resource availability. The biomass of *F. idahoensis* was greatly reduced in high nutrient conditions due to the presence of a heterospecific *C. maculosa* neighbor, regardless of the elicitation treatment. However, in low nutrient conditions *F. idahoensis* exhibited *more* biomass (-JA) or showed no change in biomass (+JA) when growing with a *C. maculosa* neighbor versus another *F. idahoensis*.

This result was surprising, in that rangeland soils collected from spotted knapweed field sites are often resource poor (see Table 7.2 of this dissertation; (Thorpe et al., 2006)). Over 75% of Montana soils collected from 2000-2001 fell into medium or low range of soil phosphorus, which is defined by the author as inadequate or barely adequate for plant nutrition, and much of the soil phosphorous may be unavailable due to high soil pH (Fixen, 2002). Although there is a large amount of variability between soils, on average, soils I collected from spotted knapweed infested sites in Montana contained 24 ppm nitrate and 14.3 ppm phosphorus (Table 7.2). The high nutrient condition used in the current study contained 105 ppm nitrate and 15.5 ppm phosphorus; whereas the low nutrient condition had 10.5 ppm nitrate and 1.55 ppm phosphorus. Other studies indicate that knapweed infested sites contain higher levels of phosphorus (ranging from ~2-23 ppm) than non-infested sites (ranging from ~2-10 ppm) (Thorpe et al., 2006). I found no significant differences between any soil characteristics measured between sites of high and low density knapweed infestation (Chapter 7).

North American rangelands are home to native perennial grasses such as *F. idahoensis*, but are prone to infestation by *C. maculosa* which tends to out-compete native grasses. Thus, it is surprising that *F. idahoensis* biomass was higher with a conspecific neighbor in the low nutrient condition, which is most similar to resource levels found in Montana rangeland soils. In addition, the overall biomass values of *F. idahoensis* (~0.3 grams dry weight) were larger than those of *C. maculosa* in the low nutrient condition (~0.2 grams dry weight). This may suggest that measures of biomass are not a good indicator of overall fitness or competitive ability in this species. Alternatively, as my experiment was conducted in a greenhouse setting, I controlled for a variety of factors that may be important or variable in the field setting. Other studies suggest that interactions between *C. maculosa* and *F. idahoensis* are modulated by local soil microbial communities (Callaway et al., 2004), which were excluded from the current study. Similarly, resource availability was controlled in this experiment by adding nutrient solution to plants which does not reflect the heterogeneity of soils in a natural setting. For instance, rangeland ecosystems are often prone to disturbances that may make nutrients more available, giving *C. maculosa* a competitive edge. Further, *C. maculosa* is known to enhance disturbance by promoting erosion in the areas it infests due to its characteristic taproot (Lacey et al., 1989).

Some of the trends in phenolics accumulation that were identified in *C. maculosa* were also found in *F. idahoensis*. Under elicited conditions, the presence of a conspecific neighbor resulted in a greater accumulation of *F. idahoensis* total phenolics than a heterospecific neighbor. Non-elicited, *F. idahoensis* individuals grown under low nutrient conditions exhibited no differences in total phenolics due to plant neighbor

identity. These results parallel those found for *C. maculosa*. However, in non-elicited high nutrient conditions, *F. idahoensis* individuals with a conspecific neighbor exhibited lower amounts of total phenolics than those with a heterospecific neighbor.

Unlike *C. maculosa*, the trends of total phenolics and biomass accumulation in *F. idahoensis* were not suggestive of a trade-off between growth and defense. For instance, when *F. idahoensis* plants were elicited in the high nutrient condition, those with a heterospecific neighbor exhibited more biomass and more total phenolics than those with a conspecific neighbor. This may indicate that the construction costs associated with the production of phenolic compounds in *F. idahoensis* is low, although phenolics are generally thought to have a high initial construction cost (Feeny, 1976; Coley et al., 1985). A study of willow failed to identify a trade-off between growth and production of phenolic compounds at early time points during the growing season, although there was some evidence for a trade-off from data taken at later time points (Matsuki, 1996). Thus, it is possible that *F. idahoensis* undergoes a trade-off between growth and production of phenolics, but evidence of such a trade off was not captured in the current experiment. Finally, it is important to note that phenolic compounds may not be indicative of antiherbivore defense in *F. idahoensis*, as has been suggested for other grasses (Theunissen, 1995).

5.6 CONCLUSIONS

Based on my results I suggest that the two plant species studied here are able to perceive the identity of their plant neighbors and induce what could be considered a species-specific metabolic response. This response is based primarily on an interaction

between plant neighbor identity and elicitation, and the extent of this response is modulated by resource availability. Although the response to these factors sometimes differed between *C. maculosa* and *F. idahoensis*, the importance of plant neighbor identity on metabolism was demonstrated in both species.

The mechanism through which plants might sense specific plant neighbors is currently unknown. The ability of many plant species to sense the presence versus absence of other plant neighbors by detecting changes in light quality is mediated predominately through phytochrome pathways (Vandenbussche et al., 2005), and can impact plant defense response and secondary metabolite accumulation (Stamp et al., 2004; Izaguirre et al., 2006). Other potential recognition mechanisms could involve the perception of plant volatile organic compounds (Karban et al., 1989; Karban et al., 2000; Karban et al., 2006; Kessler et al., 2006), root exudates (Mahall and Callaway, 1991), oscillatory signals (Gruntman and Novoplansky, 2004), tactile stimuli, or some other undescribed signaling system.

Regardless of the recognition mechanism, the results indicate that two very different plant species are able to change their metabolic response in relation to the identity of their plant neighbor under elicited conditions. Although this type of perception and response system is widely recognized in other taxa, it has to a large extent been neglected in studies of plants (but see (Mahall and Callaway, 1991; Schenk et al., 1999; Gruntman and Novoplansky, 2004; Karban, 2008)). For instance, in microbial communities, the perception of conspecific neighbors by an individual bacterium can elicit specific biochemical and behavioral responses that impact bacterial virulence, whereas perception of other bacterial species, or even different strains of the same species

can lead to entirely different, and often antagonistic responses (Diggle et al., 2007; Lyon, 2007). In a similar way, social insects, such as ants, are able to recognize and differentially respond to colony members versus colony invaders (Greene and Gordon, 2007). This recognition is modulated at least in part by chemical signals that impact the fitness of individual ants and the colony as a whole (Greene and Gordon, 2007; Martin et al., 2008). Perception and recognition of conspecifics by mammals affects mate recognition, antagonism, and immune response, impacting individual fitness (Sapolsky, 2005; Dulac and Wagner, 2006). Thus, organisms ranging from the simplest to the most complex differentially perceive and respond to self and non-self interactions and to conspecific and heterospecific neighbors. My results contribute to the small, but growing body of evidence that plants are also capable of such interactions.

In sum, my findings suggest that differences in plant neighbor identity, a parameter which is generally not incorporated into laboratory and greenhouse studies of plant physiology or metabolism, can have important influences on individual plant biochemistry that are likely to impact individual plant fitness and overall community success. I believe these findings necessitate further study.

5.7 REFERENCES

Appel HM (1993) PHENOLICS IN ECOLOGICAL INTERACTIONS - THE IMPORTANCE OF OXIDATION. Journal of Chemical Ecology **19:** 1521-1552

Broeckling C (2008) Primary and secondary metabolism in Centaurea maculosa and their potential roles in invasion biology. Dissertation. Colorado State University, Fort Collins

Broz AK, Manter DK, Callaway RM, Paschke MW, Vivanco JM (2008) A molecular approach to understanding plant-plant interactions in the context of invasion biology. Functional Plant Biology **35:** 1123-1134

Callaway RM, Ridenour WM (2004) Novel weapons: invasive success and the evolution of increased competitive ability. Frontiers in Ecology and the Environment **2:** 436-443

Callaway RM, Thelen GC, Barth S, Ramsey PW, Gannon JE (2004) Soil fungi alter interactions between the invader Centaurea maculosa and North American natives. Ecology **85:** 1062-1071

Cappuccino N, Arnason JT (2006) Novel chemistry of invasive exotic plants. Biology Letters **2:** 189-193

Cipollini DF, Bergelson J (2002) Interspecific competition affects growth and herbivore damage of Brassica napus in the field. Plant Ecology **162:** 227-231

Coley PD, Bryant JP, Chapin FS (1985) RESOURCE AVAILABILITY AND PLANT ANTIHERBIVORE DEFENSE. Science **230:** 895-899

Connell J (1971) On the role of natural enemies in preventing competitive exclusion in some marine animals and in rain forest trees. *In* P den Boer, G Gradwell, eds, Dynamics of populations. Center for Agricultural Publishing and Documentation, **Wageningen, The Netherlands**, pp 298–312

Diggle SP, Griffin AS, Campbell GS, West SA (2007) Cooperation and conflict in quorum-sensing bacterial populations. Nature **450:** 411-U417

Dudt JF, Shure DJ (1994) THE INFLUENCE OF LIGHT AND NUTRIENTS ON FOLIAR PHENOLICS AND INSECT HERBIVORY. Ecology **75:** 86-98

Dulac C, Wagner S (2006) Genetic analysis of brain circuits underlying pheromone signaling. Annual Review of Genetics **40:** 449-467

Elliger CA, Wong Y, Chan BG, Waiss AC (1981) GROWTH-INHIBITORS IN TOMATO (LYCOPERSICON) TO TOMATO FRUITWORM (HELIOTHIS-ZEA). Journal of Chemical Ecology **7:** 753-758

Feeny P (1976) Plant apparency and chemical defense. *In* J Wallace, R Mansell, eds, Biochemical Interactions Between Plants and Insects, Recent Advances in Phytochemistry, Vol 10. Plenum, New York, pp 1-40

Fixen P (2002) Soil test levels in North America. Better Crops **86:** 12-15

Folin O, Ciocalteu V (1927) On tyrosine and tryptophane determinations in proteins. Journal of Biological Chemistry **73:** 627-650

Fonteyn PJ, Mahall BE (1978) COMPETITION AMONG DESERT PERENNIALS. Nature **275:** 544-545

Greene MJ, Gordon DM (2007) Structural complexity of chemical recognition cues affects the perception of group membership in the ants Linephithema humile and Aphaenogaster cockerelli. Journal of Experimental Biology **210:** 897-905

Gruntman M, Novoplansky A (2004) Physiologically mediated self/non-self discrimination in roots. Proceedings of the National Academy of Sciences of the United States of America **101:** 3863-3867

Inderjit, Callaway RM, Vivanco JM (2006) Can plant biochemistry contribute to understanding of invasion ecology? Trends in Plant Science **11:** 574-580

Izaguirre MM, Mazza CA, Biondini M, Baldwin IT, Ballare CL (2006) Remote sensing of future competitors: Impacts on plant defenses. Proceedings of the National Academy of Sciences of the United States of America **103:** 7170-7174

Janzen DH (1970) HERBIVORES AND NUMBER OF TREE SPECIES IN TROPICAL FORESTS. American Naturalist **104:** 501-&

Karban R (2008) Plant behaviour and communication. Ecology Letters **11:** 727-739

Karban R, Baldwin IT, Baxter KJ, Laue G, Felton GW (2000) Communication between plants: induced resistance in wild tobacco plants following clipping of neighboring sagebrush. Oecologia **125:** 66-71

Karban R, Brody AK, Schnathorst WC (1989) CROWDING AND A PLANT ABILITY TO DEFEND ITSELF AGAINST HERBIVORES AND DISEASES. American Naturalist **134:** 749-760

Karban R, Shiojiri K, Huntzinger M, McCall AC (2006) Damage-induced resistance in sagebrush: Volatiles are key to intra- and interplant communication. Ecology **87:** 922-930

Kessler A, Halitschke R, Diezel C, Baldwin IT (2006) Priming of plant defense responses in nature by airborne signaling between Artemisia tridentata and Nicotiana attenuata. Oecologia **148:** 280-292

Lacey JR, Marlow CB, Lane JR (1989) INFLUENCE OF SPOTTED KNAPWEED (CENTAUREA-MACULOSA) ON SURFACE RUNOFF AND SEDIMENT YIELD. Weed Technology **3:** 627-631

Lankau RA, Strauss SY (2007) Mutual feedbacks maintain both genetic and species diversity in a plant community. Science **317:** 1561-1563

Lyon P (2007) From quorum to cooperation: lessons from bacterial sociality for evolutionary theory. Studies in History and Philosophy of Science Part C: Studies in History and Philosophy of Biological and Biomedical Sciences **38:** 820-833

Mahall BE, Callaway RM (1991) ROOT COMMUNICATION AMONG DESERT SHRUBS. Proceedings of the National Academy of Sciences of the United States of America **88:** 874-876

Maher EA, Bate NJ, Ni WT, Elkind Y, Dixon RA, Lamb CJ (1994) INCREASED DISEASE SUSCEPTIBILITY OF TRANSGENIC TOBACCO PLANTS WITH SUPPRESSED LEVELS OF PREFORMED PHENYLPROPANOID PRODUCTS. Proceedings of the National Academy of Sciences of the United States of America **91:** 7802-7806

Martin SJ, Vitikainen E, Helantera H, Drijfhout FP (2008) Chemical basis of nest-mate discrimination in the ant Formica exsecta. Proceedings of the Royal Society B-Biological Sciences **275:** 1271-1278

Matsuki M (1996) Regulation of plant phenolic synthesis: From biochemistry to ecology and evolution. Australian Journal of Botany **44:** 613-634

Perry LG, Johnson C, Alford ER, Vivanco JM, Paschke MW (2005) Screening of grassland plants for restoration after spotted knapweed invasion. Restoration Ecology **13:** 725-735

Pimentel D, Lach L, Zuniga R, Morrison D (2000) Environmental and economic costs of nonindigenous species in the United States. Bioscience **50:** 53-65

Ridenour WL, Callaway RM (2003) Root herbivores, pathogenic fungi, and competition between Centaurea maculosa and Festuca idahoensis. Plant Ecology **169:** 161-170

Sapolsky RM (2005) The influence of social hierarchy on primate health. Science **308:** 648-652

Schenk HJ, Callaway RM, Mahall BE (1999) Spatial root segregation: Are plants territorial? *In* Advances in Ecological Research, Vol 28, Vol 28, pp 145-180

Stamp N, Bradfield M, Li S, Alexander B (2004) Effect of competition on plant allometry and defense. American Midland Naturalist **151:** 50-64

Stamp NE, Yang YL (1996) Response of insect herbivores to multiple allelochemicals under different thermal regimes. Ecology **77:** 1088-1102

Stoll P, Prati D (2001) Intraspecific aggregation alters competitive interactions in experimental plant communities. Ecology **82:** 319-327

Theunissen JD (1995) PHENOLIC-COMPOUNDS IN THE LEAVES OF ECOTYPES OF 3 GRAMINOIDES IN THE SEMIARID GRASSLANDS OF SOUTHERN AFRICA. Journal of Arid Environments **31:** 45-53

Thorpe AS, Archer V, DeLuca TH (2006) The invasive forb, Centaurea maculosa, increases phosphorus availability in Montana grasslands. Applied Soil Ecology **32:** 118-122

Tirado R, Pugnaire FI (2003) Shrub spatial aggregation and consequences for reproductive success. Oecologia **136:** 296-301

Vandenbussche F, Pierik R, Millenaar FF, Voesenek LA, Van der Straeten D (2005) Reaching out of the shade. Current Opinion in Plant Biology **8:** 462-468

Wentzell AM, Kliebenstein DJ (2008) Genotype, age, tissue, and environment regulate the structural outcome of glucosinolate activation. Plant Physiology **147:** 415-428

CHAPTER 6

Soil fungal abundance and diversity;

another victim of the invasive plant *Centaurea maculosa*

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Soil fungal abundance and diversity; another victim of the invasive plant *Centaurea maculosa*

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AKB: designed and performed research, analyzed data, wrote manuscript
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JMV: designed research, edited manuscript

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6.1 ABSTRACT

Interactions between plants and soil microbes are important determinants of both above- and belowground community composition, and ultimately ecosystem function. As exotic plants continue to invade and modify native plant communities, there has been increasing interest in determining the influence of exotic invasives on native soil microbial communities. Here, using highly sensitive molecular techniques, we examine fungal abundance and diversity in the soil surrounding a particularly aggressive invasive plant species in North America, *Centaurea maculosa* Lam. In mixed stands, we show that this invasive weed can alter the native fungal community composition within its own rhizosphere and that of neighboring native plants. At higher densities, the effect of *C. maculosa* on native soil fungal communities was even greater. Our results demonstrate that this invasive weed can have significant effects not only on visible aboveground biodiversity but also on the native soil microbial community that extends beyond its rhizosphere.

6.2 INTRODUCTION

Plant-microbe interactions are an influential part of ecosystem functioning, and have recently gained attention in the context of invasion biology (Callaway et al., 2004; van der Putten et al., 2007). Previous research credits invasive weeds with decreasing the aboveground biodiversity of the invaded range, by displacing or even eradicating native plants and disrupting the migration patterns of animals that are associated with those native plants (Hierro and Callaway, 2003). However, there is only limited information concerning the impact of invasive plants on the belowground biodiversity in soil microbial communities. Invasive plant species are thought to benefit from a lack of co-evolved pathogens in their non-native range (Keane and Crawley, 2002), yet few studies comprehensively examine soil microbial community dynamics and their response to novel plant influences. As below and above ground communities are linked through feedback interactions, assessing the impacts of invasive plants on soil microflora may provide new insight into the mechanism by which some invaders are able to dominate landscapes and displace native plants (Klironomos, 2002; Wardle et al., 2004; Wolfe and Klironomos, 2005).

To improve our understanding of the influence of non-native plants on soil fungal communities, we collected soils from sites populated by the aggressive North American invasive weed *Centaurea maculosa* Lam. Fungal species diversity in high density stands of *C. maculosa* were compared to those found in adjacent low density stands at two sites. High density *C. maculosa* stands were near monocultures, consisting almost entirely of *C. maculosa* plants, whereas low density stands contained isolated *C. maculosa* ($\geq$ 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 (Manter and Vivanco, 2007).

6.3 MATERIALS AND METHODS

HIGH AND LOW DENSITY STANDS OF CENTAUREA MACULOSA

Field site sampling

Soils were collected in September 2005 from two field sites near Missoula MT (Mount Jumbo; 11T 0698990 5205230, and Petty Mountain; 12T 0274070 5195422). Each site contained adjacent areas where *Centaurea maculosa* Lam. plants were distributed at both high and low densities. High density stands were near monocultures of *C. maculosa*, whereas low density stands contained isolated adult *C. maculosa* plants one meter or more apart. Differences in vegetation between the adjacent high density and low density stands of *C. maculosa* may be due to the movement of the *C. maculosa* plant community, which is constrained by seed dispersal distances (ie; the low density stand would be considered the 'invasion front' and is predicted to transition to a higher density stand over time when more seeds reach the area). Thus, the low density stand could be considered younger than the high density stand. Alternatively, there may be some inherent differences between the adjacent sites which dictate *C. maculosa* density (soil characteristics, aspect, slope, etc). Within high and low density stands at each site, five mature *C. maculosa* plants (crown at least 5 mm in diameter) were randomly selected. In low density stands five representatives of a native grass species (*Pseudoroegneria spicata,* bluebunch wheatgrass) were also randomly selected. All plants were removed by digging a 15 cm radius around each plant, approximately 15 cm deep. Each soil ball was placed

into a 2 L plastic bag and stored at ambient temperature until processing (soil was processed <24 hours after sampling).

Soil processing

C. maculosa and *P. spicata* plants were carefully removed from the soil balls in an attempt to leave the root system intact. Remaining soil from the soil ball was pooled by site and stand density, shaken through a 2mm sieve, placed into a fresh plastic bag and homogenized by hand. Two hundred grams of this "bulk soil" from each site was removed for basic soil analyses and 50 g of soil was used to determine water content. Soil within 1 mm of plant roots was considered to be rhizosphere soil. Plants were shaken to remove loose soil and rhizosphere soil was then collected by brushing the remaining soil off of plant roots with a gloved hand. Rhizosphere soil was placed into sterile whirlpack bags. All soil samples were kept at 4° C until DNA extraction.

Soil DNA extraction

Rhizosphere (250 mg) and bulk soil (500 mg) were subject to DNA extraction using the UltraClean Soil DNA Kit (Mo Bio, Carlsbad CA) according to the manufacturer's instructions except for the addition of one extra wash with ethanol to remove excess humic acid. Samples for each site were; 1) low density bulk, 2) high density bulk, 3) low density *C. maculosa* rhizosphere, 4) high density *C. maculosa* rhizosphere, and 5) grass rhizosphere. Soil DNA was quantified using a nanodrop spectrophotometer (Nanodrop technologies, Wilmington DE) and diluted with distilled water to a concentration of 20 ng/μL.

qPCR and length heterogeneity analysis

Estimates of total fungal abundance and community composition (i.e., abundance and diversity of phylotypes) was determined by qPCR and length heterogeneity analysis using the methods of Manter and Vivanco (2007) and the highly conserved fungal primers, 2234C and 3126T (Sequerra et al., 1997; Ranjard et al., 2001). All reactions were done in duplicate. A dilution series (1x, 2x, 4x) was preformed to assess if PCR amplification was inhibited in the soil samples due to humic acids or other soil inhibitors. No inhibition was found in any samples tested.

Data analysis

All analyses were conducted in SAS 9.1 using the mixed model procedure. Total fungal abundance (fungal DNA estimated by qPCR) and individual phylotype abundance (peak heights obtained from the length heterogeneity analysis) were analyzed by ANOVA with site as a blocking factor. Differences among means were determined by Fisher's protected least significant difference ($P < 0.05$).

DISTANCE EXPERIMENT

Field site sampling

Soils were collected in late May 2006 from one roadside field site East of Missoula MT (UTM 12T 0301244 5177747). The site contained *C. maculosa* plants spaced at least 5 meters apart, with the other vegetation consisting mostly of native grasses. Five mature (crown at least 5 mm in diameter) *C. maculosa* plants were randomly selected and removed by digging a 10 cm radius around each plant,

approximately 15 cm deep. Randomly selected *Poa secunda* (a native grass) growing at different distances (15, 30, 60, and 90 cm) from the mature *C. maculosa* plants were also removed by digging. Each sample was placed in separate 2 L plastic bags and stored in the dark at ambient temperature until processing.

Soil processing

Rhizosphere soil (within 1 mm of plant roots) was collected from a single *P. secunda* growing at each distance point (0 [within *C. maculosa* sample], 15, 30, 60, and 90 cm). Plants were shaken to remove loose soil and rhizosphere soil was then collected by brushing the remaining soil off of plant roots with a gloved hand. Soil samples were kept separate and stored in Eppendorf tubes at 4°C until DNA extraction.

Soil DNA extraction

Between 100-250 mg of rhizosphere soil was subject to DNA extraction using the UltraClean Soil DNA Kit (Mo Bio, Carlsbad CA) according to the manufacturer's instructions, except for the addition of one extra wash with ethanol to remove excess humic acid. Samples consisted of *C. maculosa* rhizosphere soil and rhizosphere soil of *Poa secunda* at five different distances from each *C. maculosa* plant (0, 15, 30, 60 and 90 cm). Soil DNA was quantified using a nanodrop spectrophotometer (Nanodrop technologies, Wilmington DE) and diluted with distilled water to a concentration of 10 ng/μL.

qPCR and length heterogeneity analysis

Estimates of total fungal abundance and community composition (i.e., abundance and diversity of phylotypes) was determined by qPCR and length heterogeneity analysis using the methods of Manter and Vivanco (2007) and the highly conserved fungal primers, 2234C and 3126T (Sequerra et al., 1997; Ranjard et al., 2001). All reactions were done in duplicate.

Data analysis

All analyses were conducted in SAS 9.1 using the mixed model procedure. Total fungal abundance (fungal DNA estimated by qPCR) and individual phylotype abundance (peak heights obtained from the length heterogeneity analysis) were analyzed by repeated measures ANOVA with distance a repeated measure and individual *C. maculosa* plants serving as the subject. Significant differences from a control (*Poa secunda* rhizosphere 90 cm from the nearest *Centaurea maculosa* plant) were determined by Fisher's protected least significant difference ($P < 0.05$). Total abundance (Σ of peak heights) was determined for all phylotypes in each of two categories, significantly increasing or decreasing, based on the pairwise comparison between the 0 and 90 cm distances.

6.4 RESULTS AND DISCUSSION

Total fungal biomass (estimated by qPCR) and fungal phylotype richness varied between the two sites; however, at both sites the high-density *C. maculosa* stand was associated with significant declines in fungal abundance and diversity. Bulk soil from high-density stands of *C. maculosa* contained over 80% less fungal DNA, associated with the decline in abundance of six phylotypes, compared to low density stands (Figure 6.1, panels A and B). In soils obtained from *C. maculosa* rhizospheres, fungal biomass was reduced nearly seven times in high density stands compared to low density stands (Figure 6.1A).

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 *C. maculosa* rhizosphere, but was greater than that of the *C. maculosa* 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 *C. maculosa* low density and sixteen in the *C. maculosa* high density when compared to *P. spicata* (Figure 6.1C). Conversely, six phylotypes increased in abundance in both the high and low density *C. maculosa* rhizosphere as compared to the rhizosphere of *P. spicata*. Though previous work revealed that *C. maculosa* disrupts the arbuscular mycorrhizal fungal community of native and naturalized grasses (Mummey et al., 2005; Mummey and Rillig, 2006), our results present the first analysis of the effects of *C. maculosa* on the broader soil fungal community.

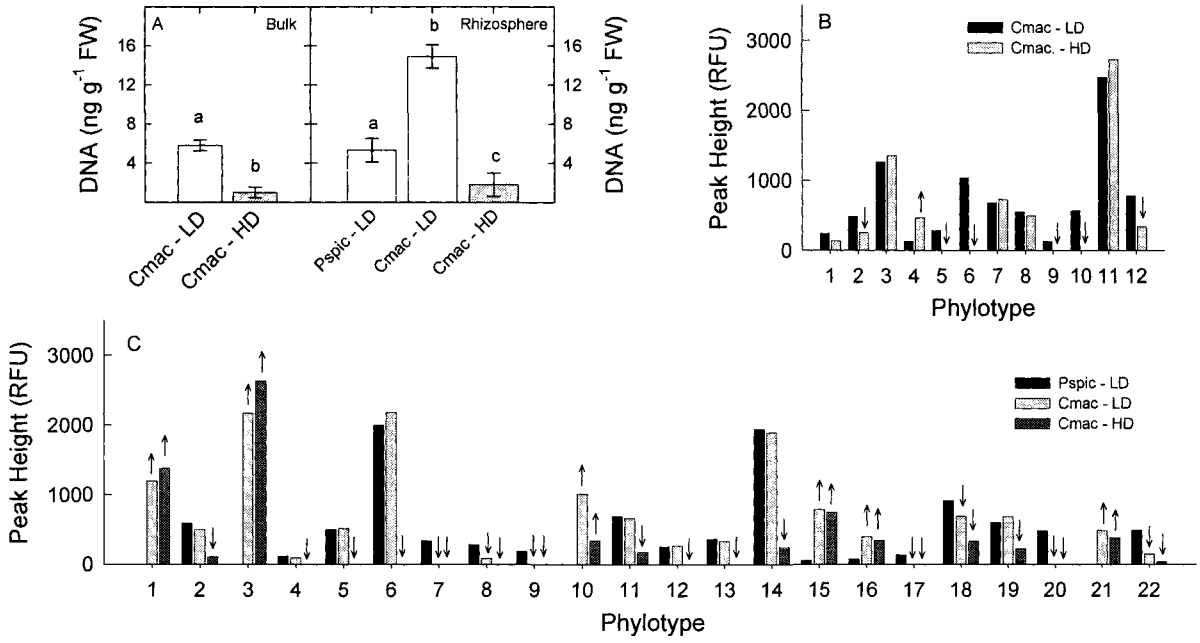

Figure 6.1. Analysis of fungal communities from high and low density stands of *C. maculosa*. Total fungal DNA (panel A) and individual phylotype abundance (peak height) from bulk (panel B) and rhizosphere soil (panel C) from two sites in Montana that contained adjacent high and low density stands of *C. maculosa*. Pspic – LD = *Pseudoroegneria spicata* in the low density stands, Cmac – LD = *Centaurea maculosa* in the low density stands, Cmac – HD = *C. maculosa* 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)

It is possible that the reduction in plant diversity in HD *C. maculosa* stands leads to the observed reduction in fungal diversity. However, recent reports suggest that soil characteristics, such as resource availability (Carney et al., 2004; Waldrop et al., 2006) and pH (Fierer and Jackson, 2006), are better predictors of microbial community diversity than the existing plant community. Soils from HD and LD stands had similar C/N ratios and similar amounts of humic acids (data not shown) however, soil characteristics that were not tested may also play a role in the modification of fungal communities between high and low density *C. maculosa* stands.

To examine if and at what distance *C. maculosa* could affect the fungal community present in the soil rhizosphere of native grasses, we collected soils from another low-density stand of *C. maculosa* (> 5 m spacing), but focused on a native grass species (*Poa secunda*) growing at various distances from *C. maculosa* adults. Rhizosphere soils collected from *P. secunda* growing directly adjacent to *C. maculosa* had significantly higher fungal biomass than those collected from distances further away (Figure 6.2A). 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 *C. maculosa* 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 culmination of two different effects of *C. maculosa* on fungi in the rhizosphere (Figure 6.2B). For example, total abundance (Σ peak heights) of some phylotypes decreased within 15 cm of a *C. maculosa* plant; whereas, other phylotypes show a dramatic increase within the *C. maculosa* rhizospheric zone. When the resultant total abundance of all phylotpyes is determined (Figure 6.2B), this pattern is consistent with our observed estimates of total fungal biomass. It should also be noted that *C. maculosa* roots were not apparent in grass rhizosphere samples at a distance of 15 cm or more from the *C. maculosa* plant, suggesting that

diffusible root exudates may be partially responsible for the observed decrease in microbial biomass at this distance.

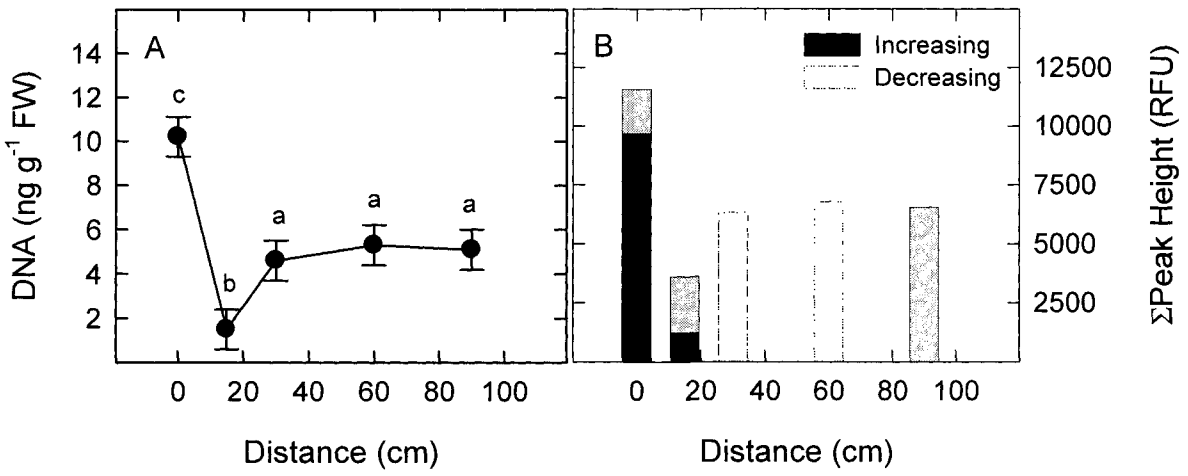

Figure 6.2. Analysis of *P. secunda* fungal communities at various distances from *C. maculosa*. Total fungal DNA (panel A) and phylotype abundance (panel B) from the rhizosphere of *Poa secunda* plants at various distances from a *Centaurea maculosa* plant. Points are LSmeans and pooled standard errors, 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) of significantly increasing or decreasing phylotypes. At each sample point, the observed phylotypes were assigned to the above categories if they were significantly different between the *P. secunda* rhizophere at 0 and 90 cm ($P < 0.05$, panel B)

Soil microbes can have a profound influence on molecular and biochemical processes in individual plants, plant communities and ultimately the ecosystem (Klironomos, 2002; Callaway et al., 2004; Stinson et al., 2006), but the consequences of modification of microbial biodiversity in the rhizospheric and nonrhizospheric soil are not well understood. Disruption of the balance between native plant and microbial communities has been shown to have a negative effect on native plants but a positive effect on invasive plants (Klironomos, 2002; Stinson et al., 2006), and it is possible that such a disruption could cause an imbalance to arise between plant species, causing some to flourish at the expense of others. Here we show that the aggressive invasive weed *C. maculosa* alters soil fungal communities in its own rhizosphere and bulk soil when it is found in high density versus low density stands, and provide evidence that these microbial community alterations extend to neighboring native grass species. In addition, we show that *C. maculosa* favored phylotypes that were rare, and often undetectable, in the native soil microbial community. A better understanding of the interactions between invasive plants, native plants, and soil microbial communities is essential for developing appropriate land management and restoration strategies.

6.5 REFERENCES

Callaway RM, Thelen GC, Rodriguez A, Holben WE (2004) Soil biota and exotic plant invasion. Nature **427:** 731-733

Carney KM, Matson PA, Bohannan BJM (2004) Diversity and composition of tropical soil nitrifiers across a plant diversity gradient and among land-use types. Ecology Letters **7:** 684-694

Fierer N, Jackson RB (2006) The diversity and biogeography of soil bacterial communities. Proceedings of the National Academy of Sciences of the United States of America **103:** 626-631

Hierro JL, Callaway RM (2003) Allelopathy and exotic plant invasion. Plant and Soil **256:** 29-39

Keane RM, Crawley MJ (2002) Exotic plant invasions and the enemy release hypothesis. Trends in Ecology & Evolution **17:** 164-170

Klironomos JN (2002) Feedback with soil biota contributes to plant rarity and invasiveness in communities. Nature **417:** 67-70

Manter DK, Vivanco JM (2007) Use of the ITS primers, ITS1F and ITS4, to characterize fungal abundance and diversity in mixed-template samples by qPCR and length heterogeneity analysis. Journal of Microbiological Methods **71:** 7-14

Mummey DL, Rillig MC (2006) The invasive plant species Centaurea maculosa alters arbuscular mycorrhizal fungal communities in the field. Plant and Soil **288:** 81-90

Mummey DL, Rillig MC, Holben WE (2005) Neighboring plant influences on arbuscular mycorrhizal fungal community composition as assessed by T-RFLP analysis. Plant and Soil **271:** 83-90

Ranjard L, Poly F, Lata JC, Mougel C, Thioulouse J, Nazaret S (2001) Characterization of bacterial and fungal soil communities by automated ribosomal intergenic spacer analysis fingerprints: Biological and methodological variability. Applied and Environmental Microbiology **67:** 4479-4487

Sequerra J, Marmeisse R, Valla G, Normand P, Capellano A, Moiroud A (1997) Taxonomic position and intraspecific variability of the nodule forming Penicillium nodositatum inferred from RFLP analysis of the ribosomal intergenic spacer and random amplified polymorphic DNA. Mycological Research **101:** 465-472

Stinson KA, Campbell SA, Powell JR, Wolfe BE, Callaway RM, Thelen GC, Hallett SG, Prati D, Klironomos JN (2006) Invasive plant suppresses the growth of native tree seedlings by disrupting belowground mutualisms. Plos Biology **4:** 727-731

van der Putten WH, Klironomos JN, Wardle DA (2007) Microbial ecology of biological invasions. Isme Journal **1:** 28-37

Waldrop MP, Zak DR, Blackwood CB, Curtis CD, Tilman D (2006) Resource availability controls fungal diversity across a plant diversity gradient. Ecology Letters **9:** 1127-1135

Wardle DA, Bardgett RD, Klironomos JN, Setala H, van der Putten WH, Wall DH (2004) Ecological linkages between aboveground and belowground biota. Science **304:** 1629-1633

Wolfe BE, Klironomos JN (2005) Breaking new ground: Soil communities and exotic plant invasion. Bioscience **55:** 477-487

CHAPTER 7

Plant-soil feedback effects from microbial communities present in spotted knapweed infested soils

7.1 ABSTRACT

Soil microbial community composition may be an important factor influencing exotic plant invasion. Feedback effects between native soil microbial communities and introduced exotic plants tend to be positive or neutral; whereas feedbacks with either rare or native plants tend to be negative. There is a good body of evidence describing alterations to native soil microbial communities that result from infestation by the invasive exotic plant spotted knapweed. In particular, decreases in fungal community biomass have been associated with increasing spotted knapweed density in the field. However, the relevance of these observed microbial community modifications on plant community structure through feedback effects remains unknown in this system. Here I provide evidence that both spotted knapweed and the native grass Idaho fescue experience either negative or neutral feedbacks from spotted knapweed infested soils when grown under greenhouse conditions. Additionally, when spotted knapweed planting density was manipulated in the greenhouse, soil fungal biomass increased with increased planting density of spotted knapweed. These results suggest that factors other than, or in combination with knapweed density are driving the observed reductions in fungal community biomass in field collected soils. I identified a variety of inconsistencies in my experimental results and those published in the literature, highlighting the difficulty of making robust conclusions from greenhouse experiments designed to determine the impact of microbial communities on plant community structure.

7.2 INTRODUCTION

Although the economic and biological costs associated with exotic plant invasion are large, the mechanisms of plant invasion remain poorly understood (Pimentel et al., 2000; Levine et al., 2003). The introduction of exotic plant species is often devastating to native ecosystem biodiversity, often leading to extinction of native plant species (Pimentel et al., 2000) and 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 et al., 2004; Wolfe and Klironomos, 2005; Wardle, 2006).

Multiple lines of evidence demonstrate linkages between above and below ground community structure in natural ecosystems (Wardle et al., 2004; Wardle, 2006). The interaction between introduced plant species and native soil microbial communities may therefore result in important plant-soil feedbacks that either facilitate or inhibit invasion of exotic plant species. Recent evidence suggests that soil microbial communities can play an important role in exotic plant invasion through plant-soil feedbacks (Klironomos, 2002; Callaway et al., 2004; Reinhart and Callaway, 2004; Wolfe and Klironomos, 2005). Exotic plants tend to experience positive or neutral feedbacks from soil communities, whereas rare native plants tend to experience negative feedbacks, likely due to increased levels of soil pathogens (Klironomos, 2002). The linkages between microbial

communities and plant invasion may have important implications for the management of exotic weeds and restoration of native plant communities (Klironomos, 2002; Reinhart and Callaway, 2004; Wardle et al., 2004).

Previous work indicates that the invasive plant species spotted knapweed (*Centaurea maculosa* Lam., also known as *C. stoebe* L. spp. *micranthos* (Gugler) Hayek, or *C. biebersteinii*) alters fungal community composition in the introduced range (Mummey et al., 2005; Mummey and Rillig, 2006; Broz et al., 2007). Reductions were found in both fungal biomass and richness in soils collected from areas dominated by spotted knapweed (high density) compared to areas with lower densities of knapweed (Broz et al., 2007). This effect was particularly strong in rhizosphere soils collected from spotted knapweed plants, but was observable in bulk soils as well (Broz et al., 2007). Similarly, reductions and alterations in arbuscular mycorrhizal fungi (AMF) were identified when comparing spotted knapweed infested sites to grass dominated sites (Mummey and Rillig, 2006). Further, the alterations in soil fungal communities due to knapweed infestation were also found to extend to the rhizospheres of neighboring grass species (Mummey et al., 2005; Broz et al., 2007). This work demonstrates that microbial communities are modified by spotted knapweed, but the relevance of this finding in regards to feedback effects on plant fitness was not determined in these experiments. Other studies suggest that spotted knapweed experiences positive feedbacks in North American soils that have been preconditioned by spotted knapweed in the greenhouse (Callaway et al., 2004; Reinhart and Callaway, 2004). Similarly, the native grass Idaho fescue is reported to perform better in soils that were preconditioned by other fescue plants versus soils preconditioned by spotted knapweed (Reinhart and Callaway, 2004).

In this study I utilized soils collected from high and low density stands of spotted knapweed in greenhouse experiments to determine if there were any positive or negative feedbacks on biomass of spotted knapweed, and the native North American bunchgrass Idaho fescue (*Festuca idahoensis*). I also utilized soils collected from a low density stand of spotted knapweed and manipulated spotted knapweed planting density in the greenhouse to determine the impact of plant density on soil microbial community composition.

Based on work by other authors (Klironomos, 2002; Callaway et al., 2004; Reinhart and Callaway, 2004), I hypothesized that invasive spotted knapweed plants grown in soils collected from both high and low density stands of spotted knapweed would result in a positive feedback (increase in biomass compared to sterilized control soils). Similarly, I expected that adding soil slurries from these soil types under different nutrient conditions would lead to increases in spotted knapweed biomass compared to sterile controls. Conversely, I hypothesized that native Idaho fescue plants grown with spotted knapweed infested soils or soil slurries would experience a negative feedback (decrease in biomass compared to sterilized controls). Due to the extreme alteration of soil communities correlated with high density spotted knapweed infestation, and the overall success of spotted knapweed in the field, I also hypothesized that soils from high density infestations would have a greater negative impact on Idaho fescue biomass than those from low density infestations.

Additionally, I was interested in determining if I could recreate the observed decrease in fungal biomass due to spotted knapweed infestation in the greenhouse. I planted spotted knapweed at different densities in soils collected from a low density

infestation of spotted knapweed. I hypothesized that I would see a decrease in fungal biomass and diversity that correlated with increased planting density of spotted knapweed.

7.3 MATERIALS AND METHODS

FIELD SITE ASSESSMENT AND SOIL COLLECTION

Field site assessment and vegetation survey:

Soils from spotted knapweed infested areas near Missoula, Montana were sampled using a transect method in early September 2007. Soils were collected from four field sites (Site 1, Waterworks Hill; 12UTM 0272985 5196383; Site 2, roadside; 12T 0272632 5188218; Site 3, Mount Petty; 11T 0699060 5205077; Site 4, near Mount Petty; 11T 0699717 5203723). Each site contained adjacent plots where spotted knapweed plants occurred at high and low relative densities. High density (HD) stands were near monocultures of spotted knapweed, whereas low density (LD) stands contained isolated adult spotted knapweed plants (*C. stoebe* L. spp. *micranthos* (Gugler) Hayek, *C. maculosa* Lam). A 50-meter long transect was run across the length of each plot. To determine percent cover of spotted knapweed and other vegetation, a 0.5- x 0.75-meter rectangle was placed next to the transect every five meters, and the percent cover of spotted knapweed, grass, forbes, legumes, woody plants, moss and bare ground within the rectangle was estimated. This resulted in ten vegetation cover measurements per plot.

Soil sampling protocol:

A protocol similar to the one used for the vegetation survey was used for the collection of spotted knapweed plants and soils. In the low density plots, one adult

spotted knapweed plant (crown at least 5 mm in diameter) and surrounding soil falling within the 0.5- x 0.75-meter rectangle was collected at every 5 meter point along the 50-meter transect. If no spotted knapweed plants were present within the rectangle at a given point, the closest plant to the rectangle was sampled. Ten plant-soil samples were collected from each low density plot, resulting in 40 total samples. The sampling protocol differed slightly for high density sites of spotted knapweed because a smaller amount of soil was desired for use in experimentation. Within the high density sites, one spotted knapweed adult and surrounding soil falling within the 0.5- x 0.75-meter rectangle was collected at three points along the 50-meter transect; the starting point (0 m), the middle point (25 m) and the end of the transect (50 m). Three plant-soil samples were collected from each high density plot, resulting in 12 total samples.

All plants and their surrounding soil were removed by digging a 10-15-cm radius around each plant, approximately 15 cm deep. Each plant and its surrounding soil was placed into a 1-gallon plastic bag, which was then sealed and stored in a cooler at ambient temperature until processing. Soils were returned to Colorado State University and allowed to air dry at room temperature. Bags of soil were kept separate based on the plot (site-density) that they were collected from during drying to prevent cross-contamination. Bags of soil were dried in boxes or coolers with the lids propped open just slightly to reduce the possibility of microbial contamination from the air. Once the soils were completely dry they were all separately sifted through a sterile 2-mm screen to remove rocks, plant material and other large objects. The soil sifting device was cleaned between sifting of each soil type by washing, and after washing the device was sprayed down with 75% ethanol and wiped clean. Sifted soil from each plot was pooled, resulting

in eight total soil types (HD or LD from sites 1-4). Once soils were pooled, they were extensively homogenized by hand, using separate pairs of latex gloves to prevent cross contamination. Three hundred grams of each of the eight soil type were submitted to the Colorado State University Plant-Water-Soil Testing Facility for a general soil analysis. A small amount of soil was removed for DNA extraction and kept at 4° C. The remaining soil was left at room temperature, with each soil type kept in a separate closed cooler.

EXPERIMENT 1: SOIL FEEDBACK EFFECTS

Soil DNA extraction and qPCR analysis

In a preliminary experiment, DNA was extracted from approximately 250 mg of soil from each plot (the four HD and four LD spotted knapweed sites) using the UltraClean Soil DNA Kit (Mo Bio, Carlsbad CA). The concentration of each DNA sample was quantified using a spectrophotometer with a 5-μL cell (Biospec Mini, Shimadzu, Columbia MD), and all samples were diluted with distilled sterile water to a concentration of 1 ng/μL. Estimates of total fungal abundance were determined by qPCR using the highly conserved fungal primers, 2234C and 3126T (Sequerra et al. 1999, Ranjard et al. 2001) in the protocol outlined in Manter and Vivanco (2007).

Soil set up in greenhouse

Soils from all eight plots were kept separate for this experiment (HD sites 1-4 and LD sites 1-4) due to variation in soil fungal abundance between plots. A portion of each soil type was sterilized by autoclaving for 30 minutes, freezing at -20° C overnight and autoclaving again for 30 minutes. The autoclaving parameters for this and all following

experiments were 20 PSI at 250° F. Sterilized soils were cooled to room temperature before use in the greenhouse experiment. Each soil type was then mixed with a sterile media that was made from two parts play sand (US Mix, Denver CO) and one part clay soil conditioner (100% arcillite, Schultz, Bridgeton MO). This resulted in sixteen different soil mixtures, each containing 30% soil and 70% sterile media (HD sites 1-4 sterile and non-sterile; LD sites 1-4 sterile and non-sterile). Sterile glass wool was placed into the bottom of 288 sterile conetainers (Stuewe and Sons, Tangent OR, USA) that each had an approximate volume of 164 cm^3 (16 soil types x 2 plant types x 9 repetitions = 288 units). The conetainers were then labeled, filled with the appropriate soil type and were wetted to saturation with water in the greenhouse.

Plant growth and harvest

Two plant types, spotted knapweed and Idaho fescue, were used in this experiment to determine feedback effects of soils. Seeds of spotted knapweed (*Centaurea stoebe* spp *micranthos*) were collected from invasive field populations in Missoula Valley, MT, USA by the laboratory of Ragan Callaway. Seeds of Idaho fescue (*Festuca idahoensis* (Elmer)), a native perennial bunchgrass that commonly occurs in rangelands invaded by spotted knapweed were obtained from Wind River Seed Company (Manderson, WY, USA). For each plant type, three seeds were placed directly into the appropriate conetainers filled with soil mixtures, watered briefly with a misting wand, and covered with plastic wrap to retain a humid environment for germination. Seedlings of both species emerged after approximately two weeks. Plastic wrap was removed from the tops of the conetainers and seedlings were thinned to one plant per conetainer.

Conetainers were then completely randomized into racks, and were placed in racks in a staggered fashion. The experiment was monitored to ensure that only one plant was present in each conetainer for the duration of the experiment. Plants were watered every Monday, Wednesday and Friday with a misting wand, excepting days that they were given Hoagland's nutrient solution.

The recipe used for Hoagland's solution is as follows: separate 1M stock solutions were made for $Ca(NO_3)_2$-$4H_2O$ (236.1 g/L), KNO_3 (101.1 g/L), KH_2PO_4 (136.1 g/L), and $MgSO_4$-$7H_2O$ (246.5 g/L). Trace elements were made in a separate stock solution with H_2BO_3 (2.8 g/L), $MnCl_2$-$4H_2O$ (1.8 g/L), $ZnSO_4$-$7H_2O$ (0.2 g/L), $CuSO_4$-$5H_2O$ (0.1 g/L), and $NaMoO_4$ (0.025 g/L). A final stock solution was made containing an EDTA Iron (III) Sodium Salt ($C_{10}H_{12}FeN_2NaO_8$) (9.71 g/L). All stock solutions were stored at 4° C when not in use. To make 1 L of full-strength Hoagland's solution from these stocks, 7 mL of 1M $Ca(NO_3)_2$-$4H_2O$, 5 mL of 1M KNO_3, 2mL of 1M KH_2PO_4, 2mL of 1M $MgSO_4$-$7H_2O$, 1 mL of trace element stock and 1 mL of iron stock were combined and brought to 1 L with water.

Each conetainer received approximately 20 mL of full-strength Hoagland's nutrient solution every week and a half. A small watering pot was used to put Hoaglands solution into each conetainer, and after receiving the nutrient solution plants were briefly misted with water. Plants were grown in the greenhouse under this watering regime for eleven weeks (February 18 – May 5, 2008).

At harvest (May 5-6, 2008), each plant was removed from its conetainer and shaken to remove loosely attached soil media. The roots of each plant were then washed in water to remove as much soil media as possible. Each plant was cut with a razor blade

to separate above and below ground biomass. All biomass samples were placed into separate envelopes and dried at 60° C for at least 48 hours. Dry biomass samples were weighed to the nearest mg, and weights of both above and below ground biomass were recorded for each plant sample.

Statistical analysis

General soil characteristics were analyzed with Statistical Analysis Software (SAS) 9.1 (Cary, NC, USA) using a mixed model ANOVA. Each soil variable (pH, OM, Cu, etc; see Table 1 for full list of characteristics) was analyzed in relation to density (high or low density plots).

Total biomass and root to shoot ratios were calculated for each plant. The Statistical Analysis Software (SAS) 9.1 (Cary, NC, USA) was used to analyze data for each plant type using a mixed model ANOVA. Soil density (HD or LD), sterility (sterile or non-sterile) and density*sterile were used as fixed variables in the analysis. Soil site (1-4), site*density, and site*density*sterile were analyzed as random factors to account for differences between sites. All data were normal without transformation and fit the assumptions of the ANOVA analysis.

EXPERIMENT 2: SOIL SLURRY FEEDBACK EFFECTS AT DIFFERENT NUTRIENT LEVELS

Soil slurry set up in greenhouse

A sterile media was made by mixing two parts play sand (US Mix, Denver CO) with one part clay soil conditioner (100% arcillite, Schultz, Bridgeton MO) and

autoclaving for 30 minutes. Sterile glass wool was placed into the bottom of 140 sterile conetainers that each had an approximate volume of 164 cm^3 (5 soil types x 2 plant types x 2 nutrient conditions x 7 repetitions = 140 units). The conetainers were then labeled, filled with the sterile soil media, placed into racks and wetted to saturation with water in the greenhouse.

Soils from each of the four plots within a density type (HD or LD) were mixed in equal proportions and homogenized by hand. Half of each soil type (HD or LD) was sterilized by autoclaving for 30 minutes (as described in experiment 1), freezing at -20° C overnight and autoclaving again for 30 minutes. Sterilized soils were cooled and stored at room temperature until use in slurries. Five "soil slurry treatments" were used in this experiment; HD sterile, HD non-sterile, LD sterile, LD non-sterile, and sterile water as a control. To prepare the soil slurries, five one-liter bottles, each containing 400 mL water, were autoclaved for 30 minutes and then allowed to cool to room temperature. For each soil type, forty grams of soil was added to one of the bottles of sterile water and shaken to mix. Ten mL of soil slurry (or water control) was applied to each appropriate cone. Before applying the soil slurry, the mixture was allowed to settle briefly, as large particles tended to clog the pipette. Soil slurries were applied to the conetainers three times over the course of the experiment; before germination, immediately after germination and during vegetative growth (September 8, September 22 and November 3, 2008).

Plant growth and harvest

Two plant types, spotted knapweed and Idaho fescue, were used in this experiment to determine feedback effects of soil slurries under different nutrient conditions. Refer to the methods for experiment 1 for details on seed source. On September 5, 2008, three seeds of the appropriate plant type were placed directly into their respective conetainers which had been filled with the sterile soil media. Seeds were watered briefly with a misting wand and covered with plastic wrap to retain a humid environment for germination. Soil slurries were applied on September 8, before seeds had germinated. Seedlings of both species emerged after approximately two weeks. Plastic wrap was removed from the tops of the conetainers and seedlings were thinned to one plant per conetainer. Soil slurries were applied again after the seedlings were thinned on September 22. At this point the conetainers were randomized in a complete block design, with blocking factors as nutrient condition (high or low) and sterility (sterile or non-sterile). Conetainers were randomized into racks and placed in a staggered fashion, resulting in four total racks. Rack position in the greenhouse was randomized every other week during the course of the experiment.

Starting on September 24, plants were given approximately 20 mL of a nutrient solution once per week (every Wednesday). The plants in the high nutrient condition received full-strength Hoagland's solution (for recipe see experiment 1), and the plants in the low nutrient condition received 1/10 strength Hoagland's solution. Plants were watered thoroughly with a misting wand every Monday and Friday over the course of the experiment, except on Monday November 3, because soil slurries were applied that day.

All plants were harvested on December 8, 2008 and biomass samples were collected, dried and weighed as in experiment 1.

Statistical analysis

Total biomass and root to shoot ratios were calculated for each plant. The Statistical Analysis Software (SAS) 9.1 (Cary, NC, USA) was used to analyze data for each plant type using a mixed model ANOVA. Soil density (HD, LD or NA for water control), sterility (sterile or non-sterile) and density*sterile were fixed variables in the analysis. All data were normal without transformation and fit the assumptions of the ANOVA analysis.

EXPERIMENT 3: DENSITY MANIPULATION EXPERIMENT

Soil set up in greenhouse

At the end of April 2008, 10 L of soil collected from the site 3 low density plot was mixed with 5 L of a sterile soil media made from two parts play sand (US Mix, Denver CO) and one part clay soil conditioner (100% arcillite, Schultz, Bridgeton MO). Results from the preliminary fungal community analysis showed the largest reduction between the high and low density soils collected at this site. A small piece of germination paper was placed at the bottom of each of 40 (4 planting densities x 10 repetitions) black 2.5- x 2.5- x 3-inch pots to ensure that the soil media did not leak out. The pots were taken to the greenhouse and each was filled with the site 3 low density soil mixture and watered until saturation.

Plant growth and harvest

Planting density of spotted knapweed was manipulated in this experiment, with pots containing a total of 1, 2, 4 or 16 plants (with 10 repetitions per density). Seeds of spotted knapweed (*Centaurea maculosa* Lam.) were collected from invasive field populations in Missoula Valley, MT, USA by the laboratory of Ragan Callaway. For seeding purposes, the desired number of plants per pot was multiplied by three, and this number of seeds was added to the pot (for instance, 6 seeds were added to pots with a final planting density of two). Pots were randomized on the greenhouse bench and covered with plastic wrap to retain humidity and enhance germination. After approximately two weeks many seedlings had germinated in the pots. Plastic wrap was removed from the pots and seedlings were thinned to the desired density (1, 2, 4 or 16 plants per pot). Three balls (approximate total weight of 45 mg) of Osmocote outdoor and indoor slow release plant food (The Scotts Miracle Grow Company, Marysville OH, USA) were added to each pot as the sole source of fertilizer in the experiment. Plants were watered thoroughly every Monday, Wednesday and Friday with water using a misting wand over the course of the experiment.

In mid October 2008, plants were un-randomized in the greenhouse. The rosette diameter and number of leaves per plant were measured for each individual plant per pot, except for those in planting density 16, in which four plants were measured per pot. Shortly thereafter, all plants were harvested. Pots from all of the planting densities were filled with roots, making it difficult to assess below ground biomass. The tops of the plants were cut with a razor blade at soil level and combined into a single sample of above ground biomass per pot. All 40 samples of above ground tissues were frozen at -

80° C for at least 4 hours and then freeze dried under a vacuum at -75° C for 24 hours, after which the tissue was stored at 4° C. All samples of above ground biomass were weighed to the nearest mg, ground to a fine powder using a coffee grinder and placed in falcon tubes for storage at 4° C.

The soil was removed and sampled from each pot separately by breaking open each root ball and taking a sample of the soil from inside. A few grams of soil was removed from each pot, placed in a labeled whirl-pack bag, and stored at -80° C until DNA extraction and subsequent quantitative PCR analysis. The soil from each pot was sampled in such a way that it was relatively free of roots, but it would likely be considered "rhizosphere soil" because it came from within the root ball and was within 2 mm of the root system. The remaining soil and roots were pooled by experimental planting density and stored at 4° C.

The dry weight of each soil was estimated by taking two 25-gram samples of the pooled soil collected from the pots, recording an initial weight, drying the soil at 60°C for at least two days and recording the dry weight.

qPCR analysis

Soil DNA was extracted from approximately 200 mg of soil from each pot (giving 10 repetitions per density) using the UltraClean Soil DNA Kit (Mo Bio, Carlsbad CA). The concentration of each DNA sample was quantified using a spectrophotometer with a 5-μL cell (Biospec Mini, Shimadzu, Columbia MD), and all were diluted with distilled sterile water to a concentration of 1 ng/μL. Estimates of total fungal abundance were determined by qPCR using the highly conserved fungal primers, 2234C and 3126T

(Sequerra et al. 1999, Ranjard et al. 2001) in the protocol outlined in Manter and Vivanco (2007). A similar protocol was utilized to determine bacterial abundance, using conserved primers to 16S ribosomal DNA regions. Technical replications were not done because samples were biologically replicated ten times for each planting density.

Total phenolics assay on plant material

For each plant sample (one sample per pot), one mL of 80% acetone was added to 20 mg of ground leaf tissue, vortexed 30 seconds, rotated at 250 rpm in the dark for 15 minutes, vortexed briefly, and centrifuged at 10,000 rpm at 4° C for 15 min. A 100-μL aliquot of supernatant was removed and stored at -20° C overnight. The remaining supernatant was dried under a continuous flow of air. The 100-μL aliquot was brought to a total volume of 1 mL with distilled water, vortexed, and used in a colorimetric microplate assay for phenolics determination.

The Folin-Ciocalteu assay was used to determine the amount of total phenolics in all samples, using gallic acid to create a standard curve (Folin and Ciocalteu, 1927). Briefly, 35 μL of each sample was mixed with 150 μL of 0.2 M Folin-Ciocalteu reagent and incubated at room temperature for 5 minutes, after which 115 μL of saturated (7.5% w/v) sodium bicarbonate was added to the reaction. The reaction was mixed briefly, incubated at 45° C for 30 minutes, and then allowed to cool to room temperature for 60 minutes. Absorbance at wavelength 765 nm was read in a SPECTRA max plus 384 microplate reader (Molecular devices, Sunnyvale CA). All samples were run in triplicate. Total phenolics were calculated as gallic acid equivalents (GAE) per gram of sample dry weight.

Statistical analysis

The rosette diameter (cm) and number of leaves per plant were measured for each individual plant per pot, except for those in planting density 16, in which four plants were measured per pot. Above ground biomass (mg) and concentration of total phenolics (mg GAE per gram dry weight) were calculated for each pot. The Statistical Analysis Software (SAS) 9.1 (Cary, NC, USA) was used to perform separate mixed model ANOVA for each response variable. Regression analysis was done using Excel.

7.4 RESULTS

FIELD SITE VEGETATION AND SOILS ASSESSMENT

Vegetation survey

The vegetation survey of high and low density spotted knapweed stands revealed approximately 70% knapweed cover in the high density stands versus 11.5% knapweed cover in low density stands (Table 7.1, Figure 7.1). Grass cover was reduced in high versus low density stands by nearly 30% on average. In addition, high density sites tended to have less bare ground (7.5%) than low density sites (25%); however, percent bare ground varied widely both within and between sites (Table 7.1).

Table 7.1. Vegetation Survey. Vegetation cover in spotted knapweed infested areas were sampled using a transect method. Each site (1-4) contained adjacent plots where spotted knapweed plants occurred at high (HD) and low (LD) relative densities. A 50-meter long transect was run across the length of each of eight plots. To determine percent cover of spotted knapweed and other vegetation, a 0.5- x 0.75-meter rectangle was placed next to the transect every five meters, and the percent cover of spotted knapweed, grass, forbes, legumes, woody plants, moss and bare ground within the rectangle was estimated. Mean percent cover by site is reported below. Legend: HD, high density spotted knapweed; LD, low density spotted knapweed. Knapweed refers to spotted knapweed.

density	site	knapweed	grass	forbes	legumes	bare	woody	moss
HD	1	63.0	25.9	4.9	0.0	6.2	0.0	0.0
HD	2	72.5	20.5	0.1	4.5	1.9	0.0	0.0
HD	3	76.0	7.0	4.4	0.0	11.3	0.0	0.0
HD	4	67.0	19.3	1.6	0.1	10.9	0.1	0.0
LD	1	3.9	89.0	5.2	0.5	0.0	0.0	0.0
LD	2	14.5	48.5	9.7	1.9	12.0	2.2	8.3
LD	3	16.5	15.0	15.1	5.0	48.4	0.0	0.0
LD	4	11.5	35.8	4.9	0.5	40.2	3.5	0.0

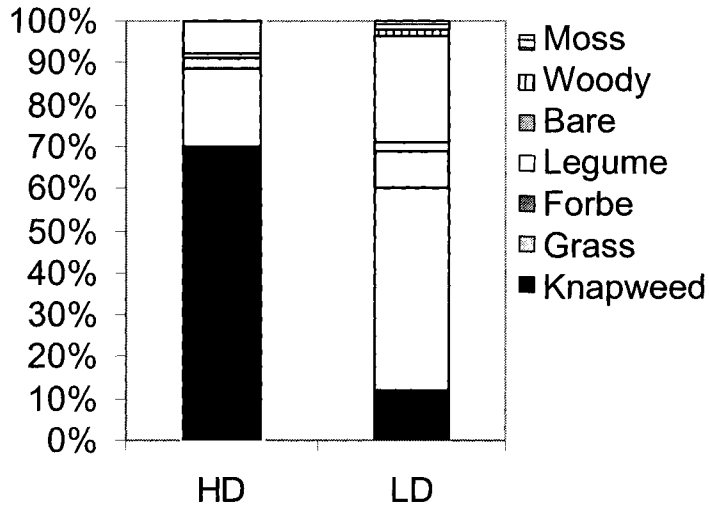

Figure 7.1. Vegetation cover at field sites. Mean percent cover of vegetation types in high density (HD) and low density (LD) stands of spotted knapweed averaged over four sites. Vegetation cover in spotted knapweed infested areas were sampled using a transect method. A 50-meter long transect was run across the length of each of eight plots (sites 1-4, HD and LD). To determine percent cover of spotted knapweed and other vegetation, a 0.5- x 0.75-meter rectangle was placed next to the transect every five meters, and the percent cover of spotted knapweed, grass, forbes, legumes, woody plants, moss and bare ground within the rectangle was estimated.

Soil analysis

All soils were designated as having either a loam or sandy-loam texture, and had low amounts of lime (Table 7.2). Soils from each plot were analyzed to determine if there were any consistent differences in pH, EC, %OM, NO3-N, P, K, Zn, Fe, Mg and Cu. No significant differences between high and low density sites were found for any of these variables in an ANOVA.

Table 7.2. Properties of soils collected from sites with high or low density spotted knapweed infestations. Soil properties are given for each of the eight plots that were sampled. Legend: ID, site and density are given (HD is high density, LD is low density; site numbers 1-4); EC, electrical conductivity; %OM, percent organic matter; all values given for elemental analysis are in parts per million (ppm).

ID	pH	EC	lime	% OM	NO3-N	P	K	Zn	Fe	Mn	Cu	Texture
HD1	6.8	0.3	Low	6.7	2.6	7.4	418	3.4	15.3	5.39	3.04	Loam
HD2	6.0	1.1	Low	9.8	85.2	25.5	491	3.0	73.9	4.57	1.85	Loam
HD3	6.3	0.2	Low	5.9	5.0	17.4	451	4.2	39.7	11.6	1.46	Loam
HD4	6.3	0.3	Low	3.9	6.4	16.8	241	0.9	26.8	5.97	1.95	Sandy Loam
LD1	6.3	0.3	Low	6.4	5.7	7.4	298	4.7	31.4	8.87	2.55	Loam
LD2	5.7	0.7	Low	14.2	75.1	14.9	381	8.5	127	11.7	2.50	Loam
LD3	5.6	0.3	Low	2.5	3.0	16.8	302	1.9	35.7	6.44	1.13	Sandy Loam
LD4	6.5	0.3	Low	6.3	10.3	8.1	299	2.4	69.1	24.5	1.03	Loam

EXPERIMENT 1: SOIL FEEDBACK EFFECTS

Soil DNA extraction and qPCR analysis

Total fungal biomass in pooled soils from each plot was estimated using qPCR. As found previously (Broz et al. 2007, Chapter 6 of this dissertation), high density stands of spotted knapweed exhibited a reduction in total fungal biomass compared to low density stands (Figure 7.2a). The extent of the reduction in fungal biomass due to spotted

knapweed density varied greatly by site (Figure 7.2b). Sites 1 and 4 showed around a 20% reduction in total fungal biomass associated with high knapweed densities, whereas sites 2 and 3 exhibited near 65% reduction (Figure 7.2b).

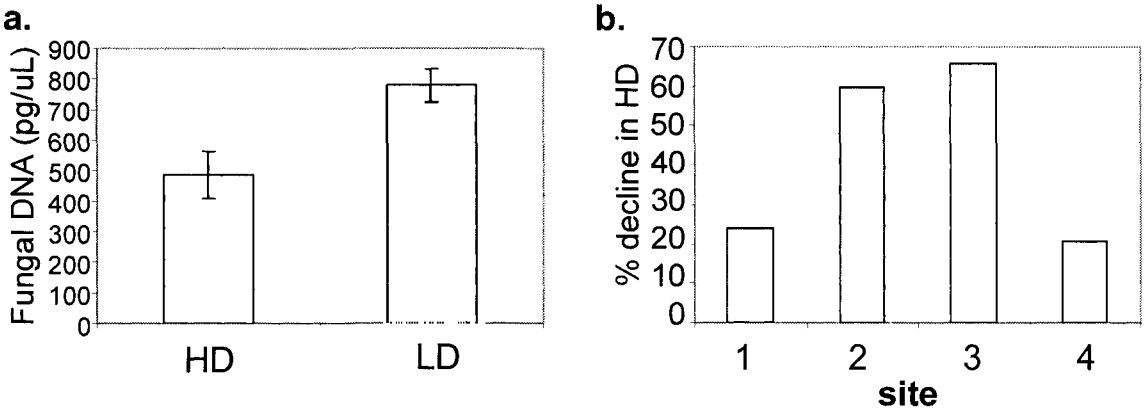

Figure 7.2. Total soil fungal DNA from high and low density knapweed infested sites. qPCR amplification of total fungal DNA extracted from high density (HD) and low density (LD) soil types confirms a reduction of fungal biomass in soils from sites with high rates of spotted knapweed infestation (a). Bars represent means with standard errors. The extent of the reduction in soil fungal DNA amplified by qPCR between high and low density sites of spotted knapweed varies by site (b). The x axis denotes each site (comparing the two adjacent plots of high and low density within a site), and the y axis represents the percent reduction of fungal DNA in high density (HD) versus low density (LD) sites using the equation: % decline in HD =((LD-HD)/LD)*100).

Greenhouse experiment

I hypothesized that spotted knapweed would experience positive feedbacks from both soil types compared to sterile controls. Contrary to my hypothesis, spotted knapweed accumulated significantly more biomass in sterilized soils versus non-sterile soils, suggesting a negative feedback (Figure 7.3a, Table 7.3, Table 7.4). This negative feedback was observed in both HD and LD soils (Table 7.5).

I also hypothesized that Idaho fescue would experience negative feedbacks from both soil types compared to sterile controls, and this effect would be higher in soils

collected from HD sites. I found that Idaho fescue tended to accumulate more biomass in sterile versus non-sterile soils, indicating a negative feedback from the knapweed infested soils (Figure 7.3a, Table 7.3, Table 7.4). However, in an analysis of pair-wise comparisons of means, this effect was only significant in soils collected from the HD sites (Table 7.5).

The main model effect in the ANOVA analysis was related to whether or not the soils were sterilized, and the original knapweed density in the soils had no significant effect for either plant type (Table 7.4). To determine the mean percent increase in biomass due to soil sterilization, I calculated the 'inoculation effect' for each plant in each soil type (Figure 7.5). In general, both plants accumulated roughly 10-25% more biomass when grown in sterilized soils versus non-sterile soils (Figure 7.5).

I also calculated root to shoot ratios for each plant type to determine if the soil treatments impacted this parameter. Idaho fescue showed no differences in root to shoot ratios due to soil type (HD or LD) or sterility (Figure 7.3b, Table 7.3, Table 7.4). Spotted knapweed root to shoot ratios varied due to soil sterilization, but not due to soil type (Figure 7.3b, Table 7.3, Table 7.4). Pair-wise comparisons of means indicated that sterilization of HD soils led to a higher root to shoot ratio in spotted knapweed ($p = 0.0021$), but there was no significant effect of sterilization on root to shoot ratio in LD soils ($p = 0.0632$) (p values represent Fischers LSD).

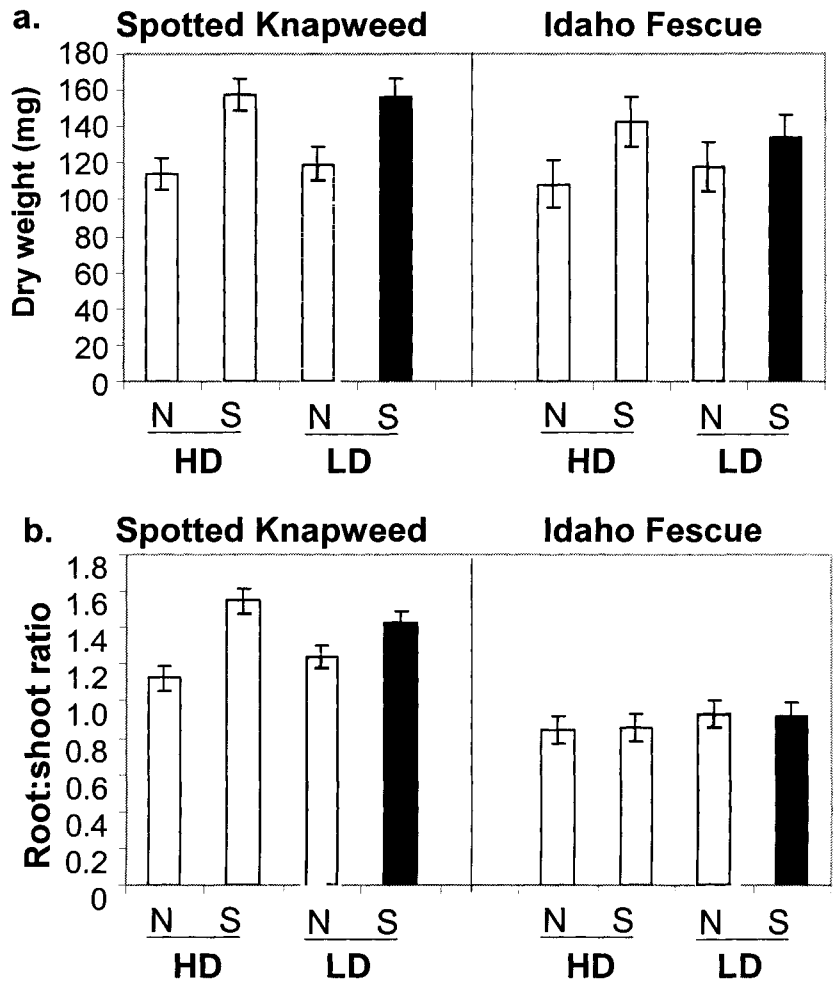

Figure 7.4. Total dry weight and root to shoot ratios of spotted knapweed and Idaho fescue tissue grown in different soil types (experiment 1). Spotted knapweed and Idaho fescue were grown for three months under greenhouse conditions in a 30% mixture of different soil types. Soils were originally collected from high (HD) and low (LD) density stands of spotted knapweed. Half of the soils were sterilized (S) to remove microbial communities. Non-sterile (N) soils were presumed to contain active microbial communities. Roots and shoots of each plant were collected, dried and weighed to obtain total biomass measurements (a). Bars represent means with standard errors. Biomass for roots was divided by biomass for shoots to obtain root to shoot ratios (b). Bars represent means with standard errors.

Table 7.3. Means values for total dry weight and root to shoot ratios of spotted knapweed and Idaho fescue tissue grown in different soil types (experiment 1). Spotted knapweed and Idaho fescue were grown for three months under greenhouse conditions in a 30% mixture of different soil types. Soils were originally collected from high (HD) and low (LD) density stands of spotted knapweed. Half of the soils were sterilized (S) to remove microbial communities. Non-sterile (N) soils were presumed to contain active microbial communities. Roots and shoots of each plant were collected, dried and weighed to obtain total biomass measurements. Mean dry weight is given in mg. Biomass for roots was divided by biomass for shoots to obtain root to shoot ratios.

Total Biomass Measurements				
Plant type	**Soil density**	**Sterile or non-sterile**	**Mean total dry weight (mg)**	**Standard error (n=9)**
Spotted knapweed	HD	N	114.46	8.98
		S	157.77	8.98
	LD	N	119.61	9.02
		S	156.82	8.98
Idaho Fescue	HD	N	108.33	13.15
		S	142.75	13.15
	LD	N	117.8	13.21
		S	133.88	13.15
Root to shoot ratios				
Plant type	**Soil density**	**Sterile or non-sterile**	**Root to shoot ratio**	**Standard error (n=9)**
Spotted knapweed	HD	N	1.12	0.07
		S	1.55	0.07
	LD	N	1.24	0.07
		S	1.43	0.07
Idaho Fescue	HD	N	0.85	0.07
		S	0.85	0.07
	LD	N	0.93	0.07
		S	0.92	0.07

Table 7.4. ANOVA model type 3 tests of fixed effects on total biomass and root to shoot ratios by plant type (experiment 1). Spotted knapweed and Idaho fescue were grown for three months under greenhouse conditions in a 30% mixture of different soil types. Soils were originally collected from high and low density stands of spotted knapweed. Half of the soils were sterilized and half remained non-sterile. Roots and shoots of each plant were collected, dried and weighed to obtain total biomass measurements (upper portion of table) and root to shoot ratios (lower portion of table). Values were analyzed using a mixed model ANOVA, with soil collection site (1-4) as a random factor. Fixed effects from the model are as follows: 'density' refers to the effect of soils collected from either high or low density spotted knapweed stands, 'sterile' refers to sterile or non-sterile soils, and 'D*S' refers to the interaction between soil density and sterility. DF refers to degrees of freedom.

Total Biomass Measurements					
Plant	**Effect**	**Numerator DF**	**Denominator DF**	**F value**	**P value**
Spotted knapweed	Density	1	3	0.15	0.7209
	Sterile	1	6	56.75	0.0003
	D*S	1	6	0.33	0.5887
Idaho Fescue	Density	1	3	0.00	0.9762
	Sterile	1	6	12.55	0.0122
	D*S	1	6	1.65	0.2458
Root to shoot ratio					
Plant type	**Effect**	**Numerator DF**	**Denominator DF**	**F value**	**P value**
Spotted knapweed	Density	1	3	0.00	0.9884
	Sterile	1	6	27.66	0.0019
	D*S	1	6	4.12	0.0887
Idaho Fescue	Density	1	3	0.65	0.4799
	Sterile	1	6	0.01	0.9251
	D*S	1	6	0.05	0.8280

Table 7.5. Pair-wise comparisons for each plant-soil combination of interest. Spotted knapweed and Idaho fescue were grown for three months under greenhouse conditions in a 30% mixture of different soil types. Soils were originally collected from high (HD) and low (LD) density stands of spotted knapweed. Half of the soils were sterilized (S) and half remained non-sterile (N). Roots and shoots of each plant were collected, dried and weighed to obtain total biomass measurements. Values were analyzed using a mixed model ANOVA, with soil collection site (1-4) as a random factor. Fixed effects from the model are given in Table 7.4. Pair-wise comparisons of interest are reported by plant, and compare differences due to original knapweed density in soils (HD or LD) or sterility (N or S). Absolute t values are given and the p values are LSD between means.

Plant type	Density	Sterility	Density	Sterility	Total Biomass	
					t	p
Spotted knapweed	HD	N	LD	N	0.68	0.5223
	HD	S	LD	S	0.13	0.9034
	HD	N	HD	S	5.75	0.0012
	LD	N	LD	S	4.91	0.0027
Idaho Fescue	HD	N	LD	N	0.81	0.4470
	HD	S	LD	S	0.77	0.4722
	HD	N	HD	S	3.43	0.0140
	LD	N	LD	S	1.59	0.1630

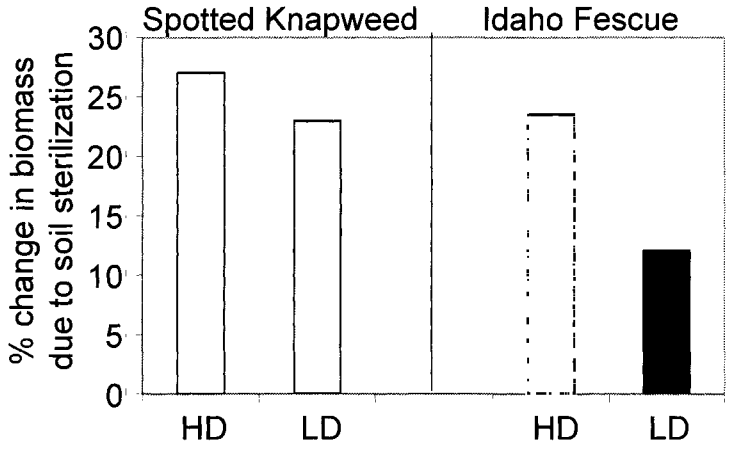

Figure 7.5. Inoculation effect of soil sterilization on total biomass (experiment 1). Spotted knapweed and Idaho fescue were grown for three months under greenhouse conditions in a 30% mixture of different soil types. Soils were originally collected from high (HD) and low (LD) density stands of spotted knapweed. Half of the soils were sterilized (S) and half remained non-sterile (N). Roots and shoots of each plant were collected, dried and weighed to obtain total biomass measurements. The relative effect of soil sterilization on total plant biomass accumulation (inoculation effect) was determined by putting mean biomass values into the following equation: inoculation effect = (Sterile soil biomass – Non sterile soil biomass) / Sterile soil biomass * 100.

EXPERIMENT 2: SOIL SLURRY FEEDBACK EFFECTS AT DIFFERENT NUTRIENT LEVELS

I designed a second experiment to investigate the feedback effects of adding different soil slurries to spotted knapweed and Idaho fescue plants, as opposed to growing plants in the different soil types. This experimental procedure should minimize any chemical differences between sterile and non-sterile soils due to the process of sterilization. I was also interested to see if nutrient availability would impact the experimental results. I expected to find results similar to experiment 1 using slurries in the two different nutrient conditions if the observed feedback effects from the soil microbial communities were robust.

The results from the second experiment were not in agreement with the results from the first experiment. Here, in the low nutrient condition, spotted knapweed showed no differences in biomass accumulation due to any of the factors tested (Figure 7.6a, Tables 7.6 and 7.7). However, in the high nutrient condition, soil sterilization led to an increase in spotted knapweed biomass, similar to the results from experiment 1 (Figure 7.6b). Interestingly, this increase in biomass was only observed when comparing sterile and non-sterile slurries within soil types (HD or LD), as the entirely sterile control had a median value that was not significantly different from either sterile or non-sterile slurry types. Additionally, the relative increase in biomass due to sterilization, or inoculation effect, was on the order of 40% in the high nutrient condition, versus ~20% in experiment 1 (Figure 7.7). The inoculation effect was much smaller for spotted knapweed in the low nutrient condition, and surprisingly, data suggested a potential positive feedback from non-sterile LD soil slurry (Figure 7.7), the opposite of the results from experiment 1 and

the high nutrient condition in the current experiment. However, the 'inoculation effect' merely depicts differences between means, and should not be regarded as a significant value.

Sterilization of soil slurries resulted in a higher root to shoot ratio for spotted knapweed, in the high nutrient condition, but not the low nutrient condition (Table 7.8, Table 7.9). Again, the density factor was not significant, but the sterilization factor was (Table 7.9). The largest difference in root to shoot ratios was seen between the LD sterile and non-sterile soil slurries (Table 7.8), which showed a significant difference in a pair-wise comparison ($p = 0.0114$). This was different from the result of experiment 1, where sterilization of the HD, but not the LD soil led to an increase in spotted knapweed root to shoot ratio. In general, spotted knapweed had a higher root to shoot ratio in the low nutrient conditions (ratio greater than 1) compared to the high nutrient conditions (ratio less than 1) (Table 7.8).

In the high nutrient conditions, Idaho fescue showed no differences in biomass accumulation due to any of the factors tested (Table 7.7). However, the large variation in biomass accumulation within treatments in the high nutrient condition (Figure 7.6b, Table 7.6) may have masked any significant effects. In the low nutrient condition, Idaho fescue biomass accumulation trends were similar to experiment 1, in that sterilization was a main effect in the model (Table 7.7). However, in an analysis of pair-wise comparisons, Idaho fescue accumulated more biomass due to sterilization in the LD soil slurry, but not the HD soil slurry ($p = 0.0179$, comparing LD sterile to LD non-sterile; $p=0.4050$, comparing HD sterile to HD non-sterile). In experiment 1, Idaho fescue accumulated more biomass due to sterilization in HD soil slurries, but not in LD soil slurries. In other

words, the results were opposite between the two experiments in regards to soil type (HD or LD). Using the entirely sterile control for pair-wise comparisons, I still found a significant increase in biomass for Idaho fescue grown in the sterile condition versus the LD non-sterile soil slurry ($p = 0.0444$). In general, sterilization of soil slurries resulted in an increase in Idaho fescue biomass compared to non-sterile soils, with an inoculation effect ranging from ~5-30% (Figure 7.7).

Sterilization of soil slurries resulted in a higher root to shoot ratio in Idaho fescue in the low nutrient condition, but not the high nutrient condition (Table 7.8, Table 7.9). This difference was the largest in a comparison of LD sterile and non-sterile soil slurries ($p = 0.0016$), where sterilization more than doubled the root to shoot ratio. Although neither sterilization or soil type were found to impact Idaho fescue root to shoot ratio in experiment 1, this is likely due to the level of available nutrients, as the root to shoot ratios observed in the high nutrient condition in experiment 2 are similar to those found in experiment 1 (root to shoot ratios are near 1). The low nutrient condition resulted in higher root to shoot ratios in Idaho fescue ranging from 1.5 to 3.5 (Table 7.8).

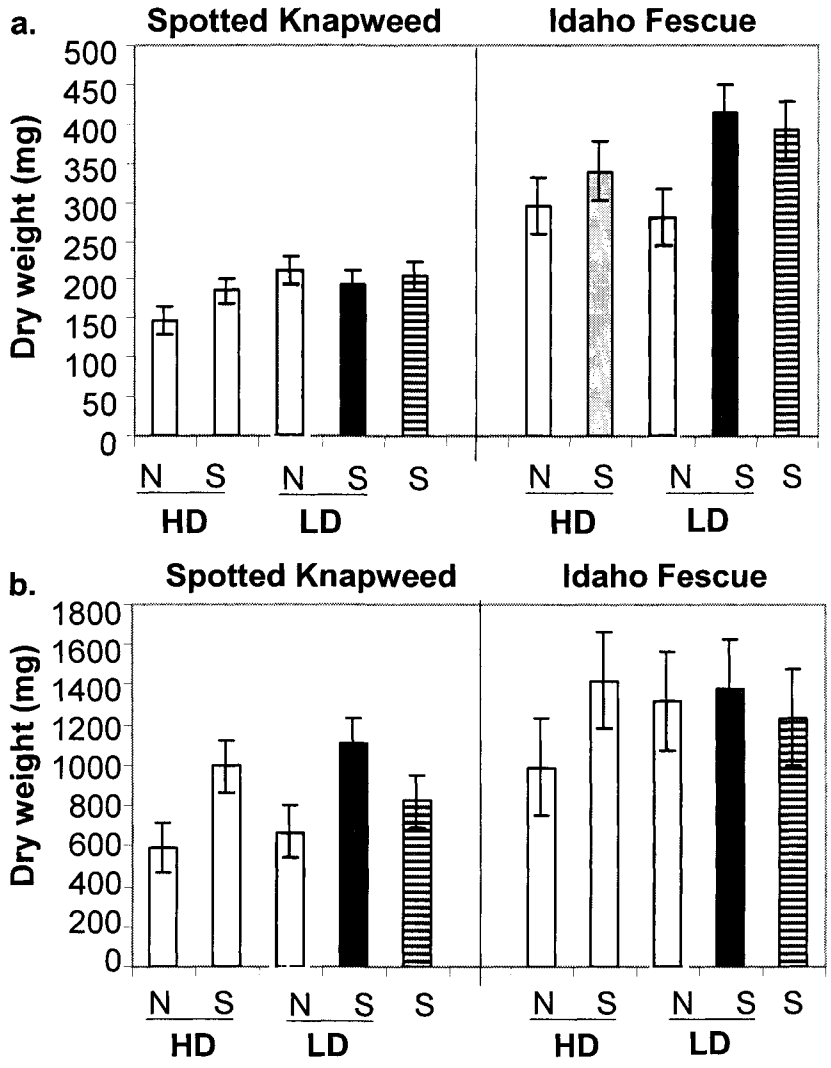

Figure 7.6 Total dry weight and root to shoot ratios of spotted knapweed and Idaho fescue tissue grown in different soil slurries at different nutrient levels (experiment 2). Spotted knapweed and Idaho fescue were grown for three months under greenhouse conditions in sterile media, and soil slurries were added 3 times during the experiment. Soils used to make slurries were originally collected from high (HD) and low (LD) density stands of spotted knapweed. Half of the soils were sterilized (S) to remove microbial communities, and an additional entirely sterile control was also performed. Non-sterile (N) soils were presumed to contain active microbial communities. Plants were grown with either low (a) or high (b) levels of nutrients. Roots and shoots of each plant were collected, dried and weighed to obtain total biomass measurements. Bars represent means with standard errors.

Table 7.6. Total biomass means and standard errors of plants grown at different nutrient levels with various slurry additions (experiment 2). Spotted knapweed and Idaho fescue were grown for three months under greenhouse conditions in sterile media, and soil slurries were added 3 times during the experiment. Soils used to make slurries were originally collected from high (HD) and low (LD) density stands of spotted knapweed. Half of the soils were sterilized (S) to remove microbial communities, and an additional entirely sterile control was also analyzed (soil density not applicable (NA)). Non-sterile (N) soils were presumed to contain active microbial communities. Plants were grown with either low or high levels of nutrients. Roots and shoots of each plant were collected, dried and weighed to obtain total biomass measurements.

Plant type	Nutrient level	Soil density	Sterile or non-sterile	Mean total dry weight	Standard error (n=7)
Spotted knapweed	Low	HD	N	147.43	16.90
			S	185.86	16.90
		LD	N	211.57	16.90
			S	195.29	16.90
		NA	S	204.67	18.25
	High	HD	N	591.86	128.41
			S	995.00	128.41
		LD	N	667.14	128.41
			S	1107.57	128.41
		NA	S	821.57	128.41
Idaho Fescue	Low	HD	N	295.14	37.07
			S	339.43	37.07
		LD	N	281.00	37.07
			S	412.29	37.07
		NA	S	391.00	37.07
	High	HD	N	990.57	243.79
			S	1423.00	243.79
		LD	N	1317.00	243.79
			S	1386.29	243.79
		NA	S	1236.43	243.79

Table 7.7. ANOVA model type 3 tests of fixed effects on total biomass by plant type and nutrient level (experiment 2). Spotted knapweed and Idaho fescue were grown for three months under greenhouse conditions in sterile media, and soil slurries were added 3 times during the experiment. Soils used to make slurries were originally collected from high (HD) and low (LD) density stands of spotted knapweed. Half of the soils were sterilized (S) to remove microbial communities, and an additional entirely sterile control was also analyzed (soil density not applicable (NA)). Non-sterile (N) soils were presumed to contain active microbial communities. Plants were grown with either low or high levels of nutrients. Roots and shoots of each plant were collected, dried and weighed to obtain total biomass measurements. Results were analyzed using a mixed model ANOVA. Soil density (HD, LD or NA for water control), sterility (sterile or non-sterile) and density*sterile were fixed variables in the analysis. 'Density' refers to the effect of soil slurries collected from either high or low density spotted knapweed stands, 'sterile' refers to sterile or non-sterile soil slurries, and 'D*S' refers to the interaction between soil density and sterility. DF refers to degrees of freedom used in the analysis.

Plant type	Nutrient level	Effect	Numerator DF	Denominator DF	F value	P value
Spotted knapweed	Low	Density	2	29	2.58	0.0932
		Sterile	1	29	0.43	0.5175
		D*S	1	29	2.62	0.1163
	High	Density	2	30	1.33	0.2785
		Sterile	1	30	10.79	0.0026
		D*S	1	30	0.02	0.8855
Idaho Fescue	Low	Density	2	30	0.37	0.6944
		Sterile	1	30	5.61	0.0245
		D*S	1	30	1.38	0.2499
	High	Density	2	30	0.34	0.7178
		Sterile	1	30	1.08	0.3117
		D*S	1	30	0.56	0.4622

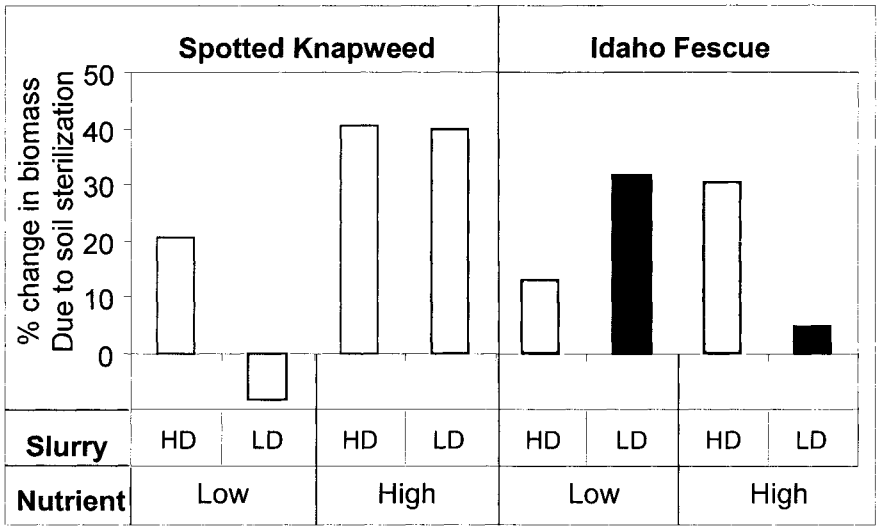

Figure 7.7. Inoculation effect of soil slurry sterilization on total biomass (experiment 2). Spotted knapweed and Idaho fescue were grown for three months under greenhouse conditions in sterile media, and soil slurries were added 3 times during the experiment. Soils used to make slurries were originally collected from high (HD) and low (LD) density stands of spotted knapweed. Half of the soils were sterilized, the other half remained non-sterile. Plants were grown with either low or high levels of nutrients. Roots and shoots of each plant were collected, dried and weighed to obtain total biomass measurements. The relative effect of soil slurry sterilization on total plant biomass accumulation (inoculation effect) was determined by putting mean biomass values into the following equation: inoculation effect = (Sterile soil biomass – Non sterile soil biomass) / Sterile soil biomass * 100. The entirely sterile control was not used in this calculation.

Table 7.8. Root to shoot ratios, means and standard errors of plants grown at different nutrient levels with various slurry additions (experiment 2). Spotted knapweed and Idaho fescue were grown for three months under greenhouse conditions in sterile media, and soil slurries were added 3 times during the experiment. Soils used to make slurries were originally collected from high (HD) and low (LD) density stands of spotted knapweed. Half of the soils were sterilized (S) to remove microbial communities, and an additional entirely sterile control was also analyzed (soil density not applicable (NA)). Non-sterile (N) soils were presumed to contain active microbial communities. Plants were grown with either low or high levels of nutrients. Roots and shoots of each plant were collected, dried and weighed and the biomass of roots was divided by the biomass of shoots to obtain a ratio.

Plant type	Nutrient level	Soil density	Sterile or non-sterile	Root to shoot ratio	Standard error (n=7)
Spotted knapweed	Low	HD	N	1.64	0.14
			S	1.44	0.14
		LD	N	1.80	0.14
			S	1.56	0.14
		NA	S	1.47	0.15
	High	HD	N	0.63	0.07
			S	0.74	0.07
		LD	N	0.56	0.07
			S	0.85	0.07
		NA	S	0.78	0.07
Idaho Fescue	Low	HD	N	2.15	0.39
			S	2.99	0.39
		LD	N	1.59	0.39
			S	3.51	0.39
		NA	S	3.24	0.39
	High	HD	N	0.98	0.14
			S	1.19	0.14
		LD	N	0.89	0.14
			S	0.97	0.14
		NA	S	1.33	0.14

Table 7.9. ANOVA model type 3 tests of fixed effects on root to shoot ratios by plant type and nutrient level. Spotted knapweed and Idaho fescue were grown for three months under greenhouse conditions in sterile media, and soil slurries were added 3 times during the experiment. Soils used to make slurries were originally collected from high (HD) and low (LD) density stands of spotted knapweed. Half of the soils were sterilized to remove microbial communities, and an additional entirely sterile control was also analyzed (soil density not applicable (NA)). Non-sterile soils were presumed to contain active microbial communities. Plants were grown with either low or high levels of nutrients. Root biomass was divided by shoot biomass to obtain a ratio. Results were analyzed using a mixed model ANOVA. Soil density (HD, LD or NA for water control), sterility (sterile or non-sterile) and density*sterile were fixed variables in the analysis. 'Density' refers to the effect of soils collected from either high or low density spotted knapweed stands, 'sterile' refers to sterile or non-sterile soils, and 'D*S' refers to the interaction between soil density and sterility. DF refers to degrees of freedom used in the analysis.

Plant type	Nutrient level	Effect	Numerator DF	Denominator DF	F value	P value
Spotted knapweed	Low	Density	2	29	0.50	0.6129
		Sterile	1	29	2.63	0.1158
		D*S	1	29	0.03	0.8566
	High	Density	2	30	0.04	0.9609
		Sterile	1	30	7.10	0.0123
		D*S	1	30	1.31	0.2609
Idaho Fescue	Low	Density	2	30	0.00	0.9988
		Sterile	1	30	12.74	0.0014
		D*S	1	30	1.91	0.1772
	High	Density	2	30	1.65	0.2086
		Sterile	1	30	1.13	0.2966
		D*S	1	30	0.23	0.6320

EXPERIMENT 3: DENSITY MANIPULATION EXPERIMENT

Based on my previous results (Broz et al., 2007) I hypothesized that increased planting density of spotted knapweed would reduce the abundance and diversity of soil fungal communities in soils collected from a low density infestation site. Contrary to my hypothesis, I found that fungal abundance (amount of PCR amplicons) tended to increase with increased planning density of spotted knapweed (Figure 7.8a). The abundance of

soil bacteria showed a somewhat opposite trend, with a general decrease in bacterial abundance with increased planting density (Figure 7.8b). However, pots containing two plants showed the lowest relative amount of bacterial abundance, so this was not a linear decline.

Percent soil moisture differed somewhat between planting densities. This was taken into account when calculating fungal and bacterial abundance per gram soil dry weight. Soils from planting density 1 had an average soil moisture of 8.68%, where planting density 2 had 9.05% soil moisture, planting density 4 had 6.72% soil moisture, and planting density 16 had 7.98% soil moisture. However, drying temperature may not have been adequate to remove all hygroscopic water trapped within soil particles, and thus may not be entirely representative of total soil water content.

I also measured physical characteristics of plants in the density manipulation experiment. As expected, I found that the average number of leaves per plant and the average rosette diameter per plant decreased with increased planting density (Figure 7.9a). There was a linear correlation between leaf number and rosette diameter with an R^2 value of 0.9939 (Figure 7.9b). Due to the correlation between these factors, it may be sufficient to measure one of these parameters in future experiments, as the other can be inferred using the calculation: rosette diameter in cm = 0.9577 * leaf number + 2.4181.

As expected, above ground biomass of individual spotted knapweed plants decreased with increasing planting density (Figure 7.10). Above ground biomass per pot was highest in the highest density planting (Figure 7.10a), but this correlates with a larger number of smaller individual plants (Figure 7.10b).

Accumulation of total phenolics (mg GAE per gram dry weight) showed no differences due to planting density (Figure 7.11). In general, mean values for phenolic accumulation increased with planting density, but values were not significantly different between planting densities ($p = 0.074$ in a comparison between densities 1 and 16 which exhibited the largest difference between means).

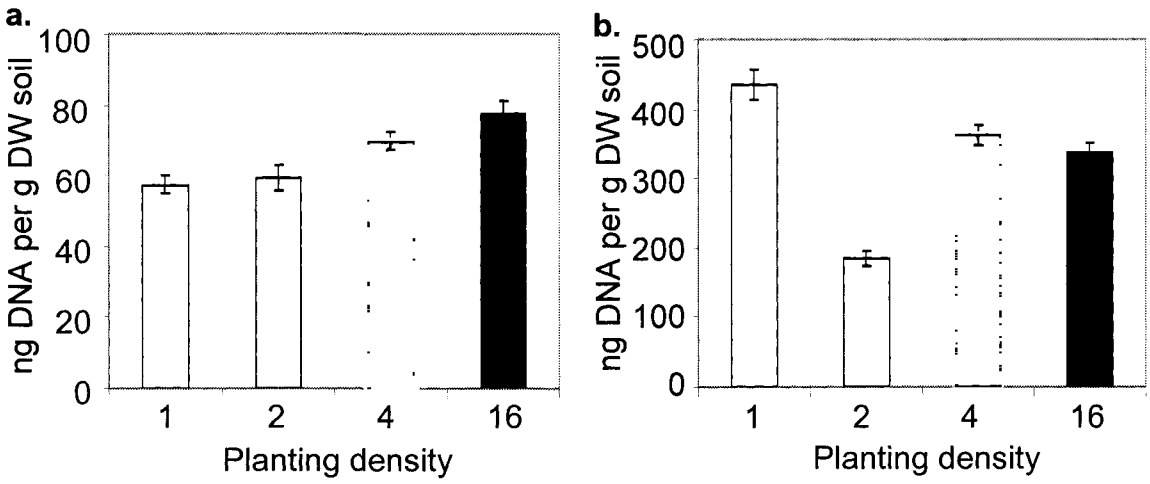

Figure 7.8. Impact of spotted knapweed planting density on soil microbial community abundance. Spotted knapweed was planted at different densities (1, 2, 4 or 16 plants per pot) in the greenhouse in 2 parts soil 1 part sterile media. Soil was originally collected from a site (3) that contained low densities of spotted knapweed. Plants were grown for approximately six months, at which time rhizposhere soil from each pot was collected. DNA was extracted from rhizosphere soils and soil fungal and bacterial DNA abundance was assessed using quantitative PCR. Total abundance of fungi (a) and bacteria (b) were assessed from soils with either 1, 2, 4 or 16 spotted knapweed plants growing per pot. Bars are means with standard errors.

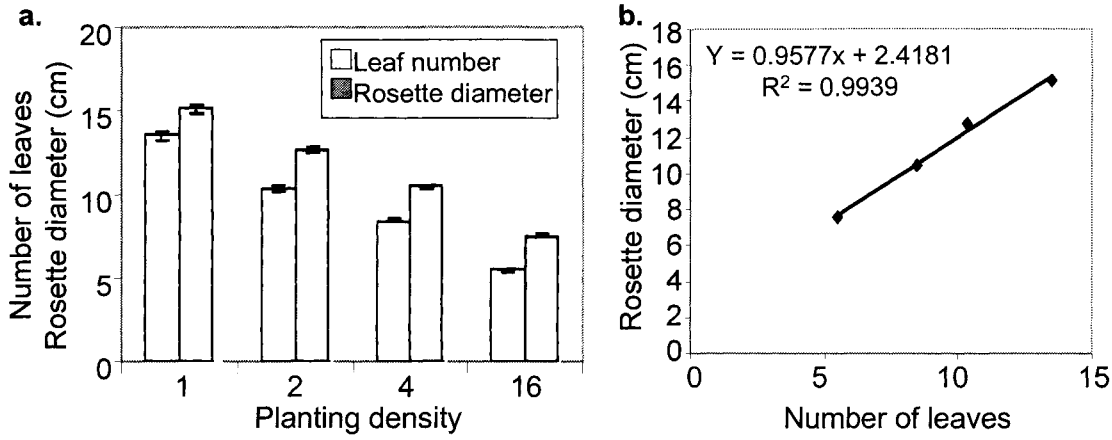

Figure 7.9. Impact of spotted knapweed planting density on plant leaf number and rosette diameter. Spotted knapweed was planted at different densities (1, 2, 4 or 16 plants per pot) in the greenhouse in 2 parts soil 1 part sterile media. Soil was originally collected from a site that contained low densities of spotted knapweed. Plants were grown for approximately six months, at which time leaf number per plant and average rosette diameter per plant (cm) were measured for each planting density (a). A linear correlation between average rosette diameter (cm) and average number of leaves per plant was identified (b). Each dot represents a different planting density; higher density is correlated with fewer leaves per plant and smaller rosette diameters.

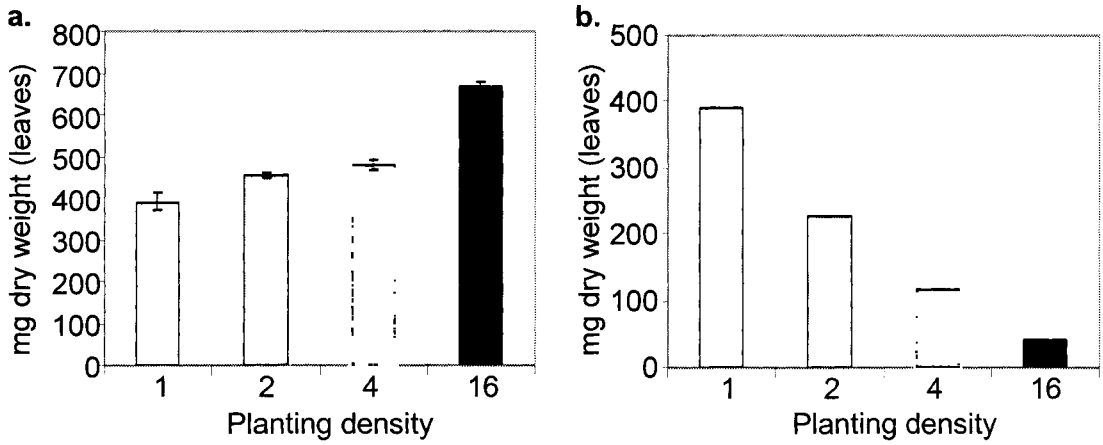

Figure 7.10. Above ground biomass of spotted knapweed changes with planting density. Spotted knapweed was planted at different densities (1, 2, 4 or 16 plants per pot) in the greenhouse in 2 parts soil 1 part sterile media. Soil was originally collected from a site (3) that contained low densities of spotted knapweed. Plants were grown for approximately six months, at which time above ground biomass was harvested from each pot, freeze dried, and weighed. Above ground biomass per pot was measured (a) (n=10 per density, bars are means with standard errors), and ground biomass per plant was calculated (b) (the value from panel a divided by number of plants per pot).

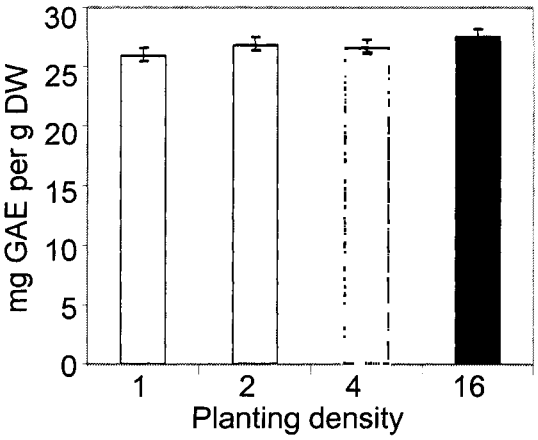

Figure 7.11. Accumulation of total phenolics does not change with planting density. Spotted knapweed was planted at different densities (1, 2, 4 or 16 plants per pot) in the greenhouse in 2 parts soil 1 part sterile media. Soil was originally collected from a site that contained low densities of spotted knapweed. Plants were grown for approximately six months, at which time above ground biomass was harvested from each pot and freeze dried. Freeze dried tissue was weighed and a portion was extracted in 80% acetone. Total phenolics were assessed using the Folin Ciocalteu method using gallic acid (GA) as a standard. Values for total phenolics are given in GA equivalents (GAE) per gram dry weight of plant leaf tissue. No significant differences were found between planting densities.

7.5 DISCUSSION

Previous studies suggest that spotted knapweed alters soil fungal community composition in North American grasslands it has invaded (Mummey et al., 2005; Mummey and Rillig, 2006; Broz et al., 2007). I was interested in determining the relevance of these microbial community alterations on feedback effects with spotted knapweed and the native grass Idaho fescue. In addition, I wanted to determine if knapweed planting density was the main factor impacting microbial community alteration, so I manipulated this factor in a greenhouse experiment.

SPOTTED KNAPWEED: FEEDBACK EFFECTS

I hypothesized that spotted knapweed would experience positive feedbacks from soil microbial communities in the soils I collected; however there was no evidence for this in either of the greenhouse studies I conducted. In fact, I often found the opposite to be true. Soil microbial communities had either no effect on spotted knapweed biomass accumulation (experiment 2 low nutrient HD and LD soil slurry) or a negative effect (experiment 1 HD and LD soils, experiment 2 high nutrient HD and LD soils) in comparison to sterile controls. Additionally, in the experiment 2 high nutrient condition, a comparison between the entirely sterile control (no slurry added) and the non-sterile soil slurries shows no significant difference. Regardless, the results did not indicate a positive feedback effect of the soil communities on spotted knapweed biomass.

It is important to note that my results differed between the experimental conditions. This appears to be at least partially due to nutrient level, which was part of the design of experiment 2, but may also be related to the soil treatment protocol. It is quite probable that I sampled different components of the soil microbial community when using soils versus soil slurries. Soil slurries are made using only a small amount of soil (compared to growing plants directly in soil), which reduces the likelihood that all types of microbial community members are represented. In addition, it is possible that many constituents of the microbial community are bound to soil particles and thus, they would be less likely to be found in the liquid fraction that is added as the 'slurry'.

Other experiments suggest that spotted knapweed experiences positive feedbacks (more biomass) when grown in soils it has preconditioned compared to sterile controls (Callaway et al., 2004; Reinhart and Callaway, 2004), which is the opposite of what I

found in the current studies. However, although Callaway et al. (2004) conclude that in the introduced range spotted knapweed "cultivates soil biota with increasingly positive effects on itself", I believe this is only partially supported by their data. In their studies, spotted knapweed accumulated more biomass in soils preconditioned by spotted knapweed in the greenhouse in comparison to both the sterile control or soil preconditioned by Idaho fescue (Figure 2, lower panel from Callaway et al.), indicating a positive feedback (Callaway et al., 2004). However, when spotted knapweed was grown in soils collected from spotted knapweed rhizospheres in the field (North America), the plants accumulated more biomass in the sterile control compared to non-sterile conditions (Figure 1 from Callaway et al.), indicating a *negative* feedback effect from the resident soil microbial community (Callaway et al., 2004). Interestingly, spotted knapweed emergence was enhanced in field soils collected from knapweed infested sites versus sterile controls, but spotted knapweed biomass was not different between soil types (Meiman et al., 2006), indicating a neutral feedback effect on biomass but a positive feedback on emergence.

My results generally suggest that spotted knapweed infested soils collected from the field contain factors that lead to reductions in spotted knapweed biomass accumulation in the greenhouse. These factors could be pathogens of spotted knapweed, or possibly some type of phytotoxic chemical that was removed during sterilization. Alternatively, it is possible that a chemical change happened during sterilization that was beneficial to spotted knapweed. However, I attempted to minimize any chemical differences due to sterilization in experiment 2 by using small amounts of soil slurries as opposed to whole soils. Negative feedbacks between plants and soil microbial

communities are typically thought to be due to a build-up of plant pathogens; however, most exotic plants (like spotted knapweed) are presumed to have escaped the majority of their co-evolved plant pathogens upon introduction to a new environment (Elton, 1958; Klironomos, 2002). However, some soil-borne fungal pathogens of spotted knapweed have been identified in the introduced range (Caesar, 2003; Ridenour and Callaway, 2003). Thus it is possible that the field soils I collected contained pathogens of spotted knapweed that were removed by sterilization.

Due to the inconsistency in the results from the current experiment and the other studies discussed above, it is difficult to draw conclusions concerning soil feedback effects on spotted knapweed. Slightly different experimental conditions tend to lead to widely different results (and thus, conclusions) in this system. These inconsistencies highlight the difficulty of studying a complex system under greenhouse conditions, and raise questions about which experimental techniques are the most relevant. It is important to note that none of these studies examined specifically which microbial community components change and how they change over time. More work is clearly needed to address the issue of plant-soil feedbacks in spotted knapweed invasion.

IDAHO FESCUE: FEEDBACK EFFECTS

I hypothesized that native Idaho fescue plants would experience negative feedbacks from soil microbial communities present in spotted knapweed stands, and that this negative effect would be more pronounced in soils from high density stands of knapweed. This hypothesis was partially supported. Soil microbial communities had a negative effect (experiment 1 HD soil and experiment 2 low nutrient LD soil slurry) on

Idaho fescue biomass accumulation in comparison to sterile controls, but all other experimental conditions showed no effect (experiment 1 LD soil, experiment 2 high nutrient HD and LD and low nutrient HD). Additionally, experiment 1 suggested that the negative effect of microbial communities on Idaho fescue biomass was indeed more pronounced in HD soils compared to LD soils as I predicted, but experiment 2 suggested that LD soil slurries had a greater negative effect on Idaho fescue than HD soil slurries in the low nutrient condition, contrary to my prediction.

Other studies have investigated feedback effects on Idaho fescue by comparing biomass accumulation in soils preconditioned by other plants. Reinhart and Callaway (2006) state that Idaho fescue "performed much better in soil conditioned by conspecifics than in soils conditioned by *C. maculosa*" (p 488); however the data presented in Figure 2 (lower panel) of their manuscript suggests that Idaho fescue does better in soils preconditioned by spotted knapweed than those of conspecifics. Although, this sheds a bit of doubt on their findings (or at least their editing skills); Figure 2 also suggests that when Idaho fescue was grown in competition with spotted knapweed it consistently performed better in sterile controls than non-sterile soils (Reinhart and Callaway, 2004), suggesting a negative feedback effect.

Other authors suggest that investigating the interaction between spotted knapweed, Idaho fescue and the native soil microbial community is more revealing than studying the direct effects of the microbial community on either plant type in isolation (Marler et al., 1999; Carey et al., 2004). Marler et al. (1999) found that soil AMF had no effect on biomass of either plant type when plants were grown in isolation; however, when spotted knapweed and Idaho fescue were grown in competition with each other the presence of

AMF tended to enhance spotted knapweed biomass and reduce Idaho fescue biomass. Interestingly, in a follow up to this experiment, Carey et al. (2004) found that the presence of AMF led to direct increases in spotted knapweed biomass when plants were grown in isolation. However, the presence of both AMF and Idaho fescue led to the largest increase in spotted knapweed biomass; again, suggesting the interaction of these factors (plant competitor identity and soil microbial community) are important in this system (Carey et al., 2004). In the experiments of Carey et al. (2004), Idaho fescue showed no difference in biomass due to competition with spotted knapweed, the presence of AMF or a combination of both factors (Carey et al., 2004). The authors suggest that this may be due to the age of the plants, as Idaho fescue was well established prior to the planting of spotted knapweed; whereas Marler et al. (1999) seeded both plants at the same time (Carey et al., 2004). This is likely the case, as Schultz (2008) found that, in sterile soil media, spotted knapweed competition reduced Idaho fescue biomass when plants were established at the same time, but had no impact on Idaho fescue that had been established for 12 weeks (Schultz, 2008).

Again, the inconsistencies in results make it difficult to draw conclusions about soil feedback effects on Idaho fescue. My experiments suggest a neutral or slightly negative feedback of the soil microbial community on Idaho fescue biomass. However, as noted by other researchers, it may be more enlightening to investigate interactions between Idaho fescue, competing plants and the soil microbial community (Marler et al., 1999; Carey et al., 2004).

DENSITY MANIPULATION EXPERIMENT

I hypothesized that increased planting density of spotted knapweed would cause a reduction in fungal community abundance, based on my previous findings and those of other authors (Mummey et al., 2005; Mummey and Rillig, 2006; Broz et al., 2007). There was no support for this hypothesis, as fungal abundance tended to increase with planting density. This suggests that factors other than, or combined with spotted knapweed density are altering soil fungal communities in spotted knapweed infested field sites. In field studies spotted knapweed biomass has been found to be inversely related to both total and active amounts of filamentous fungi in native plant roots (Klein et al., 2006). Although Klein et al. (2006) did not investigate fungi present in spotted knapweed rhizosphere soils, it suggests that spotted knapweed biomass is an important parameter impacting native soil fungal communities (Klein et al., 2006), and biomass may differ depending on planting density, as I found in experiment 3.

Although increased spotted knapweed density has been correlated with reductions in fungal community abundance and diversity, my results suggest that planting density alone does not explain this observation. However, it is important to realize the limitations of a greenhouse experiment in this regard. For instance, increasing densities of spotted knapweed in the field are likely to change multiple site parameters that may be important in microbial community structure. These site parameters could include, but are not limited to, the amount of disturbance (particularly through erosion), soil structure (tap root of knapweed versus fiberous root system of native grasses) or changes to the type and quality of plant litter, root exudates, and other compounds that are likely to be substrates for microbial communities. In addition, this study took place over a relatively

short amount of time and the plants remained in rosette form through the length of the study without flowering. Other research suggests that detectable modifications to soil microbial communities in the greenhouse require multiple generations of plant growth (Broeckling et al., 2008).

The fact that soil microbial communities exhibited changes due to spotted knapweed planting density in the greenhouse may have important implications for greenhouse experiments on plant-soil feedback effects. Many studies precondition field soils in the greenhouse by planting a particular plant species in the soil for a certain amount of time (Klironomos, 2002; Callaway et al., 2004; Reinhart and Callaway, 2004). The general assumption is that plants will culture specific soil microbial communities, similar to those they culture in the field. My results suggest that planting density alone can alter the soil microbial community biomass in ways that would not be expected based on analysis of field soils. Thus, the ways in which soils are preconditioned in the greenhouse may have large or unexpected impacts on soil microbial communities, which in turn may lead to different plant-soil feedback effects. This finding may help to explain some of the inconsistencies in spotted knapweed-soil feedbacks (discussed above) that have been found by authors, as some studies used field soils and others used soils preconditioned in the greenhouse.

7.6 CONCLUSIONS

My results suggest that both spotted knapweed and Idaho fescue experience negative to neutral feedback effects from soil microbial communities present in areas of spotted knapweed infestation. This may suggest that pathogens of both plant types are

present in field soils. However, my results were not entirely consistent in the various greenhouse conditions tested, and were also often not consistent with previously published results. Soil microbial community biomass was impacted by spotted knapweed planting density, but the changes did not reflect those found at different densities of spotted knapweed in natural field conditions. This suggests that the density of spotted knapweed is not the sole factor driving the observed differences between microbial communities collected in the field. Although the impact of soil microbial community composition on exotic plant invasion has garnered much recent interest, my studies highlight the difficulty of making robust conclusions about this process through the use of greenhouse experiments.

7.7 REFERENCES

Broeckling CD, Broz AK, Bergelson J, Manter DK, Vivanco JM (2008) Root exudates regulate soil fungal community composition and diversty. Applied and Environmental Microbiology **74:** 738-744

Broz AK, Manter DK, Vivanco JM (2007) Soil fungal abundance and diversity: another victim of the invasive plant Centaurea maculosa. Isme Journal **1:** 763-765

Caesar AJ (2003) Synergistic interaction of soilborne plant pathogens and root-attacking insects in classical biological control of an exotic rangeland weed. Biological Control **28:** 144-153

Callaway RM, Thelen GC, Barth S, Ramsey PW, Gannon JE (2004) Soil fungi alter interactions between the invader Centaurea maculosa and North American natives. Ecology **85:** 1062-1071

Callaway RM, Thelen GC, Rodriguez A, Holben WE (2004) Soil biota and exotic plant invasion. Nature **427:** 731-733

Carey EV, Marler MJ, Callaway RM (2004) Mycorrhizae transfer carbon from a native grass to an invasive weed: evidence from stable isotopes and physiology. Plant Ecology **172:** 133-141

Elton C (1958) The Ecology of Invasions by Animals and Plants. Metheun, London

Folin O, Ciocalteu V (1927) On tyrosine and tryptophane determinations in proteins. Journal of Biological Chemistry **73:** 627-650

Klein DA, Paschke MW, Heskett TL (2006) Comparative fungal responses in managed plant communities infested by spotted (Centaurea maculosa Lam.) and diffuse (C-diffusa Lam.) knapweed. Applied Soil Ecology **32:** 89-97

Klironomos JN (2002) Feedback with soil biota contributes to plant rarity and invasiveness in communities. Nature **417:** 67-70

Levine JM, Vila M, D'Antonio CM, Dukes JS, Grigulis K, Lavorel S (2003) Mechanisms underlying the impacts of exotic plant invasions. Proceedings of the Royal Society of London Series B-Biological Sciences **270:** 775-781

Marler MJ, Zabinski CA, Callaway RM (1999) Mycorrhizae indirectly enhance competitive effects of an invasive forb on a native bunchgrass. Ecology **80:** 1180-1186

Meiman PJ, Redente EF, Paschke MW (2006) The role of the native soil community in the invasion ecology of spotted (Centaurea maculosa auct. non Lam.) and diffuse (Centaurea diffusa Lam.) knapweed. Applied Soil Ecology **32:** 77-88

Mummey DL, Rillig MC (2006) The invasive plant species Centaurea maculosa alters arbuscular mycorrhizal fungal communities in the field. Plant and Soil **288:** 81-90

Mummey DL, Rillig MC, Holben WE (2005) Neighboring plant influences on arbuscular mycorrhizal fungal community composition as assessed by T-RFLP analysis. Plant and Soil **271:** 83-90

Pimentel D, Lach L, Zuniga R, Morrison D (2000) Environmental and economic costs of nonindigenous species in the United States. Bioscience **50:** 53-65

Reinhart KO, Callaway RM (2004) Soil biota facilitate exotic Acer invasions in Europe and North America. Ecological Applications **14:** 1737-1745

Ridenour WL, Callaway RM (2003) Root herbivores, pathogenic fungi, and competition between Centaurea maculosa and Festuca idahoensis. Plant Ecology **169:** 161-170

Schultz M (2008) Soil ecological interactions of spotted knapweed and native plant species. Colorado State University, Fort Collins

Wardle DA (2006) The influence of biotic interactions on soil biodiversity. Ecology Letters **9:** 870-886

Wardle DA, Bardgett RD, Klironomos JN, Setala H, van der Putten WH, Wall DH (2004) Ecological linkages between aboveground and belowground biota. Science **304:** 1629-1633

Wolfe BE, Klironomos JN (2005) Breaking new ground: Soil communities and exotic plant invasion. Bioscience **55:** 477-487

CHAPTER 8

Conclusions

Small parts contained in this chapter are awaiting publication.

Weedy and Invasive Plant Genomics
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"The Genomics of Plant Invasion; a Case Study in Spotted Knapweed"

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Authors' contributions

AKB: wrote book chapter
JMV: edited book chapter

Each chapter of this dissertation provides its own sets of conclusions, which I will not belabor here. Instead, this section of my dissertation will address conclusions and considerations in regards to the larger idea of linking ecological data with molecular research using spotted knapweed as an example. I discuss some opportunities and limitations of this type of research that I have gained from my experience at Colorado State University. Overall, I conclude that experiments using molecular techniques can provide interesting insights into biological or ecological phenomena that are often not revealed using traditional measures of plant performance. However, molecular studies must be coupled with classical biological and ecological techniques to determine the larger significance of the findings.

8.1 SPOTTED KNAPWEED, A MODEL PLANT?

Although spotted knapweed is often referred to as a model plant in ecological research, I do not believe the same can be said for this weed in regards to molecular research. Even though limited sequence information is now available for this organism, spotted knapweed continues to present a variety of challenges for researchers in both the physiological and molecular sense. In choosing a plant system to work in, I would recommend that researchers fully understand the life history of the plant of interest. I have found that general life history characteristics of the plant are extremely relevant to the challenges I have faced in working with spotted knapweed, both molecularly and physiologically. This is not to say that a plant with 'undesirable' characteristics cannot be researched. However, I would suggest that scientists interested in understanding a

plant at both molecular and ecological scales consider certain life history factors and their associated challenges.

Arabidopsis thaliana is a model plant for many reasons. It is small, easy to grow in the laboratory, is self-fertile, has a short life cycle, is easy to transform, and produces a large amount of seed. It is a diploid plant with a relatively small genome, and there are a variety of natural ecotypes that can be studied in wild populations. Although working with *Arabidopsis* can have its disadvantages, its life history characteristics are very important factors in why it was chosen as a model system for genetic and molecular research.

Spotted knapweed does not exhibit many of the desirable life history characteristics that are associated with *Arabidopsis*, providing multiple challenges for laboratory researchers. For instance, invasive spotted knapweed is an out-crossing, insect pollinated tetraploid plant that spreads predominately by seed. These issues are compounded by the perennial nature of spotted knapweed, its highly variable morphology and the fact that it often does not flower in its first year of growth. These characteristics make spotted knapweed difficult to research through traditional genetic techniques that utilize specific crosses between known genetic backgrounds, and they limit the ways in which individual lines of plants can be created and used as tools in genetics or genomics research. These life history characteristics also provide challenges for greenhouse and field investigations that attempt to assess spotted knapweed fitness under different conditions. Below I discuss the issues I have found most relevant in my work with spotted knapweed.

8.2 OBTAINING AND UTILIZING SEQUENCE INFORMATION

Although invasive weeds are widely regarded as threats to native ecosystems, and are often associated with large economic costs (Pimentel et al., 2000), these characteristics have not made them priorities for genome sequencing projects. Currently there is no ongoing effort aimed at sequencing and assembling the genome of spotted knapweed, which makes studies of the genome itself extremely difficult. Because invasive spotted knapweed occurs predominately as a tetraploid and has a large genome (over 30 times the size of *Arabidopsis*, see Chapter 1 for estimates), it is unlikely that efforts in this area will be initiated in the near future. In addition, because spotted knapweed is predominately out-crossing, it provides difficulties in generating recombinant inbred lines (RILs) which can be of great use in molecular mapping studies and classical genetic work (Broman, 2005). Combining information from physical genome maps with gene expression profiling can reveal a wealth of information not only about the organism of interest and its response to various environmental conditions, but also about basic transcriptional control inside the cell. However, as full genome sequences and maps are not currently available for spotted knapweed, researchers must rely on genomics resources in the form of EST libraries.

Currently the NCBI GenBank database contains nearly 45,000 ESTs derived from spotted knapweed. As EST sequencing projects often contain a large amount of redundant transcripts, the number of unique gene sequences represented in the database appears to be a closer to 28,200. My small-scale EST sequencing and annotation project revealed only 18% redundancy because it was normalized to enrich for rare transcripts (Chapter 2). Assembly of spotted knapweed sequences from the Compositae Genome

Project at UC Davis revealed approximately 40-50% redundancy. Based on homology searches to genes in the NCBI database, I was able to annotate nearly 3,400 of the unique sequences contained in my EST library (Chapter 2). Thanks to a database created by researchers at the Noble Foundation (He et al., 2007), these gene annotations are easily searchable and publicly available at http://bioinfo.noble.org/plan/, project 30060. Many of the transcripts sequenced in the Compositae Genome Project were able to be annotated based on homology searches, but the information is not currently in a user-friendly format, making it difficult to navigate (http://compgenomics.ucdavis.edu, assembly information link).

These two sequencing efforts represent a first step in examining spotted knapweed at a molecular level. Both sequence information and annotation can be extremely useful in determining candidate genes that may be involved in important physiological and ecological processes. This information can potentially be used to address a variety of hypotheses of plant invasion (outlined in Chapter 2) and may be important in developing management strategies for the eradication of invasive weeds. My work and that of others suggests that genomics resources can compliment traditional physiological and ecological studies aimed at understanding invasion biology.

8.3 DEALING WITH PLOIDY, TAXONOMY AND DIVERSITY

Although multiple life history characteristics of spotted knapweed are important to consider when designing experiments, I think the most important factor in my research has been the determination of plant ploidy. Spotted knapweed occurs in both diploid and tetraploid forms in the native range, while it occurs almost exclusively as a tetraploid in

the introduced range (Ochsmann, 2001; Treier et al., in press). Studies of invasion biology often focus on comparing plants from the native range to those from the introduced range; however the issue of plant ploidy is often neglected in those investigations (Bossdorf et al., 2005). Although identifying and comparing a particular 'taxonomic group' may not be as important for ecological comparisons of plant populations, it is critical in a comparison of genetics or gene expression between native and introduced plants (as highlighted in Chapter 3). Differences in ploidy are known to impact gene expression, secondary metabolism and plant physiology in a variety of organisms (Dhawan and Lavania, 1996; Soltis and Soltis, 2000; Chen and Ni, 2006; Chen, 2007). For spotted knapweed this issue is rather difficult to deal with because the diploids and tetraploids in the native range are morphologically indistinct (Oschmann, 2001). Thus, molecular methods are required to determine individual plant ploidy, which can be expensive and time consuming. However, the identification of diploid and tetraploid spotted knapweed plants is only one aspect of the problem that polyploidy presents for molecular investigations.

Aside from the fact that the taxonomy of spotted knapweed is generally confusing and unresolved (spotted knapweed is known as *Centaurea maculosa* L., *C. stoebe* L. (Gugler) Hayek, or *C. biebersteinii*) it is not known if the tetraploid spotted knapweed is a result of allo- or auto- polyploidization. Spotted knapweed is thought to hybridize with diffuse knapweed (*C. diffusa*) in the native range, and as mentioned in the introduction, the rare knapweed *C. corymbosa* of France and the widespread *C. albida* of Europe are thought to have been derived from a spotted knapweed ancestor (Freville et al., 1998). Due to the nature of spotted knapweed and its potential for hybridization, ecological

specialization and/or rapid evolution (Freville et al., 1998; Broennimann et al., 2007), it is difficult to know how the genomes of spotted knapweed individuals will differ within and between populations and how those differences might impact individual characteristics of plant growth and defense.

The available evidence suggests a large amount of genetic diversity within and between populations in both the native and introduced range of the weed (Hufbauer and Sforza, 2008; Marrs et al., 2008), but this evidence is also somewhat difficult to interpret due to the polyploidy issue and the limited capabilities of the analysis software (Marrs, personal communication). In this sense, spotted knapweed is much more difficult to work with than a typical crop species that has undergone a long history of human selection and contains only a fraction of the genetic diversity found in its wild relatives. Crop plants are generally more reliable to work with using genomics techniques because plants of a particular cultivar share a similar genetic background and provide reproducible phenotypes. Using these techniques to study invasive weeds may require more extensive sampling protocols, to ensure that the genetic diversity and related differences in gene expression are accurately assessed. If a founder population for an invasive plant has been identified it may be appropriate to study that population in reference to the invasive populations: however, for plants like spotted knapweed, no specific founder population has been identified (Hufbauer and Sforza, 2008; Marrs et al., 2008).

Generally speaking, tetraploid plants are assumed to have at least two copies of all genes present in their diploid progenitors. These genes may or may not be functional, and they may or may not be expressed (Chen, 2007). Genes present in polyploids may

even have taken on new functions distinct from those in their predecessor, or be lost entirely due to random mutation or activity of transposable elements (Chen, 2007). The limited amount of work I have pursued in relation to this issue (Chapter 3) suggests that spotted knapweed gene expression is influenced by both origin and ploidy; highlighting the importance of determining plant ploidy in this type of investigation.

Even when using plants of known ploidy, making conclusions based on molecular techniques provides difficulties. For instance, in my work with cross-species hybridization to microarray, I did not know how much genome coverage I was getting using spotted knapweed cDNA on an *Arabidopsis* microarray (Chapter 4). Similarly, without a genome sequence for spotted knapweed it is difficult to determine gene copy number, hybridization rate and other important factors when using microarray (Chapter 4). In this sense, I think the Q-PCR based approach was more informative, and likely more reliable than the microarray technique (Chapter 4). However, it was often challenging to design primers that only amplified a single product, and even when primer sets result in a single product, it does not necessarily mean that the primers were gene-specific. For instance, it is possible that a given primer set amplified a region of cDNA that is conserved in size, but is variable in sequence in different gene copies. Based on some of the unigene sequence data from the EST library, it appears that there may be multiple gene polymorphisms in the spotted knapweed coding regions. Gene polymorhpisms in both coding and non-coding regions can lead to differences in gene expression or protein function in plants (Plantegenet et al., 2009). Therefore, the results of both Q-PCR and microarray should be interpreted with caution when attempting to make conclusions about spotted knapweed ecology or biology.

8.4 MEASURING SUCCESS

Life history characteristics of spotted knapweed tend to complicate the matter of measuring the fitness or competitive ability (in general, spotted knapweed success), particularly in greenhouse conditions. These issues are by no means isolated to spotted knapweed, as the question of how to accurately assess individual fitness remains unanswered for a large number of organisms. However, when studying a perennial plant in short term greenhouse experiments, multiple questions can arise about how to measure success in a way that can ideally be extrapolated to the field. For instance; is biomass an appropriate measure of spotted knapweed fitness or competitive ability, or is seed set or some other characteristic more relevant? Should investigations be designed to encompass multiple years of growth because of the perennial nature of the plant, or are early indicators effective in predicting the outcome of experiments involving plant-plant competition, etc? In reviewing the literature, and based on my own experience, I remain uncertain if there are any hard and fast answers to these questions.

Because biomass is relatively easy to measure I have often used it as an indicator of spotted knapweed success (Chapters 3, 4, 5 and 7). However, the extent to which this measure of success can be extrapolated to natural field conditions remains understudied. For instance, the results from Chapter 3 indicate that there are no significant differences in vegetative characteristics between native and introduced populations of plants grown in a common garden. Similarly, seed head number, a traditional measure of fitness, showed no differences between the geo-cytotypes after one year (Chapter 3). None of the measures taken significantly differentiated between native and introduced tetraploids (Chapter 3). These studies neglected to identify a measurable physiological trait that

would be indicative of spotted knapweed fitness. The populations of spotted knapweed described in Chapter 3 will be monitored in the coming years by the laboratory of Heinz Müller-Schärer, and I expect this long-term study will provide some important information about differences in spotted knapweed characteristics that are due to plant ploidy and origin.

I am also concerned that overall biomass is not a particularly good indicator of competitive ability, because in my plant competition experiments I find that the native grass Idaho fescue accumulates more total biomass than spotted knapweed under low resource conditions (Chapter 5); yet, in the field spotted knapweed is able to out-compete and often eradicate this grass. When the plants are grown in isolation, Idaho fescue also tends to accumulate more biomass than spotted knapweed over the same amount of time (Chapter 7, experiment 2). Thus, if greater biomass was the only parameter that influenced success, I would expect that spotted knapweed would be dominated by Idaho fescue, particularly in the low resource conditions often found at my field sites (Chapters 6 and 7). However, my field sites are all thought to be former native grasslands that are now dominated by spotted knapweed (G Thelen and RM Callaway, personal communication). Thus, it is clear that plant biomass is not the only parameter impacting the ecology of these systems.

8.5 INTEGRATING ENVIRONMENTAL CONDITIONS

Some of my work indicates that spotted knapweed is able to perceive and respond to neighboring plants in what could be considered a species-specific manner (Chapters 4 and 5). This finding is extremely intriguing particularly in regards to the implications it

could have for plant success and community composition in natural field settings. The conclusions from my experiments are rather limited in that I have only investigated a small number of competitive settings in the greenhouse. However, the observation that both spotted knapweed and Idaho fescue exhibit specific metabolic responses related to plant neighbor identity suggests that this could be an important factor in multiple types of plant systems (Chapter 5).

I also found that spotted knapweed alters native soil microbial communities, and that these microbial communities sometimes lead to negative feedback effects with both spotted knapweed and Idaho fescue (Chapters 6 and 7). Thus, biotic alterations (such as microbial community modification) induced by spotted knapweed infestation may have some impact on the overall plant community structure. However, as noted in Chapter 7, it is difficult to conclude to what extent soil microbial structure might be playing a role in spotted knapweed invasion, particularly due to conflicting results described here and in the literature. In addition, other researchers note that combining aspects of plant competition and soil microbial community structure may lead to more important insights than studying any of these factors in isolation (Marler et al., 1999; Carey et al., 2004).

In general, the results from Chapters 4-7 highlight the potential difficulty of performing what researchers would like to consider 'ecologically relevant experiments' under greenhouse conditions. For instance, if plant neighbor identity is indeed a major factor impacting plant gene expression (Chapter 4) and metabolism (Chapter 5), how do we address this factor when designing experiments? Similarly, if soil microbial communities are major factors impacting plant community structure, how do we appropriately integrate this into greenhouse experiments? Additionally, how do we

combine these factors into one experiment in a feasible manner? What plant or soil characteristics do we measure in these experiments? There are no easy answers to these questions. Some clues will likely be derived from well designed experiments, but the findings need to be tested under multiple field and greenhouse conditions to determine their influence on spotted knapweed invasion biology.

8.6 FINAL THOUGHTS

When I first began working on this dissertation I posed the following question to myself: why is spotted knapweed so successful? I have continued to ponder this question over the years, and must conclude that a single factor does not exist which can, by itself, explain the success of this invasive plant. Based on the results of the experiments outlined in this dissertation, and the work of other researchers, it appears that there are multiple factors involved in the invasive success of this weed. Some of these factors are linked to the genetics, biology and physiology of the plant itself, while others appear more closely related to both biotic and abiotic conditions present in the areas where the plant occurs. Below I outline a few ideas for future research that may help identify the significance of these factors in spotted knapweed invasion.

Ploidy appears to be an important factor in the success of tetraploid populations of spotted knapweed in both the native and introduced ranges (Chapter 3). In addition, studies of gene expression suggest that tetraploid populations may have undergone selection or rapid evolution in the introduced range (Chapter 3). Gene expression data lend credence to the idea that introduced spotted knapweed is more poorly defended against herbivores than native tetraploids, and thus may have undergone enemy release

upon introduction (Chapter 3). However, future experiments should investigate the susceptibility of the different geo-cytotypes to both generalist and specialist herbivores while monitoring levels of both constitutive and induced defenses. This type of work will give researchers more clues about the potential for rapid evolution in the introduced range, and whether or not hypotheses like the evolution of increased competitive ability (EICA) apply to spotted knapweed.

Future experiments would also benefit from a better understanding of the genetic relatedness of the plant populations that are being tested. Spotted knapweed exhibits many life history characteristics that are likely to increase genetic diversity. For instance, it is out-crossing, insect pollinated, has a perennial life style, flowers for many months, and exhibits lengthy seed dormancy. Because of these characteristics and the fact that spotted knapweed is thought to harbor a large amount of genetic diversity in both the native and introduced range (Hufbauer and Sforza, 2008), it is difficult to assess how much of this diversity is actually being captured in greenhouse or laboratory experiments. As genetic diversity is considered the raw material that natural selection acts upon over time, it is important to understand the genetic diversity that is present in these plant populations and the resultant phenotypes that may occur due to genetic diversity. The large amount of genetic diversity that is known to exist within and between introduced spotted knapweed populations is likely to be important in its invasive success. In addition, the observed morphological diversity of the weed and its ability to tolerate a wide variety of habitats suggests that adaptive plasticity may be an important factor for success as well.

My work suggests that spotted knapweed is able to sense and respond to plant neighbors in species-specific ways that may be important in its invasive success (Chapters 4 and 5). These results may have important implications for management of the weed and plant species choice in restoration of native areas, but they are also interesting to think about in an evolutionary context. The question of whether or not these traits have been selected for in introduced populations of spotted knapweed remains undetermined. Is the observed differential response of spotted knapweed to different plant neighbors somehow ingrained in its genetic code, a product of natural selection over multiple generations, or are these observations more closely related to changes in environmental factors dictated by the presence of a specific plant neighbor? Future studies should attempt to identify the mechanism by which spotted knapweed is able to sense and respond to specific plant neighbors. Understanding this phenomenon mechanistically would help provide a clearer picture of whether or not these traits are a product of natural selection. Alternatively, because these traits may represent a plastic response of spotted knapweed presented with subtly different environmental conditions related to plant neighbor identity, future experiments should also closely examine environmental variables. These could include, but are not limited to parameters such as plant neighbor root exudation profiles, nutrient uptake rates, and quality of reflected light.

I found that plant neighbor identity can lead to modifications in secondary metabolic profiles in leaves of both spotted knapweed and Idaho fescue under certain conditions (Chapter 5). This suggests that leaf litter quality will be impacted by plant neighbor identity. The quality of leaf litter is likely to lead to modifications in some environmental parameters, as leaf litter provides substrates for soil microflora and fauna.

Because I have identified alterations in soil microbial communities related to increases in spotted knapweed infestation (Chapters 6 and 7), it would be interesting to explore whether or not there is a relationship between leaf litter quality and soil microbial community composition at high and low relative densities of the weed. By further identifying the microbial community members present in knapweed infested areas and determining their ability to utilize different substrates, researchers may be able to identify links between plant neighbor identity, leaf litter quality and microbial community composition in this system.

Because there appear to be multiple factors involved in spotted knapweeds invasive success, there are multiple research areas to pursue in regards to the question: why is spotted knapweed so successful? My research suggests that molecular techniques, when coupled with traditional measures of plant performance and success, will provide interesting and important insights into studies of plant invasion. However, as discussed above, studies must be well designed and may need to take multiple variables into account to more fully understand spotted knapweed invasion biology.

In a general sense, I think that science is moving towards a more interdisciplinary approach to understanding the natural world. I believe that science will, out of necessity, begin linking research on multiple scales, as this will be required to more fully understand the inner workings of the world we live in. In my doctoral research I was interested in trying to bridge the gap between the large and small scales. I have found this type of research to be challenging on multiple levels. However, I look forward to the new and exciting findings that will inevitably be gained by taking this multi-scale approach.

As the current year of 2009 marks the 200th birthday of Charles Darwin, I feel it appropriate to reference a quotation from his widely read and regarded publication, "The Origin of Species" (1859). Darwin commented in his work, "our ignorance of the laws of variation is profound" (Darwin, 1859). Although researchers have devised new techniques and tested multiple hypotheses to identify the laws behind variation, selection and evolution of species, I believe that this statement is as true today as when it was written. The attempt to understand the natural world that surrounds us remains a constant challenge. I regard this challenge as one of the most intriguing, yet humbling aspects of the human experience.

8.7 REFERENCES

Bossdorf O, Auge H, Lafuma L, Rogers WE, Siemann E, Prati D (2005) Phenotypic and genetic differentiation between native and introduced plant populations. Oecologia **144:** 1-11

Broennimann O, Treier UA, Muller-Scharer H, Thuiller W, Peterson AT, Guisan A (2007) Evidence of climatic niche shift during biological invasion. Ecology Letters **10:** 701-709

Broman KW (2005) The genomes of recombinant inbred lines. Genetics **169:** 1133-1146

Carey EV, Marler MJ, Callaway RM (2004) Mycorrhizae transfer carbon from a native grass to an invasive weed: evidence from stable isotopes and physiology. Plant Ecology **172:** 133-141

Chen ZJ (2007) Genetic and epigenetic mechanisms for gene expression and phenotypic variation in plant polyploids. Annual Review of Plant Biology **58:** 377-406

Chen ZJ, Ni ZF (2006) Mechanisms of genomic rearrangements and gene expression changes in plant polyploids. Bioessays **28:** 240-252

Darwin C (1859) On the Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life. John Murray, London

Dhawan OP, Lavania UC (1996) Enhancing the productivity of secondary metabolites via induced polyploidy: A review. Euphytica **87:** 81-89

Freville H, Colas B, Ronfort J, Riba M, Olivieri I (1998) Predicting endemism from population structure of a widespread species: Case study in Centaurea maculosa Lam. (Asteraceae). Conservation Biology **12:** 1269-1278

He J, Dai XB, Zhao XC (2007) PLAN: a web platform for automating high-throughput BLAST searches and for managing and mining results. Bmc Bioinformatics **8**

Hufbauer RA, Sforza R (2008) Multiple introductions of two invasive Centaurea taxa inferred from cpDNA haplotypes. Diversity and Distributions **14:** 252-261

Marler MJ, Zabinski CA, Callaway RM (1999) Mycorrhizae indirectly enhance competitive effects of an invasive forb on a native bunchgrass. Ecology **80:** 1180-1186

Marrs RA, Sforza R, Hufbauer RA (2008) Evidence for multiple introductions of Centaurea stoebe micranthos (spotted knapweed, Asteraceae) to North America. Molecular Ecology **17:** 4197-4208

Ochsmann J (2001) On the taxonomy of spotted knapweed (Centaurea stoebe L.) *In* L Smith, ed, Proceedings from The First International Knapweed Symposium of the Twenty First Century. USDA-ARS, Albany, CA, pp 33-41

Oschmann J (2001) On the taxonomy of spotted knapweed (Centaurea stoebe L.) *In* Proceedings from The First International Knapweed Symposium of the Twenty First Century, pp 33-41

Pimentel D, Lach L, Zuniga R, Morrison D (2000) Environmental and economic costs of nonindigenous species in the United States. Bioscience **50:** 53-65

Plantegenet S, Weber J, Goldstein DR, Zeller G, Nussbaumer C, Thomas J, Weigel D, Harshman K, Hardtke CS (2009) Comprehensive analysis of Arabidopsis expression polymorphisms with simple inheritance. Molecular Systems Biology **5:** 10

Soltis PS, Soltis DE (2000) The role of genetic and genomic attributes in the success of polyploids. Proceedings of the National Academy of Sciences of the United States of America **97:** 7051-7057

Treier UA, Broennimann O, Normand S, Guisan A, Schaffner U, Steinger T, Muller-Scharer H (in press, 2009) Shift in cytotype frequency and niche space in the invasive plant Centaurea maculosa. Ecology **90**: 1366-1377