DISSERTATION

PRIMARY AND SECONDARY METABOLISM IN *CENTAUREA MACULOSA* AND THEIR POTENTIAL ROLES IN INVASION BIOLOGY

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY COREY D. BROECKLING ENTITLED 'PRIMARY AND SECONDARY METABOLISM IN *CENTAUREA MACULOSA* AND THEIR POTENTIAL ROLES IN INVASION BIOLOGY' BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

PRIMARY AND SECONDARY METABOLISM IN *CENTAUREA MACULOSA* AND THEIR POTENTIAL ROLES IN INVASION BIOLOGY

Centaurea maculosa is plant species native to Eurasia which has become invasive in North America, in part through allelopathic behavior. Allelopathy remains a highly debated subject, and a more firm understanding is necessary. *C. maculosa* is reported to secrete catechin as an allelochemical that is toxic to North American native plants. In this dissertation, a novel colorimetric assay for use in detection of catechin from soils is described and validated. This assay is highly sensitive, selective, and fast, which should allow for more detailed measures of catechin under field conditions, and ultimately a better understanding of the variation in catechin accumulation. To increase the depth of understanding of catechin biosynthesis, I report the cloning and characterization of *C. maculosa* dihydroflavonol reductase (CmDFR), a gene very likely to be necessary for catechin biosynthesis. To expand our view of metabolism beyond catechin, metabolome analysis is applied to field collected plant material, and demonstrate that the physiology of *C. maculosa* varies with the surrounding plant community – plants growing in patches with high *C. maculosa* density tend to be accumulate higher levels of secondary metabolites than plants growing in the company of few conspecifics. Finally, the results of a study that clarifies the role of root exudates in structuring the soil fungal community are presented in the context of invasion biology.

This study demonstrates that root exudates are capable of influencing the soil fungal community even in the absence of the plant in a species specific manner, and that the effect of root exudates on the soil fungal community is qualitatively similar to the effect of the whole plant.

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Abbreviations used in text:

ANOVA – analysis of variance

ANR – anthocyanidin reductase

ANS – anthocyanadin synthase

C18 – octadecyl stationary phase

CDF – cumulative distribution function

cDNA – complimentary deoxyribonucleic acid

$CHCl_3$ - chloroform

Cm –*Centaurea maculosa*

DFR – dihydroflavonol reductase

DHQ - dihydroquercetin

DMACA – dimethylaminocinnamaldehyde

DNA – deoxyribonucleic acid

EDTA – ethylenediaminetetraacetic acid

ESI – electrospray ionization

EV – empty vector

FWHM – full width at half maximum

GC-MS – gas chromatography – mass spectrometry

Gen - generation

HCA – hierarchical component analysis

HCl – hydrochloric acid

HPLC – high-pressure liquid chromatography

IPTG – isopropyl-β-D-thiogalactopyranoside

ITS – internal transcribed spacer

LAR – leucoanthocyanidin reductase

LB – Luria-Bertani media

M - molecule

mRNA – messenger ribonucleic acid

MS – mass spectrometry

MS media - Murashige and Skoog plant growth medium

MSTFA - N-Methyl-N-trifluoroacetamide

NADH - nicotinamide adenine dinucleotide, reduced form

NADPH – nicotinamide adenine dinucleotide phosphate, reduced form

NCBI – National Center for Biotechnology Information

NMR – nuclear magnetic resonance spectroscopy

NOAA – National Oceanic and Atmospheric Association

ORCA3- octadecanoid-derivative responsive *Catharanthus* P2-domain protein 3

PCA – principle components analysis

PCR – polymerase chain reaction

PLS-DA partial least squares – discriminant analysis

qPCR – quantitative polymerase chain reaction, also known as real-time PCR – polymerase chain reaction

Q-TOFMS – quadrupole time of flight mass spectrometry

RNA – ribonucleic acid

Rt – retention time

RT-PCR – reverse transcription – polymerase chain reaction

SDS – sodium dodecyl sulfate

TRIS – tri(hydroxymethyl)-aminomethane

UPLC – ultra-high-pressure liquid chromatography

UV - ultraviolet

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1. **Introduction and literature review**

Invasive species:

Exotic invasive species are continuing ecological and economic problems driven largely by the inability to prevent accidental species introductions in a globalized economy (Meyerson and Mooney, 2007). The economic and environmental costs of control of invasive species in the US alone has been estimated at approximately 120 billion dollars annually (Pimentel et al., 2005). Preventing the introduction of plant species from foreign lands is the preferred solution to the exotic species problem, but this method is difficult to implement and thousands of species have been inadvertently introduced. However, not all species introduced to the United States establish, and even fewer become problematic. Why this is true is a highly debated and researched field (Shea and Chesson, 2002). Introduced plant species that become problematic attain high densities in the invaded range, whereby they displace native species, reduce native plant biodiversity, and disrupt ecological processes that depend on those native plants. Nearly half of the endangered species in the US are threatened primarily by habitat loss due to invasive species (Pimentel et al., 2005). The superior competitive ability of invasive plants is the primary factor allowing for this behavior, but the mechanism by which competitive ability is achieved is unclear, and is likely to vary between species (Shea and Chesson, 2002).

Centaurea maculosa ***biology and control:***

Centaurea maculosa Lamarck (synonymous with *C. beibersteinii* de Candolle and *C. stoebe* L. subsp *micranthos*; Spotted knapweed) is a tap-rooted perennial aster species of the Centaureinae subtribe. The genus *Centaurea* contains between 400 and 700 species, the phylogentics of which are complex and unresolved (Garcia-Jacas et al., 2001). *C. maculosa* was introduced into the United States, possibly in alfalfa seed, in the late 1800's. Since that time, it has expanded its range throughout the US and is now considered an exotic invasive plant in most on the western states (USDA Plants database; http://plants.usda.gov/index.html).

Several biological control herbivores have been introduced in an attempt to control *C. maculosa* and other invasive *Centaurea spp.* (Maddox, 1982; Powell et al., 2000; Clark et al., 2001). While these species are moderately successful in reducing *C. maculosa* population abundance or fitness (Maddox, 1982; Story et al., 2000; Corn et al., 2006; Story et al., 2006), knapweed continues to expand in range. Some reports suggest that herbivory increases the competitive advantage that *C. maculosa* has over North America native species (Callaway et al., 1999; Ridenour and Callaway, 2003; Thelen et al., 2005; Newingham et al., 2007) and that biological control insects indirectly facilitate increased hantavirus by increasing the abundance of the mouse vector (Ortega et al., 2004; Thelen et al., 2005; Pearson and Callaway, 2006). Further, North American native ant species are contributing to the spread of spotted knapweed. Knapweed seeds contain an expendable eliasome which ants collect as a food source - once removed the viable seed is discarded (Jensen and Six, 2006).

In addition to insect biological control agents, pathogens have been examined for control of spotted knapweed. *Pseudomonas syringae* was observed to cause stem dieback disease, and the disease is apparently promoted by herbivore damage combined with a cool, wet environment (Kearing and Nowierski, 1997). The fungal pathogen *Alternaria alternata* produces a host-specific phytotoxin (Stierle et al., 1988), maculosin, which was examined as an alternative herbicide (Bobylev et al., 1996). Unfortunately, maculosin has yet to develop into a practical control measure. Thus biological control efforts, though demonstrating promise, are ineffective at controlling the expansion of spotted knapweed in the United States. Several herbicides are effective at killing *C. maculosa*, but this approach is not feasible at large scales or in ecologically sensitive lands.

C. maculosa is a particularly problematic weed in western rangelands and public parks and reserves. Several mechanisms of invasion have been examined in an attempt to explain the invasive behavior of *C. maculosa*, including greater efficiency in nitrogen acquisition and use (Blicker et al., 2002; Olson and Blicker, 2003), water use efficiency (Blicker et al., 2003; Hill et al., 2006), soil fungal relationships (Marler et al., 1999; Zabinski et al., 2002; Callaway et al., 2004; Callaway et al., 2004; Carey et al., 2004; Mummey et al., 2005; Meiman et al., 2006; Broz et al., 2007), allelopathy (Ridenour and Callaway, 2001; Bais et al., 2003), physical properties of soils (Sperber et al., 2003), and phylogenetic relationships to native plants (Pokorny et al., 2005). Evidence supports

many of the above listed mechanisms as important in invasive behavior, but it remains unclear exactly why *C. maculosa* is so successful in the western US.

C. maculosa demonstrates allelopathic behavior, and this property was proposed to be mediated by root exudates (Callaway and Aschehoug, 2000). Further research revealed that a racemic mixture of (+/-)-catechin was found in the exudates, that this compound applied exogenously to *Arabidopsis thaliana* plants at levels comparable to that found in *C. maculosa* exudates was phytotoxic, and this phytotoxicity was partially mediated through calcium signaling leading to cell death (Bais et al., 2003). Further, soils from North America supporting invasive *C. maculosa* had higher levels of catechin than did European soils supporting growth of native *C. maculosa* (Bais et al., 2003). However, accumulation of catechin varies dramatically, and the lower concentrations found in some studies are unlikely to be ecologically relevant (Blair et al., 2005; Blair et al., 2006; Perry et al., 2007). Catechin is rapidly degraded following exposure to light or to neutral to basic pH conditions (Kiatgrajai et al., 1982; Porter, 1989), and it remains unclear whether the variation in catechin accumulation patterns is due to biological variation/regulation or physiochemical degradation.

Primary and secondary metabolism:

Plants are autotrophic organisms, possessing the ability to fix CO_2 to generate the carbohydrates that provide energy and serve as substrates for growth, maintenance, and biotic and abiotic stress responses. Central metabolic pathways that are essential for

growth are referred to as 'primary metabolism' while those pathways peripheral to primary metabolism and not critical for growth under certain conditions are referred to as 'secondary metabolism.' Though this distinction has proven somewhat artificial (Berenbaum, 1995), the classification holds value in that primary metabolites typically are involved in growth and maintenance, while secondary metabolites are typically involved in biotic and abiotic stress tolerance or other ecological phenomena. Primary metabolism includes metabolic pathways including carbon fixation via photosynthesis, the tricarboxylic acid cycle, amino acid biosynthesis, redox cycling, carbohydrate production and degradation, membrane lipid synthesis, and a variety of other pathways that are largely conserved with other non-plant taxa.

While secondary metabolites were originally classified under the assumption that they were non-essential, several secondary metabolites have more recently been demonstrated to have critical roles in fundamental biological processes. For example, flavonoids are thought to play a role in the transport of auxin, a phytohormone critical for proper plant development (Peer et al., 2004; Taylor and Grotewold, 2005; Wasson et al., 2006). Flavonoids also act as floral pigments, UV protectants, in defense against herbivores and pathogens, and in communication with bacterial symbionts. These functions are critical to the plant under natural growth conditions; *Arabidopsis thaliana* plants deficient in chalcone isomerase, the first dedicated enzyme of the flavanoid pathway, are highly susceptible to UV stress and demonstrate high levels of oxidative damage (Landry et al., 1995). The additional oxidative stress generated in the absence of flavonoids results in genome instability (Filkowski et al., 2004). From an evolutionary perspective, insect

herbivores are a positive selective agent in the evolution of plant secondary metabolism, (Benderoth et al., 2006).

Secondary metabolites are necessarily derived from primary metabolic pathways (Pichersky and Gang, 2000). While the biosynthetic link between primary and secondary metabolism has been known for decades, more recent research has revealed that primary and secondary metabolism are co-regulated at the level of gene expression. This was most conclusively demonstrated with the discovery that the gene product of ORCA3 is a transcriptional regulator of genes of both primary and secondary metabolism (Stokes, 2000; van der Fits and Memelink, 2000). Elicitation experiments have also revealed coordinated changes in primary and secondary metabolite accumulation patterns (Hirai et al., 2004; Kant et al., 2004; Broeckling et al., 2005). The link between primary and secondary metabolism is through flux control and co-regulation, but has yet to be fully developed (Schwachtje and Baldwin, 2008).

Plants are capable of synthesizing an incredible array of secondary metabolites. A database dedicated to secondary metabolites from the Asteraceae family (of which *C. maculosa* is a member) contains approximately 24,000 chemical structures (BGBM, The Bohlman Files phytochemical database, http://bohlmann.bgbm.org/bohlmann/). Enzymes of plant secondary metabolism are often moderately non-specific in their substrate preference, and often generate multiple products from a single precursor (Wise et al., 1998; Firn and Jones, 2003; O'Maille et al., 2006). This diversity is facilitated in part

through gene duplication and divergent evoloution (Helariutta et al., 1996). The evolution of such incredible diversity has spurred some to liken plant secondary metabolism to 'in-vivo combinatorial chemistry' (Osbourn et al., 2003).

The flavonoid pathway:

The flavonoids pathway is the most well characterized secondary metabolic pathway in plants (Winkel-Shirley, 2001). The products of this pathway are important in UV protection (Bieza and Lois, 2001), hormone transport (Peer et al., 2004), and defense against herbivores and pathogens (Bailey et al., 2005; Miranda et al., 2007), among other functions.

This pathway, as are all secondary metabolic pathways, is initially derived from primary metabolism. The first committed step from primary metabolism to the phenylpropanoids pathway involves the conversion of the amino acid phenylalanine to the secondary metabolite, cinnamic acid, by the enzyme phenyalanine ammonium lyase. This step provides substrates for conversion to other phenylpropoid compounds which are utilized in the synthesis and accumulation of lignins and suberins. One of the products of the phenylpropanoid pathway is coumaric acid ligated to Coenzyme A (Coumaroyl CoA). This compound serves as a substrate for the enzyme chalcone synthase which condenses Coumaroyl CoA with three molecules of another primary metabolite, malonyl CoA, to produce a chalcone, the first committed product of the flavonoid pathway. Through a

series of reduction, oxidation, condensation, glycosylation, and other reactions, this chalcone precursor gives rise to diverse flavonoids structures.

One product of the flavanoid pathway is catechin, the putative allelochemical responsible for the allelopathic traits of *C. maculosa.* Catechin is typically a precursor to condensed tannins, which have recently received increased attention due to their health promoting properties [extensively discussed in (Dixon et al., 2005; Xie and Dixon, 2005)]. The pathway leading to condensed tannins has yet to be fully characterized, but the early steps are fairly well described (Xie and Dixon, 2005). A flavonol is oxidized to a dihydroflavonol, which serves as the substrate for dihydroflavonol reductase (DFR). DFR activity generates moderately unstable leucoanthocyanins, which are demonstrated to be converted either to anthocyanins by anthocyanidin synthase (ANS) or to catechin by leucoanthocyanidin reductase (LAR). Anthocyanins are then converted to epicatechin by anthocyanidin reductase (ANR). Both LAR and ANR have been characterized at the molecular and biochemical levels (Tanner et al., 2003; Xie et al., 2003), but there is no apparent LAR gene in *A. thaliana*, which contains only epicatechin in it condensed tannins, and questions remains as to the relevance of LAR to tannin biosynthesis in some legumes (Pang et al., 2007). Catechins (catechin or epicatechin) serve as a substrate in an uncharacterized condensation reaction to produce condensed tannins (synonymous with proanthocyanidins). Whereas DFR has been cloned from numerous plant species, ANR and LAR have been cloned from only a few, none of which are asters. Whether racemic (+/-)-catechin from *C. maculosa* is generated through the activity of LAR or ANR, these pathways must pass through DFR.

***Metabolomics as a tool for studying metabolism*:**

At the start of the new millennium, plant biologists began to embrace a new approach in the study of plant metabolism, physiology, and functional genomics termed metabolomics (Fiehn et al., 2000). The concept behind the approach is adapted from proteomics and transcriptomics technologies, which strive to simultaneously quantify hundred(s) to thousands of proteins or mRNA transcripts, respectively. Metabolomics aims to obtain relative quantitative data on 'all' of the metabolites in a given sample simultaneously, and use this approach to compare genotypes (Fiehn et al., 2000; Robinson et al., 2005; Tohge et al., 2005), mutants (Fiehn et al., 2000), developmental states (Jeong et al., 2004), or stress conditions (Hirai et al., 2004, 2004; Kant et al., 2004; Broeckling et al., 2005) as a means of understanding the physiology underlying a phenotype. Typically, metabolomics studies use chromatographic methods coupled to mass spectrometry, though NMR is also used (Sumner et al., 2003). Metabolomics relies heavily on computational tools for peak detection, quantification, data visualization and statistical analysis and numerous approaches and tools have been developed (Duran et al., 2003; de la Fuente et al., 2004; Jonnson et al., 2004; Kopka et al., 2004; Jonsson et al., 2005; Katajamaa and Oresic, 2005; Schauer et al., 2005; Shellie et al., 2005; Tikunov et al., 2005; Broeckling et al., 2006; Sumner et al., 2007).

***Description of proposed research*:**

I wished to isolate the biosynthetic gene for catechin production from *C. maculosa* in an effort to clarify the role of catechin in allelopathy and plant invasion. To this end, a colorimetric assay was developed to rapidly detect the presence of catechin in soils and/or exudates. The DFR gene was then cloned using a degenerate PCR approach, tissue-specificity of expression is reported, and the gene product is biochemically characterized following heterologous expression in bacterial cells. Allelopathy is unlikely to be the only mechanism used in invasion, and therefore alternative mechanisms were explored that might be involved in invasion biology. The first examined the density dependence of plant physiology using metabolome techniques of field collected plants. The second examined the role of root exudates in regulating the soil microbial community using two model plant species, *Arabidopsis thaliana* and *Medicago truncatula*.

2. **Development of a selective, sensitive, and rapid in-field assay for soil catechin, an allelochemical of *Centaurea maculosa*.**

Summary

Centaurea maculosa is a plant species native to Eurasia and invasive in many regions of North America that secretes a phytotoxin from its roots, (+/-)-catechin, which is thought to serve an allelopathic role by reducing fitness of neighboring species. However, catechin is a relatively unstable compound, is often found at relatively low concentrations, interacts with soil cations to form insoluble complexes and/or degradation products, and exhibits extremely variable accumulation patterns in the soil. These factors, coupled with a lack of knowledge of the regulation of catechin production by *C. maculosa*, confound our understanding of the importance of catechin as an allelochemical. The time and cost of current sampling procedures effectively limit the observations made on catechin accumulation patterns, without which it is extremely difficult to conclusively attribute allelopathic significance to this compound. Thus, a highly sensitive, fast, inexpensive and reliable method for soil catechin analysis is needed. A novel method is described that utilizes the colorimetric reagent dimethylamino-cinnamaldehyde (DMACA) in an acidic ethanol solution for detection of soil catechin. This method is selective and extremely sensitive and can be used in the field for qualitative, but not quantitative, analysis. This assay will allow for a greater understanding of the role of catechin as an allelochemical.

Introduction

Numerous mechanisms for successful invasion by exotic plant species in novel habitats have been suggested (Shea and Chesson, 2002). One conjecture for the success of invasive species posits that plants that are introduced from a distant region possess phytochemical 'weapons' that are novel, and therefore more detrimental, to pathogens, parasites, or competitors in the introduced range. The novelty of these weapons allows for successful establishment and ultimately range expansion and invasive behavior; this is known as the 'novel weapons' hypothesis (Callaway and Ridenour, 2004). This hypothesis is based in part on results suggesting that the European species *Centaurea maculosa* has gained a competitive advantage in North America partly through use of the novel weapon (+/-)-catechin (Bais et al., 2003).

(+/-)-Catechin is thought to serve an allelochemical function, and this example has been considered one of the strongest for allelopathic behavior in plants (Fitter, 2003). *C. maculosa* root exudates have been found to possess phytotoxic properties, these exudates have been found by chemical analysis to contain catechin, and catechin supplied exogenously was found to inhibit growth and germination at concentrations reported to be exuded from *C. maculosa* under lab and field conditions (Bais et al., 2003). However, there is a great deal of variation in the levels of catechin recovered as root exudate from both lab and field studies, with some groups reporting only trace levels (Bais et al., 2003; Blair et al., 2005; Perry et al., 2005; Thelen et al., 2005; Blair et al., 2006) while others report milligram quantities per gram of soil (Bais et al., 2003; Blair et al., 2005; Perry et al., 2005; Thelen et al., 2005; Blair et al., 2006). Further, a recent study found that soil catechin concentrations can vary from very high to absent from one month to the next

(Perry et al., 2007). The interpretation of these results are complicated by the fact that catechin is a relatively unstable compound, is often found at relatively low concentrations, interacts with soil cations to form insoluble complexes and/or degradation products, and exhibits extremely variable accumulation patterns. This variation in soil catechin concentrations suggests that frequent measurements at multiple sites, monitoring at daily or even hourly intervals, may be required to understand catechin dynamics in *C. maculosa* soils, and thus the role of catechin in *C. maculosa* invasion. However, such a large study would be difficult given the time and cost of soil extraction and analysis by current methods. A faster, less expensive method for soil catechin detection is needed.

Catechins (catechin and its diastereomer, epicatechin) are products of the flavonoid pathway (Tanner et al., 2003; Xie et al., 2003; Xie et al., 2004) and can be either end-products that accumulate under certain conditions or can serve as building blocks for condensed tannins, which are polymers of catechins (Porter, 1989). Condensed tannins have drawn research interest due to physiochemical properties that make them relevant to human health, plant defense, and agriculture (Dixon et al., 2005; Xie and Dixon, 2005). However, condensed tannins are chemically complex and difficult to structurally characterize. These properties necessitated a rapid assay for their presence, a need met by several colorimetric reagents, including the compound dimethylaminocinnamaldehyde (DMACA) (Porter, 1989). This compound is yellow-pigmented, but in the presence of alcohol solvents, strong mineral acid, and condensed tannin or related compounds, binds the tannin and becomes a vivid blue stain. These properties have allowed it to be used for histology (Feucht and Schmid, 1983; Gutmann, 1993; Abrahams et al., 2002), chemical

analysis (Qureshi et al., 1981; Treutter, 1989; Nagel and Glories, 1991; Solich et al., 1996), and structural characterization (Treutter et al., 1994). DMACA recognizes the *meta*-orientated hydroxyls on the A ring, which imparts specificity (McMurrough and McDowell, 1978; Treutter, 1989), and the vivid blue color with maximal absorbance in the long wavelength region of the visible spectrum makes the assay highly sensitive. These properties make it an attractive candidate for studying soil catechin levels in studies of *C. maculosa* allelopathy and the role of catechin in invasion.

This chapter describes a novel assay that employs DMACA for qualitative and semi-quantitative analysis of soil catechin. The method is selective, sensitive, and extremely fast, and can be conducted safely under field conditions. This should allow for more samples to be analyzed, leading to a greater understanding of the regulation of catechin accumulation in soil under field conditions and ultimately for a greater understanding of the importance of catechin as an allelochemical.

Materials and methods

Assay development:

Ethanol was obtained as absolute ethanol, but no attempts were made to remove trace water; thus there is likely to be ~5% water present as an azeotrope. Dimethylaminocinnamaldehyde (DMACA) was obtained from Sigma (# 49825). HCl was fuming (~37%) and obtained from Sigma (# 84436). All reactions were prepared as 200 µL total volume containing 160 µL of HCl solution, 20 µL catechin solution (or soil extract), and 20 µL DMACA solution. All solutions were prepared in ethanol unless

otherwise noted. HCl solutions were prepared as 1, 2, 5, and 10% v/v. Catechin solutions were prepared as a 1000 μg/mL solution, and serially diluted to 100, 10, 1, and 0.1 μg/mL stocks. Catechin and DMACA solutions were diluted ten times (20 μL in 200μL final volume) for assays, resulting in final concentrations of 100, 10, 1.0, 0.1, and 0.01 μg/mL catechin. DMACA was prepared as a 4.0 mg/mL solution and serially diluted to 2.0, 1.0, 0.5, and 0.25 mg/mL. These values represent concentrations before 10-fold dilution into the assay solution. All assays were performed in 96 well plates, and the absorbance at 600nm was used for quantification. Plates were read using a OspysMR plate reader (Dynex Technologies) fitted with a 600nm filter. All reactions were set up with the 96 well plate resting on ice, to minimize variation in incubation start time and evaporative concentration of reagents.

Field samples:

Field collected soil extracts from a previous study were analyzed for catechin content by the DMACA assay to validate the method. Soils were sampled from multiple locations (Table 1), and ~1.3 g soil samples were extracted in 10 mL methanol immediately upon collection, centrifuged and the supernatants concentrated to 0.4 mL (Perry et al., 2007). Each sample was initially diluted ten times to obtain absorbance values in the linear range of the DMACA assay. 20 μL of each diluted sample was added to a well containing 160 μL of 1% HCl in ethanol and 20μL of 4mg/mL DMACA in ethanol. Values obtained using the DMACA method were then compared to those obtained using the standard HPLC protocol described previously (Perry et al., 2007).

Table 1.1. Soil collection site data.

Site	Location	Latitude	Longitude
Canyon Ferry	MT, USA	46.30745° N	111.65037° W
Nelson Gulch 1	MT, USA	46.57040° N	112.14981° W
Clearwater	MT, USA	47.00726° N	113.37319° W
Mt. Sentinel	MT, USA	46.84102° N	113.98251° W
Big Creek	MT, USA	46.45580° N	114.18236° W
Petty Mountain	MT, USA	46.97157° N	114.38408° W
Elko	BC, CAN	49.29157° N	115.12134° W
Gyor	Hungary	47.67809°N	17.63929° E

Soil extraction:

Soil was collected from beneath grasses growing near a *C. maculosa* patch on Mt. Sentinel (Table 1.1). The soil did not directly support growth of *C. maculosa* plants, but was of a similar soil type to that beneath the adjacent *C. maculosa* patch. The soil was air-dried and homogenized before use. 40 g of soil (~50 mL volume) was spiked with zero, five, or 15.0 mg of (+/-)-catechin (Sigma, C1788) for use in extractions. Dry catechin was suspended in two mL distilled water and immersed in a sonicating water bath to break catechin agglomerates into fine particles. The aqueous catechin suspension (catechin is not soluble in water at these concentrations) was added to dry soil. The vials were then rinsed with an additional two mL distilled water and this was added to the soil as well. This resulted in 40 g of soil with four mL of water, allowing realistic soil moisture conditions to be tested. Catechin-free control soil was also moistened with four mL water. The catechin-spiked soil was mixed thoroughly by hand for ten minutes to achieve as homogenous a distribution as possible.

Moistened soil (0.3 g) was transferred to a 2.0 mL eppendorf tube. The spiked soil was then extracted with 1.5 mL of either ethanol, methanol, ethanol + 1% HCl, or methanol + 1% HCl. The samples were extracted by vortexing for 15 seconds. After extraction, the samples were centrifuged for ten minutes at 13,000 rpm with a benchtop centrifuge. 180 μL of the particle free supernatant was then transferred to a 96-well plate. 20 μL of four mg/mL DMACA was then added to each well and two μL of concentrated HCl was added to the ethanol and methanol extraction samples (to achieve the same final HCl concentration). The solution was allowed to develop for 20 minutes at room temperature before measuring absorbance. The absorbance values were compared to those obtained for authentic (+/-)-catechin from 0.0 – 100 μg/μL final catechin concentration. 100% recovery would yield a final concentration of 23.8 μg/mL catechin in the DMACA assay solution for the 15 mg dose, and 7.9 μg/mL for the five mg dose. Percent recovery was calculated as the actual amount recovered divided by the theoretical 100% recovery amount times 100.

To test for the effect of water content of the soil, 10 mg of catechin was suspended in either 0.5 ml, 1.0 mL or 1.5 mL of water. After vortexing and sonicating to create a uniform suspension, the water with catechin was applied to 10 g of soil. The vials were then rinsed with a second volume for a total water content of 1.0 ml/10 g soil (low), 2.0 ml/10 g soil (moderate), or 3.0 ml/10 g soil (high water content). These soils were mixed thoroughly by hand to achieve a uniform catechin distribution. The weight of moistened

soil was adjusted such that each extracted sample contained the same amount of soil and catechin – low samples contained 0.33 g soil + water, moderate contained 0.36 g soil + water, and high contained 0.39 g soil + water. Thus the only change was in the water content, and the total soil and catechin was the same for each sample. The samples were extracted with 1.5 mL of 1% HCl in ethanol by vortexing for 10 seconds and centrifuging for five minutes. 180 μL of this solution was added to 20 μL of 4 mg/ml DMACA in ethanol, allowed to develop for 20 minutes, and the absorbance measured at 600 nm. 0.75 mL of the remaining extract was reserved for HPLC-MS analysis. HPLC-MS analysis was conducted on a Dionex system composed of P680 pump, and ASI-100 autosampler, and a PDA100 photodiode array detector. This was coupled to a Thermo Finnigan Surveyor MSQ mass spectral detector. Separation was performed on a Dionex Acclaim 120 C18 column (5μm, 4.6 x 150 mm) using gradient elution. Solvent A was water + 0.1% v/v acetic acid and solvent B was methanol + 0.1% acetic acid. Compounds were eluted at a 0.7 mL/min flow rate for 3 minutes at 10% B, a linear gradient to 90% B over 40 minutes, and held at 90% B for 8 minutes. UV detection was recorded from 200-800nm. Ionization for MS analysis was performed in both positive and negative ion mode using elecrospray ionization with a nitrogen flow at 80 psi, a cone voltage of 70V, needle voltage of 3 kV, and sheath temperature of 600°C. Mass data was collected over the range of the gradient program at a rate of one scan per 2 seconds.

Statistical analysis:

Raw absorbance values were log transformed for both graphical display and analyses of variance (ANOVA). ANOVA and Tukey's HSD post-hoc comparisons were performed in JMP v 5.1.2 (SAS Institute, Cary, North Carolina, USA).

Results

Assay development:

An alcohol solution of DMACA is yellow in color, and changes to blue in color in the presence of catechins. To guide the assay protocol development, the UV-Visible absorbance spectrum was acquired for the reagent solution in the presence and absence of catechin. This experiment revealed a large absorbance peak in the presence of catechin at 640nm which was not present in the absence of catechin (Fig 2.1). Though monitoring at 640 nm would provide optimal sensitivity, a plate reader equipped with a 600 nm filter – absorbance was used, and absorbance is only approximately 50% maximal at this wavelength. A laboratory capable of monitoring at 640nm could increase the sensitivity of this assay further. All subsequent assays are quantified using 600nm absorbance. The DMACA solution in the absence of catechin has absorbance values at 600nm that are indistinguishable from solvent controls that did not contain DMACA.

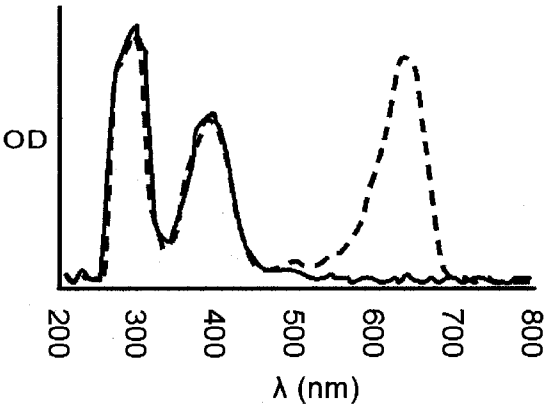


Fig 2.1. UV-visible light absorption spectrum for DMACA catechin assay solution (dashed line) of 8 parts 5% HCl in ethanol (v/v), 1 part 2.0 mg/mL DMACA in ethanol, and 1 part 100 μg/mL (+/-)-catechin hydrate in ethanol. The solid line represents the same solution, except pure ethanol replaced catechin solution. Wavelength is depicted on the x-axis while absorbance is depicted on the y-axis.

Several DMACA concentrations were examined to determine maximal sensitivity, taking into consideration sensitivity as measured both by spectrophotometric readings at 600nm and visible examination. All DMACA concentrations tested demonstrated response linearity between 0.01 and 10 μg/mL final concentration, a dynamic range of four orders of magnitude (Fig 2.2a). Visible sensitivity was slightly less, with 0.1 μg/mL of catechin being the minimum concentration reliably differentiated from the catechin-free control. Catechin concentrations of 0.01 μg/mL could often be distinguished from controls, but with less confidence. Visible sensitivity was greater when higher concentrations of DMACA were used. For subsequent assays, 20 μL of a 4.0 mg/mL DMACA stock solution was used in a reaction solution of 200 μL.

HCl is highly hazardous, and minimization of HCl levels will allow for a safer field assay. HCl concentrations were systematically varied to minimize its concentration while retaining sensitivity. All HCl tested concentrations demonstrated strong response linearity and at all HCl concentrations the lowest catechin concentration tested (0.01 μg/mL) was clearly distinguishable from catechin-free controls (Fig 2.2b). However, lower HCl concentrations (1% HCl v/v) gave slightly but consistently higher absorbance readings than did higher (5% and 10% HCl v/v) concentrations. 1% HCl was used for further assays.

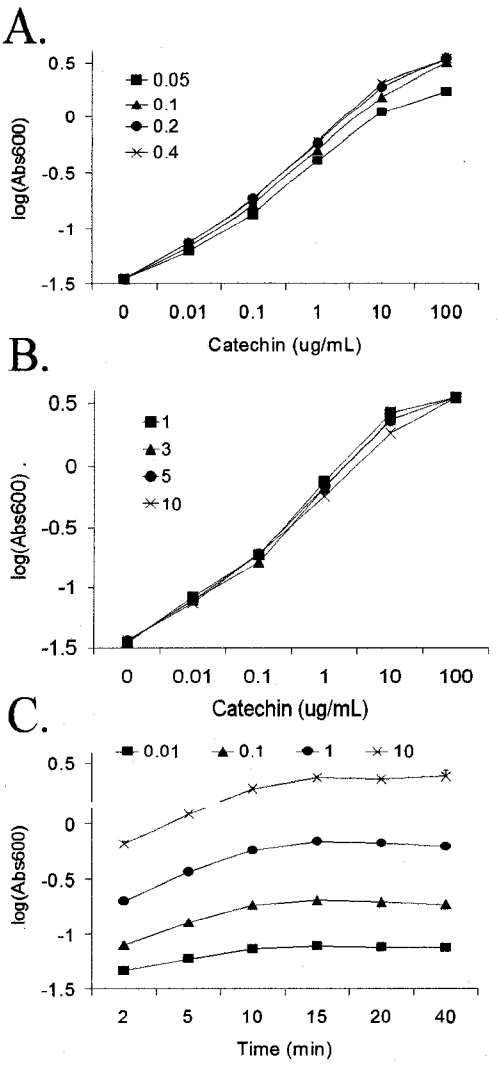


Fig 2.2 Assay development for maximal sensitivity: *a)* DMACA and *b)*HCl were adjusted to select optimal assay conditions. *a)* All DMACA concentrations (0.05 – 0.4 mg/mL) tested were sensitive enough to distinguish 0.01 ug/mL of (+/-)-catechin from the catechin free control, and all concentrations were distinguishable from each other. DMACA concentrations of 0.2 and 0.4 mg/mL are more sensitive than 0.1 and 0.05 mg/mL at all catechin concentrations except the catechin free control. Visible sensitivity was greatest at higher DMACA concentrations (0.4 and 0.2 mg/mL), at which even the lowest catechin concentrations were often visually distinguished from catechin free controls. *b)* All catechin concentrations were distinguishable from catechin-free controls at all HCl concentrations (1-10%, *v/v*). 1% HCl was more sensitive than higher concentrations at concentrations from 0.01 to 10 µg/mL. *c)* Color development was followed over the course of 40 minutes. Development is essentially complete at 15 minutes, and absorbance begins to fall slightly after 40 minutes. Different symbols indicate different (+/-)-catechin concentrations as described by the legends. Error bars represent standard error, but are often concealed beneath the symbols. $n = 4$ for all data points.

The DMACA colorimetric reaction begins to develop immediately, but is not complete for some time. To provide data to infer the minimal and optimal incubation time, an experiment was performed that monitored the development of absorbance at 600nm over time at the four intermediate catechin concentrations (0.01 – 10.0 μg/mL) in a solution of 1% HCl and 4% DMACA. The reaction was performed at room temperature (22°C) and monitored for 40 minutes. The data demonstrated that the absorbance is maximal at approximately 15-20 minutes, and thereafter begins to decay very slightly (Fig 2.2c). Over longer time frames (>12 hours), the blue pigmentation fades and a red coloration begins to develop (not shown). Thus for quantitative purposes, optimal sensitivity is achieved between 15 and 20 minutes, though this time would likely decrease with increasing temperature. The subtle decay after 20 minutes is not visibly detectable, thus visual examination for qualitative purposes can be reliably scored between 15 minutes and 2 hours.

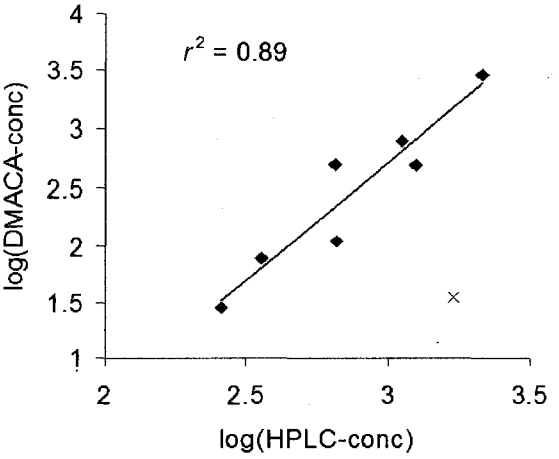
Assay validation and extraction efficiency:

To test whether the laboratory results would translate to a successful field assay, samples that had previously been prepared from field collected samples from *C. maculosa* patches from multiple soils were tested (Perry et al., 2007). These included samples previously demonstrated to contain catechin and those that did not. This allowed for a test of the sensitivity of the assay, to compare the quantitative results with those previously obtained using a more established HPLC-based method, and to determine whether other soil components may cross react with the reagent to generate false positive results. All samples that were found to contain no catechin as previously determined by HPLC

analysis demonstrated absorbance values at 600 nm below those for the lowest standard catechin concentration used (< 0.01 μg/mL – data not shown). These samples were collected from numerous sites with diverse soil properties (Table 1.1), demonstrating that false positive results are likely to be rare.

All samples that were found to contain catechin by HPLC analysis were also found to contain catechin by DMACA detection. The quantitative results as calculated by detection with DMACA and compared to a catechin dose-response curve were in general agreement with the HPLC results, but the exact quantities did deviate from the HPLC results, in one instance rather dramatically (Fig 2.3). These differences are likely due to components of the soil extract which interfere with the colorimetric reaction. It is currently unclear what those components might be.

Fig 2.3. Quantitative results obtained by using HPLC (x-axis) and DMACA (y-axis) are compared. The DMACA method was applied to previously collected field soil methanol extracts. Plotting the quantities obtained with each method demonstrates strong agreement, with the exception of the sample represented by the 'X' symbol. If this sample is included, the r^2 values drops to 0.36. Note also that the slope of the line is not 1; at lower concentrations, the DMACA method underestimates catechin concentration. The line through the scatterplot represents the linear regression, excluding the 'X' symbol data from the analysis.



Catechin extraction efficiency was then tested from field collected soils. 40 g of air-dried soil was spiked with either 0, 5, or 15 mg of (+/-)-catechin, moistened with four mL of water, mixed thoroughly, and subsamples extracted with one of four alcohol-based solvent systems: ethanol, ethanol + 1% HCl, methanol, or methanol + 1% HCl. Catechin-free samples were used to estimate the background absorbance generated by extraction with each solvent system. Inclusion of HCl in the methanolic or ethanolic extraction increased the color of the extract, both visually and as measured by absorbance at 600 nm (Fig 2.4a). This generated a brown solution, which did not appear blue visually, thus is not likely to be confused as a positive catechin test. Ethanol + 1% HCl was more effective for extracting catechin than was ethanol alone, but less effective than methanol (Fig 2.4b). Methanol is slightly more effective than ethanol at higher concentrations (15 mg catechin per 40 g soil), but less effective at lower concentrations (5 mg per 40 g soil), and methanol + HCl is more effective than is ethanol + HCl at lower concentrations, but not at higher concentrations. In all cases, extraction efficiency was low, with maximal values of ~5%.

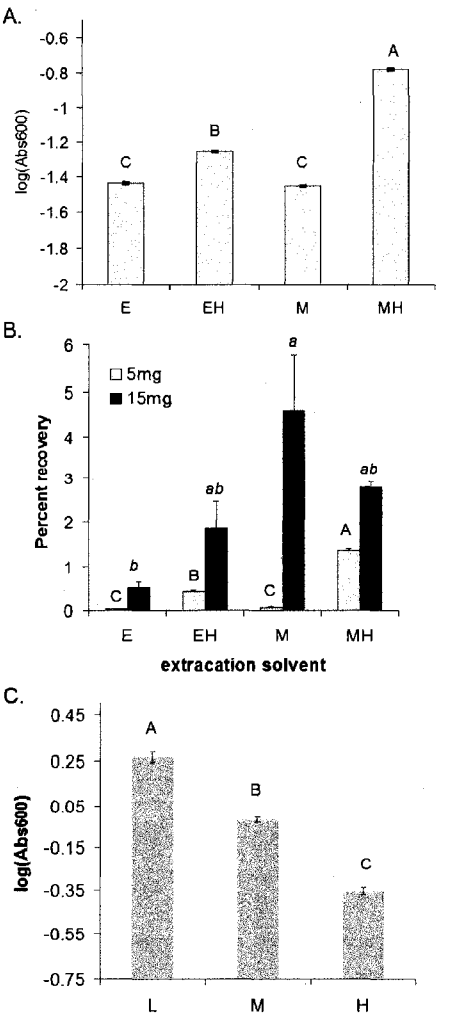


Fig 2.4. Comparison of solvents on extraction and detection of catechin. *a)* Comparison of absorbance at 600 nm when extracting from catechin-free soil with ethanol (E), ethanol + 1% HCl (EH), methanol (M), or methanol + 1% HCl (MH). Error bars represent standard error. Bars labeled with the same letter are not significantly different (Tukey's HSD, $p < 0.05$). *b)* Effect of various solvents on extraction efficiency – labeled as in part *a*. *c)* Increased soil water content results in reduced DMACA development in a manner that is dose dependent. Low (L - 1 mL per 10 g soil), moderate (M - 2 mL per 10 g soil) and high water content (H – 3 mL per 10 g soil).

Field soil moisture conditions vary considerably seasonally, from day to day and within a day. It is therefore informative to compare the extraction efficiency and reagent sensitivity under different soil water conditions. Soil spiked with catechin was wetted to low, moderate, or high water content (one, two, or three mL water per ten g soil) and a sample of the soil was extracted with 1.5 mL 1% HCL in ethanol. 180 µL of this extract was transferred to a 96 well plate and mixed with 20 µL four mg/mL DMACA in ethanol. The absorbance at 600 nm demonstrated less color development with higher soil water content (Fig 2.4*c*). HPLC analysis of the same extracts (not shown) demonstrated that the reduced absorbance at 600 nm was due not to reduced extraction efficiency, but to reduced color development in the presence of higher water content, as previously demonstrated (McMurrough and McDowell, 1978; Treutter, 1989).

Discussion

This novel method offers a rapid, highly sensitive qualitative assay for analysis of soil catechin under field conditions. The assay is somewhat sensitive to soil water content and interference from unknown soil components, thus quantitative results based solely on absorbance at 600nm should be viewed with some caution. At this time, I must conclude that use of an HPLC should be considered the superior method for quantitative results, as separation helps to reduce interference.

Catechin extractions from soil are typically performed with methanol or acetone. Acetone has been previously shown to inhibit color development (Treutter, 1989).

Methanol is compatible with DMACA reaction conditions, but is relatively toxic – a poor trait for an assay with potential use under field conditions. Ethanol was found to offer similar extraction efficiency and serves as a relatively non-toxic assay solvent. Likewise, HCl is toxic at high concentrations, and its concentration was adjusted to reduce toxicity while retaining assay sensitivity. The results from these tests suggest that ethanol + 1% HCl offers the best option, balancing extraction efficiency at both high and low concentrations, DMACA color development for maximal sensitivity, and safety and environmental concerns. DMACA itself is relatively non-toxic, and maximal sensitivity was found at higher concentrations (a final assay concentration of 0.4 mg/mL working from a 4.0 mg/mL stock solution).

Extraction efficiency of catechin from soils is low under all conditions tested. This has been previously observed for alcohol-based extraction methods in Montana soils (Blair et al., 2005), and the results obtained for the DMACA study are in general agreement with the previous reports. DMACA is reliant on alcohol as a reaction solvent and the reaction is sensitive to water, thus the acetone/water solvent system (approximately 33% extraction efficiency in soils similar to those used for this study) previously described (Blair et al., 2005) is infeasible. The poor extraction efficiency of catechin from soils could be due to chelation by catechin of di- or tri-valent cations and/or pH sensitive degradation. Previous reports describe significantly higher recovery from dry soil and from sand (Blair et al., 2005), suggesting that catechin in aqueous solution either degrades or becomes resistant to alcohol-based extraction due to interaction with soil components. These recovery issues are potentially limiting when absolute quantitation is

necessary and a relatively insensitive assay is used. However, the sensitivity of the DMACA assay allows for reliable detection despite the poor extraction efficiency of alcohol-based solvents. For example, catechin concentrations of 50 μg/g soil with an extraction efficiency of 1% would generate a solution concentration of 0.1 μg/mL – a concentration that is readily detected through either use of a spectrophotometer or visual examination. In fact levels 10 times lower than this are still readily detected by a spectrophotometer and can often be distinguished visually. For comparison, recent studies indicate that when catechin is present, it is present at levels between 140 and 2150 μg/g soil (Perry et al., 2007), indicating that levels of catechin found under field conditions can be detected using the DMACA method, despite low extraction efficiency.

For field assays, a freshly prepared solution of 1% HCl in ethanol and 4.0 mg/mL DMACA in ethanol is recommend. A small amount (< 0.5 g) of soil should be added to a 2.0 mL eppendorf tube containing 1.5 mL of acidic ethanol extraction solution. The soil extract should be vigorously shaken, and allowed to extract for ~ 10 minutes while shielded from light; during this time soil particles will fall out of solution if left undisturbed. 450 μL of this solution can be transferred to a new tube, and 50 μL of the DMACA solution added to the soil extract (volumes can be adjusted as necessary, so long as the proportions remain the same). High catechin concentrations will generate a visible blue color almost instantly, and maximal sensitivity will typically be achieved within 20 minutes. The remaining soil extract can be transported back to the lab if the DMACA assay demonstrates a positive test and the remaining soil, or an adjacent soil sample, extracted more thoroughly using previously established methods if precise and

absolute quantitation is desired. Alternatively, the entire reaction solution (1% HCl and 0.4mg/mL DMACA in ethanol) could be used as an extraction solvent. Previous reports indicate that a solution of DMACA in acidic alcohol is stable for up to a week if kept in the dark (McMurrough and McDowell, 1978; Treutter, 1989); thus containers in which this solution is stored should be kept from light. In this instance, there is no need to transfer from tube to tube; the solution will develop as the catechin extraction proceeds. In this case, a second soil sample should be collected for subsequent HPLC analysis, as the stability of catechin as a DMACA conjugate is unknown.

This method will allow for a rapid screen to guide the sampling process and allow for increased sampling that may elucidate the factors that affect soil catechin levels. The unpredictability of the presence and quantity of catechin may be due to a lack of knowledge of the regulation of its temporal production or secretion or factors influencing its degradation in soils. This efficient and rapid sampling method will allow for greater sampling regularity and more diverse sampling sites. The data generated using this method may help to clarify the regulation of catechin production by *C. maculosa* and potentially clarify the role of this putative allelochemical in *C. maculosa* invasion.

3. **Cloning and biochemical characterization of Centaurea maculosa dihydroflavonol reductase.**

Summary

Allelopathy is implicated in the successful invasion of North America by the Eurasion aster species, *Centaurea maculosa*. The allelochemical implicated in this trait is a racemic mixture of (+/-)-catechin, a product of the flavonoid pathway. The cloning of the dihydroflavonol reductase (DFR) gene from *C. maculosa* cDNA is reported. The gene encoding DFR produces an enzume that participates in all characterized biochemical pathways leading to catechin or epicatechin, and therefore a necessary enzyme in the allelopathic behavior of *C. maculosa*. The *C. maculosa* DFR sequence is compared to other plant DFR sequences at the nucleotide and amino acid levels. I demonstrate its activity through heterologous expression in bacterial cells, followed by biochemical assays and chemical characterization of the reaction product. Gene expression studies indicate that this gene is expressed at undetectable levels in roots, at low levels in rosette leaves, and at highest levels in inflorescence parts. These results suggest that the putative allelochemical, catechin, is not produced under the growth conditions used for the gene expression study.

Introduction

Centaurea maculosa is an invasive weed introduced from Eurasia that is particularly problematic in western North America. Allelopathy has been implicated in the successful establishment and spread of *C. maculosa* (Kelsey and Locken, 1987; Ridenour and Callaway, 2001) and its congener *C. diffusa* in North America (Callaway and Aschehoug, 2000). In *C. maculosa*, a racemic mixture of (+/-)-catechin has been identified as the

active allelochemical (Bais et al., 2002; Bais et al., 2003). However, recovery of catechin under field and laboratory conditions is highly variable (Blair et al., 2006; Perry et al., 2007), leading some to question the relevance of catechin as a mechanism of invasion variable (Blair et al., 2006; Perry et al., 2007).

Catechin and its diastereomer, epicatechin, are flavan-3-ols which differ only in the stereochemistry of the 2,3-positions (*cis* for epicatechin, *trans* for catechin). These compounds can be conjugated to other small molecules, such as gallic acid or sugars or polymerized to form proanthocyanidins, or condensed tannins (Dixon et al., 2005). Many compounds derived from (epi)catechin possess health-beneficial, agronomic, and economic qualities, which drives research into understanding the biosynthetic, regulation, and in-plant functions of these metabolites.

Recent research has revealed two pathways for the biosynthesis of catechins (Tanner et al., 2003; Xie et al., 2003). Both pathways are derived from the flavonoid pathway, branching after enzymatic conversion of dihidroflavonols to leucoanthocyanins by the product of the dihidroflavonol reductase (DFR) gene. The first pathway characterized converts cyanidin (an anthocyanin converted from leucocyanidin by anthocyanidin synthase) to epicatechin in an NADPH-dependent reduction reaction catalyzed by anthocyanidin reductase (Xie et al., 2003; Xie et al., 2004). An alternative pathway was described which directly converts leucoanthocyanidin to catechin in an NADPH-dependent reaction catalyzed by leucoanthocyanidin reductase (LAR) (Tanner et al., 2003). The gene encoding LAR was originally cloned from the legume, *Desmodium*

uncinatum. There is no apparent LAR in *A. thaliana*, and when a gene with high sequence similarity to LAR in *M. truncatula* was down-regulated by antisense expression there was no effect on tannin accumulation, raising questions as to its function in *M. truncatula* (Pang et al., 2007). It is thus unclear which pathway will be dominant in a given species.

Both of the characterized genes involved in the ultimate conversion to catechin or epicatechin are downstream of DFR, the enzyme responsible for the synthesis of leucocyanidin. In an attempt to improve our understanding of catechin synthesis in *C. maculosa*, *C. maculosa* DFR was cloned from cDNA, evidence of its function through heterologous expression was demonstrated, and gene expression patterns were reported in *C. maculosa* plants.

Materials and Methods

Plant material.

C. maculosa seeds were obtained from randomly selected plants from around Missoula, MT. Seeds were surface sterilized for 10 minutes in 20% bleach solution containing 0.1% SDS as a surfactant before planting. For tissue specific expression analysis, plants were grown in 6-cm x 6-cm pots containing sand as the growth medium, fertilized with Osmocote (Outdoor & Indoor Smart Release Plant Food – 19-6-12 formulation), and harvested after 10 weeks of growth under greenhouse conditions. Dissected tissues were rapidly frozen in liquid nitrogen and held at -80°C for later use in RNA extractions.

PCR amplification of *C. maculosa* DFR

Total RNA was harvested from dissected plant tissues using TRIzol reagent (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions, including all optional steps. All PCR reactions were conducted in 20-μL reactions using Taq polymerase (Takara, Madison, WI, USA). cDNA was synthesized from RNA samples using the Invitrogen SuperScript III cDNA synthesis kit.

Degenerate primers were designed based on conserved regions of nucleotide and protein DFR sequences from other aster species. Primers used for the initial fragment were CmDFRF2 (5' CCACCCCTATGGACTTTGAA 3') and CmDFRR2 (5' CACCGCTGATCAAGGAGAGT 3'). PCR was performed on an Applied Biosystems thermal cycler (GeneAmp PCR system 2700, Foster City, CA, USA) using one cycle of 120 second at 94°C, followed by 32 cycles of 94°C for 45 seconds, 46°C for 60 seconds, and 72°C for 120 seconds, and a single cycle of 120 seconds at72°C. This product was cloned and sequenced using the Invitrogen TOPO TA cloning kit and used to design internal primers for 3' RACE. A primer internal to the original fragment was used with a Poly(dT) primer to amplify the 3' end of the transcript as described (Sambrook and Russell, 2001). The 5' end of the transcript was amplified from primers designed against *C. maculosa* DFR transcripts present in NCBI databases as part of an EST project (NCBI accession numbers EH726779.1, EH732292.1, and EH733520.1). Primers were then designed to amplify the full length gene (forward 5' ATGGTACAGAATTCTCCAACCACCG 3'; and reverse 5' TTATTGGTACAATGAGACATAAATGTCTTTG 3'). The PCR product of the full

length cDNA was cloned into the TOPO TA vector system (Invitrogen) and resequenced. The complete *C. maculosa* dihydroflavonol reductase (CmDFR) coding sequence has been deposited in the NCBI database (FJ376591).

For tissue specific gene expression studies, a 600-bp fragment of CmDFR was amplified from cDNA prepared from C. maculosa tissues using the primers 5'-GCGGAACATGAAGAAAGTGA-3' and 5'- TGGCTTTCACAAAGATCATCC -3'. As a control, β-actin was amplified with the primers 5'-GATATGGAAAAGATCTGGCATCAC-3' and 5'-TCATACTCGGCCTTGGAGATCCAC-3'. PCR was performed using a program consisting of initial three minutes at 94°C, followed by 25 cycles of 94°C for 60 seconds, 57°C for 60 seconds, and 72°C for 120 seconds, and a single final ten minute extension period at 72°C. Actin and DFR amplification were performed in separate 20-μL reactions using Taq polymerase (Takara, Madison, WI, USA).

Analysis of CmDFR sequence

Clustal W and phylogenetic analysis were perfomed using MEGA 4 (Tamura et al., 2007). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 1.073 is shown. The phylogenetic tree was linearized assuming equal evolutionary rates in all lineages (Takezaki et al., 2004). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkankl

and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option).

Heterologous expression of CmDFR

Full-length CmDFR was PCR amplified from the full-length clone contained in the TA vector using primers (5'- TATGCATGCATGGTACAGAATTCTCCAACCACCG-3' and 5'- TATCCCGGGTTATTGGTACAATGAGACATAAATGTCTTTG-3') containing restriction sites for subcloning into an expression vector. This PCR product was inserted into the TA vector for propogation, and this plasmid and pQE30 bacterial expression vector was digested with XmaI and SacI (New England Biolabs, Ipswitch, MA). The pQE30 expression vector was ligated to digested CmDFR using T4 DNA ligase (New England Biolabs, Ipswitch, MA, USA). The ligated plasmid was then transferred to chemically competent *E. coli* M15 cells via heat shock, with positive transformants selected by growth on LB (Luria-Bertani) agar plates containing 25 μg/mL kanamycin and 100 μg/mL ampicillin. Plasmid was isolated from positive cultures, and restriction digested to ensure insertion of CmDFR. Cells were grown in LB containing selection antibiotics overnight, and 100 μL of overnight culture was transferred to 10 mL LB with antibiotics. This culture was then grown at 30°C while shaking until culture density reached 0.5 OD. 1.0 M IPTG was added to a final concentration of 1.0 mM, and the culture was incubated overnight at 25°C.

Total cell lysate was used to confirm DFR biochemical activity of heterologously expressed CmDFR (Xie et al., 2004). A two mL IPTG-induced culture was centrifuged at 5000g at 4°C for 15 minutes and the supernatant was decanted. The cell pellet was resuspended in 1.0 mL of lysis buffer (100 μg/mL lysozyme, 100 mM Tris-HCl, 5 mM EDTA, pH 8.0), and the cells were lysed by sonicating on ice for 10-15 seconds. The cell debris was removed by centrifugation and the supernatant used in DFR activity assays.

DFR activity assays consisted of 370 μL of TRIS-HCl (pH 7.0), 70 μL of crude protein from IPTG induced *E. coli* cultures, 50 μL of 10mM NADPH, and 10 μL of 10mg/mL (+/-)-dihydroquercetin (DHQ, or taxifolin, Sigma-Aldrich, St. Louis, MO, USA) in methanol (Xie et al., 2004). The reaction was allowed to proceed for 30 minutes at room temperature, after which 750μL of ethyl acetate was added to stop the reaction and extract the leucoanthocyanidin product. The upper ethyl acetate layer was collected, and the aqueous phase was extracted a second time with ethyl acetate. The two ethyl acetate fractions were pooled, evaporated under nitrogen, and resuspended in 100μL of methanol for analysis by HPLC-MS. For the DFR activity assays, negative controls included an empty pQE30 vector, cell culture containing CmDFR in pQE30 uninduced with IPTG, no protein, boiled protein, no NADPH cofactor, and no dihydroquercetin substrate.

HPLC-MS analysis was using a Dionex HPLC system (P580 pump, ASI-100 autosampler, PDA100 photodiode array detector – Dionex, Sunnyvale, CA) coupled to a Surveyor MSQ single quadrupole mass spectrometer (Thermo Electron, Waltham, MA). Separation was performed on a C18 reverse phase column (Dionex, 250 x 4.6 mm, 5μM)

separated on a linear gradient of 0–3 min, 90% A; 10–90% from 3–43 min; 43–51 at 90% B, with solvent A consisting of 0.1% acetic acid in purified water, and solvent B consisting of 0.1 acetic acid in Methanol. The mass spectrometer was operated in full scan positive ion mode from *m/z* 100-1000.

Results

Centaurea maculosa dihydroflavanol reductase (CmDFR)

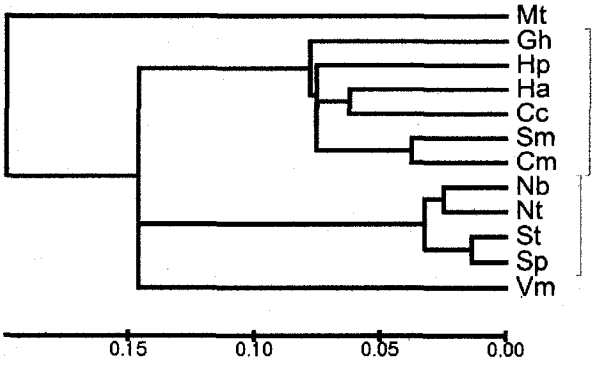
Degenerate primers were used to amplify a fragment of DFR from cDNA derived from *C. maculosa* leaf tissue. The 3' end of the cDNA was obtained using 3' RACE and the 5' end of the gene was obtained using primers designed from a recently released *C. maculosa* EST sequence project deposited in NCBI. The full length gene contains 1035 base pairs, including the stop codon, which is translated to a 344 amino acid protein with a molecular weight of approximately 39 kD. This DFR protein shows very high sequence similarity to DFR proteins from other aster species (Fig 3.1a). The putative NADPH–binding domain (Fig 3.1a, upper boxed region) is extremely highly conserved, as is the substrate recognition sequence (Fig 3.1a, lower boxed region). The most similar sequence in the NCBI database is a DFR sequence from the aster *Saussurea medusa*. The aster family is contained within the Asteridae subclass, along with families including Solanaceae and Ericaceae. DFR sequences from these families share high sequence similarity with CmDFR, and the results from ClustalW analysis of these sequences reflects the phylogenetic relationships within and between families (Fig 3.2). The N-terminus is more highly conserved than the C-terminus, as has been observed for two DFR genes from *Medicago truncatula* (Xie et al., 2004).

Fig 3.1. Cloning of DFR

Sequence alignment of *Cm*DFR with select aster sequences. The amino acid sequences for DFR proteins from Asteraceae species, *Vitis vinifera* and *Medicago truncatula* were obtained from NCBI. Clustal-W alignment was performed as described in the materials and methods. Cm – *Centaurea maculosa,* FJ376591; Sm - *Saussurea medusa,* gi148628025; Ha - *Helianthus annuus*, gi156708231;Vv – *Vitis vinifera* - Protein Data Bank with Entry 2c29; Mt - *Medicago truncatula*, gi38683951. Upper boxed region represents NADPH binding domain, lower boxed region represents the substrate specificity region.

```
Cm  MVQNSPTTVCVTGAAGFIGSWLVMRLLERGYIVRATVRDPGNMKKVKHLIQLPKAETNLT 60
Sm  MVQDSPTTVCVTGAAGFIGSWLVMKLLQRGYTVRATVRDPGNMKKVNHLIQLPKAKTNLT 60
Ha  MKEDSPATVCVTGASGFIGSWLVMRLLERGYIVRATVRDPGDMKKVKHLKELPGAETNLT 60
Vv  MGSQS-ETVCVTGASGFIGSWLVMRLLERGYTVRATVRDPTNVKKVKHLLDLPKAETHLT 59
Mt  MGSMA-ETVCVTGASGFIGSWLVMRLMERGYMVRATVRDPENLKKVSHLLELPGAKGKLS 59
    * . :  *******:*********:*::*** ******** ::***.** :** *: :*:

Cm  LWKADLTQEGSFDEAVEGCHGVFHVATPMDFESKDPENEIIKPTIQGVLSIIRSCVKAKT 120
Sm  LWKADLTQEGSFDEAIEGCHGVFHVATPMDFESKDPENEIIKPTIEGVLSIIRSCVKAKT 120
Ha  LYKADMTQEGSFDEAIEGCHGVFHVATPMDFESKDPENEIIKPTVQGVLSIVRSCAKAKT 120
Vv  LWKADLADEGSFDEAIKGCTGVFHVATPMDFESKDPENEVIKPTIEGMLGIMKSCAAAKT 119
Mt  LWKADLGEEGSFDEAIKGCTGVFHVATPMDFESKDPENEMIKPTIKGVLDIMKACLKAKT 119
    *:***: :********::** ********************:****::*:*.*:::*  ***

Cm  VKKLVYTSSAGTVNVEEHQLPVYDESNWSDLDFIYSKKMTAWMYFVSKTLAEKAAWKATT 180
Sm  VKKLVFTSSAGTVNVQEHQLPVYNELDWSDLDFIYSKKMTAWMYFVSKTLAEKAAWEATK 180
Ha  VKKLVFTSSAGTVNVQEKQLPVYDESHWSDLDFINSQKMTAWMYFVSKTLAERAAWKAAI 180
Vv  VRRLVFTSSAGTVNIQEHQLPVYDESCWSDMEFCRAKKMTAWMYFVSKTLAEQAAWKYAK 179
Mt  VRRFIFTSSAGTLNVTEDQKPLWDESCWSDVEFCRRVKMTGWMYFVSKTLAEQEAWKFAK 179
    *:::::******:*: *.* *:::*  ***::*    ***.***********: **: :

Cm  ENNIDFISIIPTLVVGPFITPSFPPSLITALSLINGAESHYSIIKQGQYVHLDDLCESHI 240
Sm  ENNIEFISIIPTLVVGPFITPSFPPSLITALSLINGAESHYSIIKQGQYVHLDDLCECHI 240
Ha  ENNIDFISIIPTLVVGPFITPIFPPSLITALSLINGMESHYSIIKQGQFVHLDDLCECHI 240
Vv  ENNIDFITIIPTLVVGPFIMSSMPPSLITALSPITGNEAHYSIIRQGQFVHLDDLCNAHI 239
Mt  EHNMDFITIIPPLVVGPFLIPTMPPSLITALSPITGNEAHYSIIKQGQFVHLDDLCEAHI 239
    *:*::**:***.******: . :********* *.* *:*****:***:*******:.**

Cm  YLYEHPKATGRYICSSHDATIHQLAKMLKEKWPEYHIPNQFPGIDKEPPIVSFSSKKLTE 300
Sm  YLYENPKAKGRYICSSHDATIHQLARMMKKKWPEYHVPNQFPGIDKEPPIVSFSSKKLTE 300
Ha  FLYENPEAKGRYICSSHDATIHQLARMIKEKWPEYHVPTQFVGIDEDIPVVSFSSKKLTD 300
Vv  YLFENPKAEGRYICSSHDCIILDLAKMLREKYPEYNIPTEFKGVDENLKSVCFSSKKLTD 299
Mt  FLFEHMEVEGRYLCSACEANIHDIAKLINTKYPEYNIPTKFNNIPDELELVRFSSKKIKD 299
    :*:*: :. ***:**: :. * ::*::::. *:***::*.:* .: .:   * *****:.:

Cm  MGFEFKYNLEDMFKGAIDSCREKGLLPYSTIKNHKKEK----DDYFHV------- 344
Sm  MGFEFKYDLEDMFKGAIDSCREKGLLPYSTIKNHKK------DDHIHV------- 342
Ha  MGFEFKYNLEEMFKGAIDCCREKGLLPYSTNEVKKRLITSPINDHVQGQECKVAN 355
Vv  LGFEFKYSLEDMFTGAVDTCRAKGLLPPSHEKPVDGKT----------------- 337
Mt  LGFEFKYSLEDMYTEAIDTCIEKGLLPKFVKSTNK------------------- 334
    :******.**:*:. *:* *  *****    . .
```

Fig 3.2. DFR sequences reflect familial phylogeny. DFR sequences from the Asteridae subclass were aligned in MEGA, and the phylogenetic relationships ascertained as described in the materials and methods. The upper bracket contains all DFR sequences from members of the aster family, the lower bracket contains sequences from solanaceous species, and the bottommost species (Vm) represents a species from the family Ericaceae. *Medicago truncaulta* DFR serves as a rooted outgroup. Cm – *Centaurea maculosa*, gi_____; Sm - *Saussurea medusa*, gi148628025; Hp - *Hieracium pilosella*, gi171906246; Cc - *Callistephus chinensis*, gi1066451, Ha - *Helianthus annuus*, gi156708231; Gh - *Gerbera hybrid cv.* 'Terra Regina', gi312777; St - *Solanum tuberosum*, gi21666730; Nb - *Nicotiana benthamiana*, gi126211541; Nt - *Nicotiana tabacum*, gi164454779; Mt - *Medicago truncatula*, gi38683951; Sp - *Solanum pinnatisectum*, gi62112681; Vm - *Vaccinium macrocarpon*, gi19526438



Biochemical activity of CmDFR

The full length cloned CmDFR gene was expressed in *E. coli* under control of an IPTG-inducible promoter. The crude protein from induced empty-vector and CmDFR cell cultures was used in DFR activity assays. After incubating protein in the presence of dihydroquercetin and NADPH, one major and one minor peak were apparent using HPLC-MS analysis. These peaks did not appear when DHQ and NADPH were incubated with IPTG-induced empty vector control protein preparations, or when either DHQ or NADPH was omitted from the incubation (Fig 3.3a) in the presence of CmDFR. The mass spectrum of the early eluting major peak contained m/z at 289, 307, and 329, which are consistent with a CmDFR reaction product of leucoanthocyanidin ($[M-H_2O+H]^+$, $[M+H]^+$, $[M+Na]^+$, respectively, Fig 3.3b). Treatment of the CmDFR reaction product with HCl, resulted in a novel peak which eluted later than DHQ substrate, coeluting with the anthocyanin, cyanidin (Fig 3.3c, top panel). Both the acid-treated CmDFR product and cyanidin posses a λmax at540 nm and a mass spectrum demonstrating a prominent ion at m/z 287 (Fig 3.3c, middle and bottom panels). These data establish CmDFR as an enzyme capable of using DHQ as a substrate in the presence of NADPH to produce leucoanthocyanidin.

Fig 3.3. Biochemical activity of in-vitro expressed DFR. *a.* CmDFR in, produces a major and minor product that is dependent on the substrate DHQ and the cofactor NADPH. The panel labeled 'DFR' contained intact protein, NADPH, and DHQ. Incubation of induced empty vector controls (EV) produced no product. Elimination of either NADPH or DHQ (bottom panels) yielded no product. In all panels, x-axis represents time (min) and y-axis represence absorbance at 280nm. *b.* Mass spectrum of CmDFR product suggests leucoanthocyanidin. The molecular weight of leucoanthocyanidin is 306, consistent with $(M+H)^+$ and $(M+Na)^+$ at *m/z* 307 and 329, respectively. The base peak of the spectrum is an ion representing $(M+H-H_20)^+$ at *m/z* 289. *c.* Acid treatment of the DFR product produces cyanidin. Acid treated DFR product coelutes with authentic cyanidin on HPLC with detection at λ540 nm detection (top left panel), shares the same λ_{max} as cyandin (top right panel, small bump at λ450 is from unreacted DHQ), and has the same molecular ion as cyanidin using positive mode MS anlaysis (bottom panels, Cyanidin-left, acid treated DFR product-right).

a.

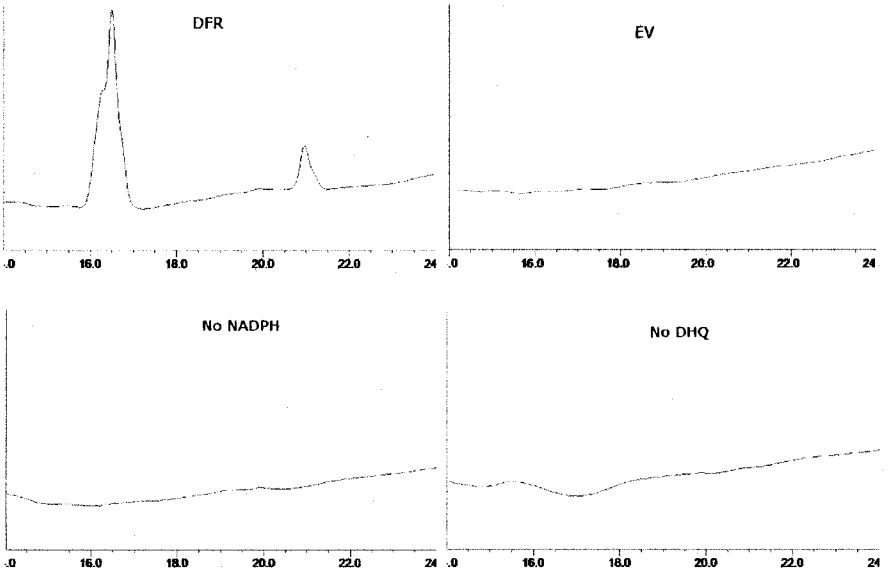
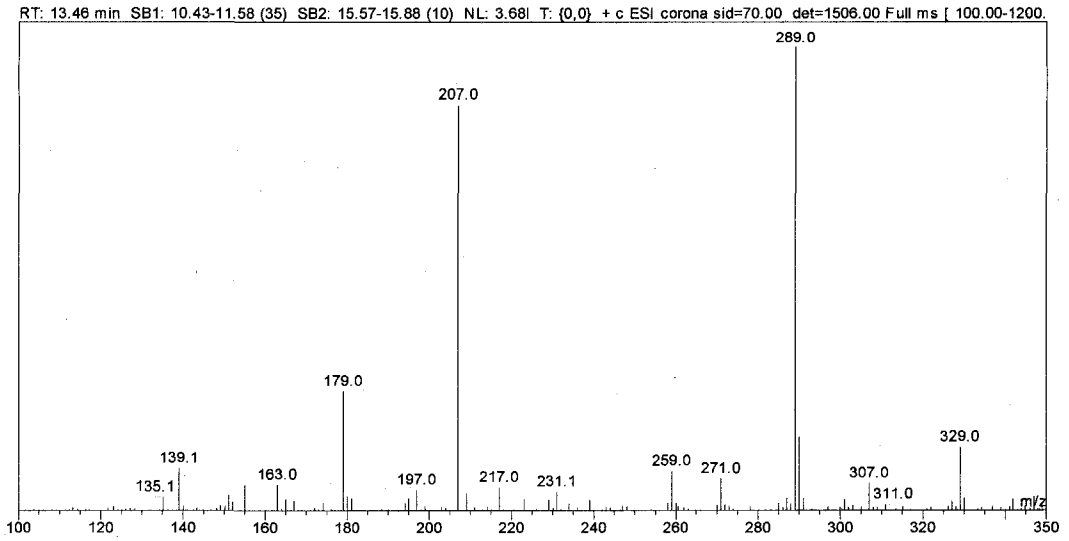
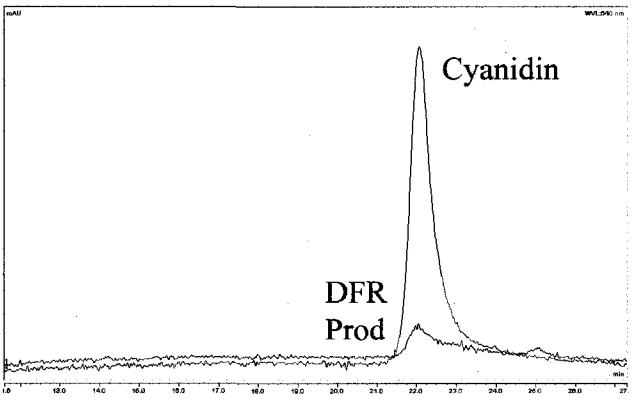


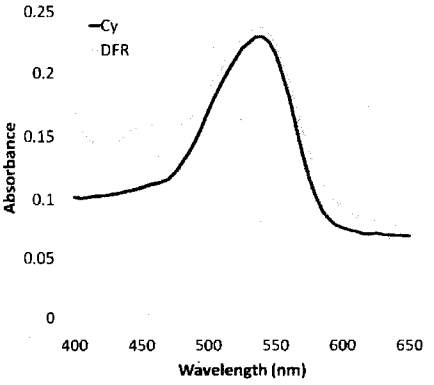
Fig 3.3 (cont)

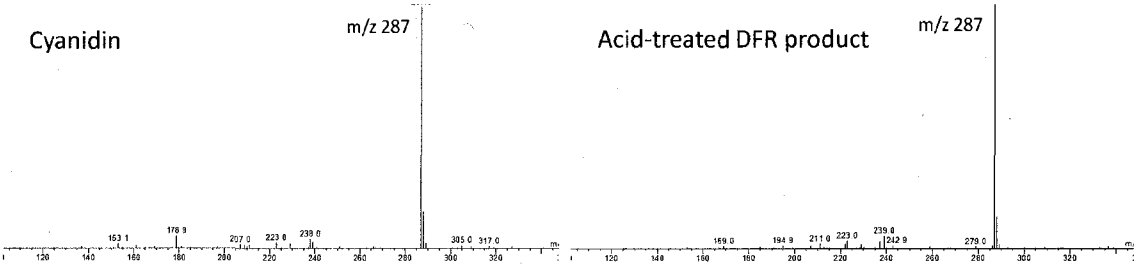
b.



c.



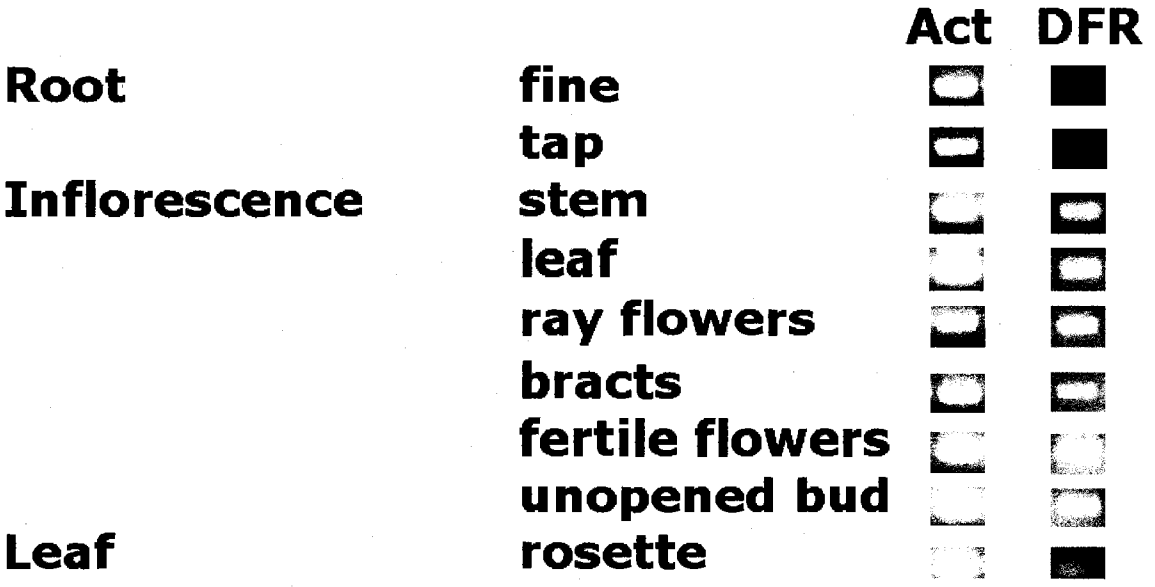




Gene expression analysis of CmDFR

To provide insight into the function of DFR and its metabolic products, RT-PCR was used to determine the presence or absence of CmDFR transcript in various tissues. Whereas CmDFR was expressed highly in all tissues of the inflorescence (notably in the purple-colored petals) and was detectable in leaf tissue samples, it was undetectable in root tissue, either fine or tap root (Fig 3.4).

Fig 3.4. *Cm*DFR gene expression analysis by RT-PCR. RNA was isolated from selected tissues, cDNA constructed from RNA samples, and DFR amplified by PCR with gene-specific primers. DFR is expressed at levels below detectable limits in roots, is highly expressed in inflorescence parts including the stem, cauline leaves, and floral parts, and only weakly expressed in rosette leaves. β-Actin (Act) serves as a positive control.



Discussion

Catechin is implicated in the alleopathic behavior of *C. maculosa* toward North American native plant species (Kelsey and Locken, 1987; Ridenour and Callaway, 2001; Bais et al., 2003). However, recovery of catechin under field and laboratory conditions is highly variable (Blair et al., 2006; Perry et al., 2007), leading to questions regarding the relevance of catechin as a mechanism of invasion. In an attempt to further elucidate the role catechin might play in *C. maculosa* invasive behavior, the characterization of a gene likely to be involved in the production of catechin in *C. maculosa* is reported. The data presented in this paper report the cloning of a *C. maculosa* DFR cDNA sequence, the translated amino acid sequence of that clone, demonstration of its activity when expressed in *E. coli* cells, and the gene expression analysis of this gene from greenhouse grown plants.

DFR is an extremely well characterized gene, with nearly 250 plant protein sequences in NCBI gene bank databases annotated as DFR enzymes. DFR is hypothesized to share a common ancestor with mammalian 3-beta-hydroxysteroid dehydrogenase enzymes, which are involved in the production of steroid hormones (Baker *et al.*, 1990). Recently, a DFR like gene was characterized from *Arabidopsis thaliana* that is directly or indirectly involved in brassinosteroid hormone metabolism (Yuan *et al.*, 2007). Thus DFR and genes of similar sequence are typically involved in metabolism of relatively non-polar secondary metabolites in plants, often transferring hydrogens from NAD(P)H to the substrate in a reduction reaction. CmDFR falls into this category, converting dihydroquercetin to leucoanthocyandin, in a reaction dependent on NADPH.

The functional domains of DFR include an N-terminal NADPH binding domain which occurs as part of a Rossman fold as well as a more variable substrate binding domain in the 130-160 positions of the protein (Johnson *et al.*, 2001; Petit *et al.*, 2007). These domains are well conserved between DFRs across vascular plants, and are particularly well conserved at smaller taxonomic units, such as within families. This holds true for the asters and CmDFR, as demonstrated in the ClustalW alignment (Fig 3.1) and phylogenic relationship with DFR sequences from other species (Fig 3.2).

Crystallographic structural analysis of Grape DFR indicated that the residues (using grape DFR numbering) G12, S14, G15, F16, I17, G18, R37, D38, K44, D64, L65, A85, T86, P87, Y163, K167, P190, V193, S205 interact with NADP+ through a series of hydrogen bonds and electrostatic interactions (Petit *et al.*, 2007). Of these residues, all are absolutely conserved in CmDFR, and only S14 (A in 2 of 3 aster species) and L65 (M in Ha) are variant in species and *M. truncatula* presented in Fig 3.1.

DHQ is bound through interactions with residues S128, A129, N133, Y163, F164, L192, I222, T208, and Q227 (Petit *et al.*, 2007). Of these residues, only N133 is variable, and it is most frequently replaced with D. The remaining residues in direct contact with DHQ are absolutely conserved. The conservation of DFR across plants species reflects the importance of downstream products such as anthocyanins and tannins in plant biology and ecology, most notably as floral pigments and in protection of the seed, respectively.

DFR is a necessary component of known biosynthetic pathways leading to the production of catechins (Abrahams *et al.*, 2003; Xie *et al.*, 2003). Catechin was not detected as a metabolite which accumulates in root tissue of *C. maculosa* but is found as a root exudate (Bais *et al.*, 2003), suggesting root-localized biosynthesis and secretion into the soil. Under greenhouse conditions, gene expression analysis failed to detect DFR expression in *C. maculosa* root tissue, but detected low level expression in rosette leaves and higher expression in inflorescence parts. The absence of CmDFR expression in the roots likely precludes catechin biosynthesis by greenhouse-grown *C. maculosa* plants. The regulation of DFR gene expression is highly species dependent, demonstrating responses to phosphate in grape cell cultures (Dedaldechamp *et al.*, 1995), nitrogen in tomato (Bonguebartelsman and Phillips, 1995), tissue-type in strawberry (Moyano *et al.*, 1998), and herbivory in trembling aspen (Peters and Constabel, 2002). DFR is also sensitive to cytokinin and circadian-periodicity in *A. thaliana* (Deikman and Hammer, 1995), and is both temporally and spatially regulated within floral organs in *Anthurium* (Collette *et al.*, 2004). The absence of DFR gene expression in root tissue could be a function of plant age, greenhouse growth condition, absence of plant competitors, nutrient availability, or other factors. These factors should be pursued in future work in an effort to understand the regulation of catechin production in *C. maculosa*. This report serves as a fundamental step forward in the understanding of catechin production in *C. maculosa*, and should allow for more detailed investigations into the regulation of catechin biosynthesis.

4. **Metabolome analysis reveals density-dependent metabolite accumulation in the invasive weed, *Centaurea maculosa* under field conditions.**

Summary

Invasive plant species often reach unusually high population densities and patch sizes in their non-native ranges. High population densities increase the intensity of intraspecific competition within a population, increase competitive effects on other species, enhance interactions with mutualists, or expose individuals to greater density-dependent consumer pressure. The influence of population density on individual plant physiology and biochemistry is poorly understood. This report describes the application of metabolome analysis to determine whether plant density impacts metabolite levels in naturally occurring field stands of the invasive weed, *Centaurea maculosa*. Metabolome analyses revealed increased accumulation of several defense-related secondary metabolites, including the sesquiterpene lactone cnicin, and decreased concentrations of numerous primary metabolites in individuals growing at high densities, demonstrating that plant physiology is dependent on plant density under field conditions. Due to these findings, specialist herbivore density in *C. maculosa* stands was also examined, evidence for increased specialist herbivore load on plants growing in high densities was found. This study indicates that the physiology and biochemistry of an individual plant in the field reflects the plant community in which it grows, and that these changes are revealed by metabolome analyses even under highly variable natural growth conditions.

Introduction

Population density, patch size, and the species diversity of the surrounding community affect the way that plants interact with each other, mutualists, consumers, and their environment. Intraspecific aggregation decreases competition with other species in ways that suppress competitively superior species (Stoll and Prati, 2001) and enhance facilitative relationships (Tirado and Pugnaire, 2003). Competition with conspecifics can be strikingly different than competition with other species (Fonteyn and Mahall, 1978), and population density can influence intraspecific competitive effects (de Wit, 1960; Robberecht et al., 1983) and interactions with mutualists (Parker, 2001; Moeller, 2004). Patch size, stand diversity, and population density affect interactions between plants and their herbivores and pathogens (Root, 1973; Bach, 1980). These interactions all have the potential to affect plant biochemistry through responses to limited resources, biochemically mediated competition (Mahall and Callaway, 1992), signals between plants and mutualists (Marx, 2004), and induced defense responses to herbivores and pathogens (Karban et al., 1997; Karban and Baldwin, 1997).

Exotic invasive plants often establish very high population densities that can approach monocultures, but may also exist at much lower densities in a matrix of other plants. This wide variation in density makes invaders good species with which to examine the effect of density on plant biochemistry under natural conditions. Furthermore, biochemical characteristics have been implicated 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).

Metabolomics is a rapidly developing area of study in which the small molecule composition of biological samples can be extracted and profiled. Because it is not dependent on previously acquired sequence information (nucleotide or amino acid), metabolomics is particularly well suited to studying non-model species. The most commonly used metabolomic techniques include gas and liquid chromatography coupled to mass spectrometric detection, which affords a unique balance of sensitivity and selectivity (Sumner et al., 2003). Metabolomic studies have been typically used in highly controlled experiments and applied to genetically uniform model species or highly inbred crop species, thereby eliminating genetic variation and natural phenotypic variation from the experimental design. These controls result in highly reproducible metabolite accumulation patterns which can be interpreted in a larger physiological or genomic context. However, as is the limitation with all highly controlled experiments, this process eliminates most natural sources of variation, which could potentially provide greater insight into the biology of the organism.

This study examines *Centaurea maculosa* (Lam.; spotted knapweed, Asteraceae, recently suggested to be *C. stoebe micranthos* L. [USDA, NRCS 2007]), a native of Eurasia that was introduced in the late 1800's to the Pacific northwest of North America. *Centaurea maculosa* is now listed as invasive in over half of the states in the USA. In this study non-targeted metabolomic analyses was applied to leaves of *C. maculosa* plants growing under natural field conditions in the introduced (North American) range in an attempt to

examine the effect of the density of conspecifics on the physiology of individual plants. Further, this experiment tests the utility of metabolomics as a tool for field biologists.

Materials and Methods

Preliminary greenhouse experiment

A pilot experiment was performed to guide the sampling procedure. *Centaurea maculosa* seeds collected from near Missoula, MT were surface sterilized with 20% bleach (v/v) + 0.1% SDS (w/v), rinsed 4x with deionized sterile water, and germinated in peat pellets for 14 days. Seedlings were then removed from peat moss, rinsed, and transferred to 9:1 mixture of sand and peat moss in 7-cm x 7-cm x 7-cm pots. Plants were grown under ambient greenhouse conditions with supplemental light to extend the photoperiod to 14L:10D. Plants were fertilized with 5 g of Osmocote (Outdoor & Indoor Smart Release, 19-6-12 formula) per pot and watered regularly. After 8 weeks of growth, leaf tissue samples were collected and submerged in liquid nitrogen. Leaf samples were divided into two categories, mature and immature. Mature leaf samples included all those that were fully expanded and showed no visible symptoms of senescence. Immature leaves included all those that were not yet fully expanded. Tissue samples were then freeze-dried and processed as described below for metabolome analysis.

Field sites

For metabolomic analyses, two field sites were chosen near Missoula, Montana and plants were collected in late May 2006 from a field site ≈30km east of Missoula (Site A,

Beavertail site: 12T 0301244E 5177747N), and from a field site on Mt. Sentinel, adjacent to University of Montana (Site B: 12T 273192E 5193062N). The Beavertail site is an abandoned agricultural field characterized by deep loamy soil (Ray Callaway, U. of Montana, *pers. comm.*). The Mt. Sentinel site is on a west facing slope and is characterized rocky clay-loam soil (Ray Callaway, U. of Montana, *pers. comm.*). At both sites the density of *C. maculosa* varies from low to very high densities. Herbivore response to *C. maculosa* density was measured at the Mt. Sentinel site and at a third site, the North Hills of Missoula, Montana. Plants sampled were categorized as growing under low density conditions if individual plants occurred at < 0.1 per m^2. Plants were categorized as high density if they occurred at > 15 per m^2.

Field experimental design

Sampling was conducted using a block design, with day and site serving as blocks. Sampling was conducted on two consecutive days (May 13 and 14, 2006). Each of two sites was visited in opposing order each day between the hours of 11:00 and 15:00 to minimize diurnal effects on metabolite accumulation data. On each sampling day, eight high density plants and eight low density plants were sampled at each site. High density plants were, for the tissue metabolome sampling, those that contained at least ten conspecifics within a 1.0 m radius of the sample plant. Low density plants were those that contained fewer than three conspecifics within a 1.0 m radius. This design resulted in a sampling regime composed of two sites visited on two consecutive days, for a total of 32 samples each of high and low density plants and a total of 64 samples. For each sample, data were collected for factors that might affect metabolism. Evidence of

herbivory (none, chewing, piercing-sucking), the distance to the nearest conspecific, the distance to the nearest other species, and number of conspecifics within a 30-cm radius were recorded. Hourly temperature and relative humidity was obtained from the NOAA website for Missoula, MT. A quadratic function was fitted to these data with respect to time to extrapolate temperatures and humidity between available time points (1 hr increments, $r2 > 0.98$). All of these ecological and climatic variables were added to the basic ANOVA model as covariates, but failed to improve the ANOVA model fit and were thus not included in model used for the final analysis.

Field tissue sampling

Individual *C. maculosa* rosettes without current-year flowering stalks were selected for metabolite analyses. However, for the sake of consistency, only plants with dried flowering stalks from the previous year were sampled, ensuring that samples were taken from individuals in at least their second year of growth. Non-senescent, fully expanded mature leaves were harvested from the rosette and frozen by immediately sandwiching them between two bricks of dry ice. Tissue samples were held on dry ice until return to the laboratory, and then transferred to an -80°C freezer until processing. Individual samples were processed by grinding them to a fine powder in liquid nitrogen and freeze drying.

Metabolome analysis

Metabolome analysis for GC-MS was conducted essentially as previously described (Broeckling et al., 2005). For GC-MS plant metabolome analysis, 6.0 mg of dried tissue was extracted with 1.5mL $CHCl_3$ (with internal standard - IS) for 60 minutes at 37°C. After 60 minutes, 1.5 mL of water (with IS) was added to form a biphasic solvent system. This mixture was thoroughly vortexed and incubated for an additional 60 minutes at 37°C. The samples were then centrifuged at 3000xg for 30 minutes to separate the solvents. One mL of the $CHCl_3$ fraction was collected and transferred to an autosampler vial – this comprises the non-polar fraction. $CHCl_3$ was evaporated under a gentle flow of nitrogen gas. The dried sample was derivatized in 70μL of pyridine and 30μL MSTFA at 50°C for one hour to generate trimethylsilyl derivatives. One mL of the aqueous fraction was collected and transferred to an autosampler vial – this comprises the polar fraction.

The aqueous extract was held at -80°C until it was dried in a vacuum centrifuge at ambient temperature. The dried aqueous extract was derivatized with 120 μL of pyridine with 15mg/mL methoxyamine HCl for 1 hr at 50°C, with occasional vortexing and sonicating in a water bath. 120 μL of MSTFA was then added and incubated at 50°C for 30 minutes to trimethylsilylate the polar compounds.

Separation was performed on a 60 m DB5-MS (J&W Scientific, 0.25 mm ID, 0.25 μm film thickness) column. Separation was achieved with a temperature program of 80°C for two min, then ramped at 5°C min-1 to 315°C and held for 12 min and a constant flow

of 1.0 ml min-1. Mass data was collected on a Agilent 5973 single quadrupole mass spectrometer using electron impact ionization. One μL of derivatized non-polar fraction was injected onto an Agilent 6890 GC using a 1:1 split ratio. A 1.0 μL portion of the aqueous fraction was analyzed in the same manner, except a 15:1 split ratio was used. All identifications were made by comparison to a custom authentic standard library by comparison of retention time and mass spectral data.

For UPLC-MS analysis, 40 mg of freeze dried and homogenized leaf tissue was extracted twice in 70% methanol in water containing 0.1 μg/μL 4-methylumbelliferone (internal standard). The extracts were centrifuged to remove particulate material and pooled. Samples were held at 10 °C during the analysis. One microliter injections were separated by reverse phase chromatography using an Acquity UPLC™ (Waters Corporation, Milford, MA, USA). Solvent and column parameters are as follows: Solvent A = 95:5 H2O:methanol (Fisher Optima LC/MS grade) + 0.1% formic acid (Fluka, LC/MS grade); Solvent B = 100% methanol + 0.1% formic acid; column = 1.0 x 100mm Waters Acquity UPLC™ BEH C18 1.71 μm particle size; column temperature = 40 °C. The solvent gradient parameters were as follows: Flow rate: 0.140 mL/min; 0 to 2 min: Solvent A 100 %; 2 to 22 min: Solvent A 100 % to Solvent B 100 %; 22 to 25 min: Solvent B 100 %; 25 to 28 min: Solvent B 100 % to Solvent A 100 %; 28 to 30 min: Solvent A 100 %. A short gradient was run between each sample to prevent any trace sample carry-over and equilibrate the column. The equilibration gradient characteristics were as follows: Flow rate: 0.140 mL/min; 0 to 0.1 min: Solvent A 100 %; 0.1 to 5 min:

Solvent A 100 % to Solvent B 100 %; 5 to 8 min: Solvent B 100 %; 8 to 11 min: Solvent B 100 % to Solvent A 100 %; 11 to 20 min: Solvent A 100 %.

Effluent from the UPLC system was infused directly into a Waters Micromass Micro quadrupole orthogonal acceleration time-of-flight mass spectrometer (Q-TOFMS) via electrospray ionization (ESI), in the positive ion mode using the following operating parameters: Capillary: 3000V; Sample cone: 35 V; Extraction cone: 2 V; Collision cell: 7 eV, 20 psi pressure (argon); Source temperature: 130°C; Desolvation temperature: 300°C; Desolvation gas flow: 400L/h. Sodium formate was used to calibrate the Q-TOF across the mass range of detection. Leucine enkaphalin was introduced via a secondary LockSprayTM positive ion ESI source as a mass standard to improve the accuracy of collected mass values. Mass data were collected in real-time centroid mode, creating centered measurements for each scan. The measured mass resolution was measured to be 5000 (FWHM). Both the UPLC and Q-TOF were controlled by Waters MassLynx software (v4.1). Identification of cnicin was based on comparison of retention time and mass spectrum to authentic standard (Phytoplan cat# 2113.98, Heidelberg, Germany).

Data extraction and statistical analysis

GC-MS data was processed using AMDIS (Halket et al., 1999) for peak detection from multiple randomly selected samples and quantitative peak area data extracted using default settings in MET-IDEA (Broeckling et al., 2006). Redundant peaks were removed and data were normalized to internal standard peak area to adjust for sensitivity drift of

the instrumentation. ANOVA, regression analysis, and HCA were conducted in JMP v.5.1 (SAS Institute, Cary, NC, USA).

UPLC-MS data were analyzed with Waters MarkerLynx software (v4.1) using the following parameters: retention time range: 0 - 24 min; mass range: 50 – 1000 Da; "apex Track peak parameters": automatically calculated peak width and baseline noise with no smoothing; "collection parameters": intensity threshold: 20 counts; mass window: 0.07 Da; retention time window: 0.1 min. Analyte features were labeled by their retention time and mass, and exported to Umetrics SIMCA-P v11 (Umetrics, Umeå, Sweeden) for multivariate analysis. Pareto scaling was applied to all data. Principal components analysis (PCA) was performed to verify the reproducibility of results among the triplicate data, and a single representative run from each experimental set was chosen for subsequent analysis. PCA was also used as an unsupervised method for observing sample grouping. Partial least-squares to latent structures-discriminant analysis (PLS-DA) was used to classify and group related samples. PLS-DA S-plots were generated to highlight the features unique to individual groupings.

Herbivore density

The relationship between *C. maculosa* density and biological control density at two other sites were explored. First, the number of larvae and pupae of seedhead gallflies was measured. Two species of *Urophora* gallflies (Insecta: Diptera: Tephritidae) are specialists on *C. maculosa*, and have been introduced from Europe as biocontrol agents.

Urophora quadrifasciata (Meigen) was released into British Columbia (Harris, 1980), Canada, but is now widely distributed throughout the northwest (Wheeler and Hoebeke, 2004); *Urophora affinis* (Frauenfeld) was introduced into the United States in 1973 (Story, 2002). Female *Urophora* flies oviposit into the knapweed seedhead, and the developing larvae eat the seeds. The larvae overwinter in the *C. maculosa* seedheads and emerge the following spring. The Urophora larvae were not identified to species, but *U. quadrifasciata* is more widely distributed than *U. affinis*. Seedheads of *C. maculosa* plants from low density areas (< 0.1 plant/m^2) and high density areas (> 15 plants/m^2) were sampled. Five seedheads per plant were dissected, and the number of Urophora larvae and pupae counted. Empty pupal cases were also included in these counts: these indicated that the larva had either already eclosed or had been parasitized. In either case, an empty pupal case indicates that a larva had survived in the seedhead through to pupation. Second, the proportion of *C. maculosa* plants either with root herbivores present or with recent signs of tunneling in roots were measured for individuals in dense stands and individuals isolated from other conspecifics. The specialist herbivores *Cyphocleonus achaetes* (Fahraeus) (Insecta: Coleoptera: Curculionidae) and *Agapeta zoegana* (Linnaeus) (Insecta: Lepidoptera: Cochylidae) have been introduced as biological controls for *Centaurea* and exist now in patchy populations throughout much of the weed's range (Story et al., 1991; Story et al., 1997; Powell et al., 2000; Story et al., 2008). The larvae of *Cyphocleonus* and *Agapeta* overwinter in the roots of *Centaurea* and do substantial physical damage to the roots. In an approximate area of 10 ha, 26 plants with > 10 other conspecific individuals in a 1 m^2 plot centered on the target plant (high density) and 26 plants with ≤ 1 other conspecific individual in a 4 m^2 plot centered

on the target plant (low density) were located. Each of these 52 plants was excavated, and the taproot dissected to look for insects or evidence of recent insect damage. The numbers of plants with insects and evidence for damage were pooled for each density class and the proportions with and without evidence of root herbivory were compared in high and low density classes with Chi-square analysis.

Soil analysis:

Soil samples were collected approximately 10 cm from the base of three randomly selected high density and low density plants at each site. Soil was sampled to a depth of approximately 10 cm, and was immediately frozen on dry ice. Soil nutrient analysis was performed by the Soil, Water and Plant testing laboratory at Colorado State University using standard methods.

Results

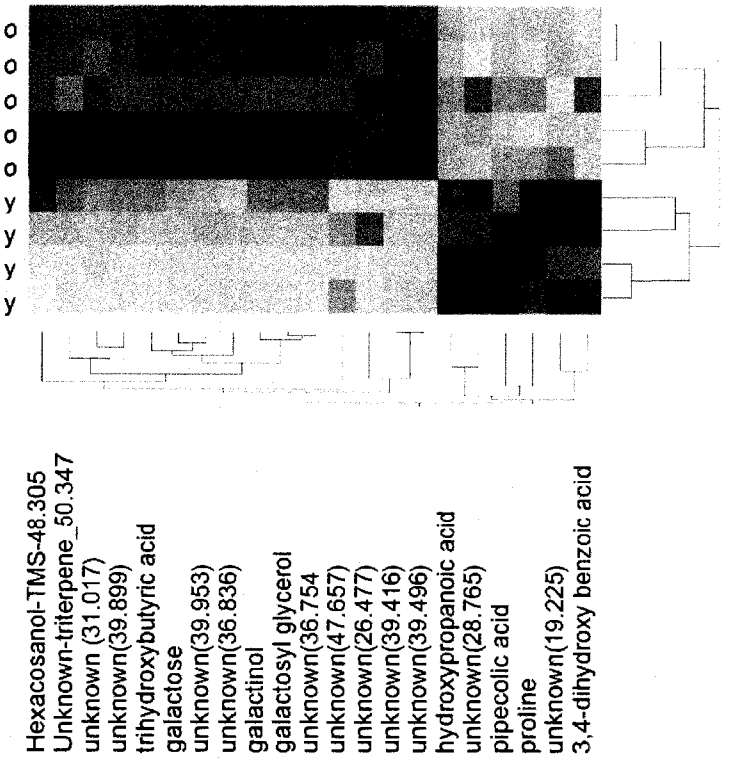
Developmental effects on the *C. maculosa* metabolome

The initial greenhouse experiment demonstrated that metabolite accumulation patterns reflect the developmental state of leaves (Figure 4.1). All metabolites detected were present in both mature and immature leaf tissue, but many demonstrated quantitative differences between mature and immature leaves. Non-polar metabolites including the two lipophilic compounds, hexacosanol and an unidentified compound with a mass spectrum similar to triterpenes such as β-amyrin (data not shown) accumulated to higher levels in mature leaves. These classes of compounds are commonly found in leaf

epicuticular waxes. To determine whether these compounds are wax components, epicuticular waxes were extracted from intact freshly harvested leaves with hexane (Zhang et al., 2005). This wax sample was found to contain both hexacosanol and the triterpene-like compound (data not shown), confirming that wax accumulation is developmentally regulated in *C. maculosa*. In addition to hexacosanol, other long chain alcohols and acids as well as alpha-tocopherol demonstrated similar trends, though ANOVA results were not significant at the 0.01 level (not shown).

Figure 4.1. Developmental age of leaves is apparent in the metabolome.

Leaves from greenhouse grown plants were prepared for GC-MS metabolome analysis. Mature ('o') and immature ('y') leaves were sampled from greenhouse grown plants, and the tissue was processed as described in materials and methods. Relative accumulation levels for those metabolites demonstrating significant ANOVA ($p < 0.01$) were used to generate a hierarchical cluster analysis heat map, with darker shades indicative of higher levels of accumulation.



Numerous polar metabolites were also developmentally regulated, including galactose, galactinol, galacosyl glycerol, and trihydroxy butyric acid (Figure 4.1). The non-targeted metabolomic approach also revealed accumulation of both free salicylic acid and a glycoside derivative in both mature and immature leaves. Further analysis of the data revealed a weak trend in which both the aglycone (ANOVA, $p = 0.113$) and the glycoside (ANOVA, $p = 0.013$) accumulate to higher levels in immature tissue. The structurally similar 3,4-dihydroxybenzoic acid also accumulated at higher levels in immature tissue ($p < 0.01$). The results of this analysis validate the metabolome approach for this species and demonstrate that development affects metabolite accumulation patterns. Based on these results, only mature non-senescent leaves were sampled for metabolome analysis of field grown plants.

Field study examining density-dependence of the metabolome

GC-MS metabolome analysis

Many polar metabolites including small organic acids and monosaccharides were found at lower levels in plants growing at high densities (Table 4.1). These include maleic acid, fumaric acid, succinic acid, and fructose. Several of the amino acids demonstrated the same trend, including six protein and one non-protein amino acid, gamma-aminobutyric acid. Further, several metabolites potentially related to membranes from both polar and non-polar fractions are found at decreased levels in samples from high-density plants.

Table 4.1. Mean, standard error, and ANOVA results for all identified compounds demonstrating significant ANOVA density effects from GC-MS data. The mean and standard error of the mean are presented for each metabolite. The ratio of the mean values for high and low density samples is presented, with values less than one indicative of higher values in low density samples, and values greater than one indicative of high values in high density plants.

Rt	cmpd	High density		Low density		Fold change	ANOVA results	
		mean	std err	mean	std err	(H/L)	p(ANOVA)	p(dens)
Polar phase								
16.152	Ethanol Amine	1.182	0.081	1.456	0.074	0.812	<.0001	<.0001
17.162	Phosphoric acid	53.460	2.251	67.764	4.396	0.789	0.0245	0.0064
17.185	Glycerol	6.190	0.249	7.233	0.334	0.856	<.0001	0.0096
17.869	L-Threonine	0.073	0.008	0.127	0.014	0.578	0.001	0.001
18.126	Maleic Acid	0.168	0.012	0.292	0.034	0.575	0.0007	0.0005
18.231	Glycine	0.899	0.070	1.281	0.108	0.702	<.0001	0.0023
18.409	Succinic Acid	0.441	0.020	0.565	0.029	0.781	0.0016	0.001
18.585	Catechol	0.053	0.005	0.065	0.004	0.818	<.0001	0.0096
19.389	Fumaric Acid	0.451	0.021	0.692	0.051	0.651	0.0004	<.0001
19.453	Cytosine	0.022	0.004	0.060	0.011	0.370	0.0048	0.0019
19.691	L-Alanine	0.157	0.013	0.326	0.039	0.483	0.0001	<.0001
23.696	L-Aspartic Acid	0.814	0.078	1.447	0.163	0.562	0.0011	0.001
23.866	Pyroglutamic acid	2.400	0.146	3.865	0.326	0.621	0.0008	0.0001
24.045	4-aminobutyric acid	2.347	0.224	3.916	0.484	0.599	<.0001	0.0029
24.957	3-hydroxybenzoate	1.594	0.075	1.727	0.058	0.923	<.0001	0.0077
25.316	L-Proline	0.181	0.030	0.494	0.098	0.367	0.0189	0.0035
27.301	Ribose	3.789	0.228	4.965	0.397	0.763	<.0001	0.0092
31.295	Quinic Acid	284.059	8.981	236.913	10.650	1.199	0.0005	0.0012
31.515	Fructose	46.607	2.745	65.131	4.668	0.716	0.0017	0.0013
31.703	Fructose	33.206	1.824	45.310	3.108	0.733	0.0016	0.0015
33.257	Inositol-like	41.923	3.597	26.426	3.169	1.586	0.0109	0.0025
34.027	Galactonic acid	0.412	0.018	0.338	0.012	1.218	<.0001	0.0002
34.649	Inositol-like	35.419	1.560	26.904	1.376	1.316	0.0019	0.0001
36.171	Galactose	2.547	0.109	2.047	0.096	1.245	0.0034	0.0009
51.811	Chlorogenic Acid	2.176	0.451	1.046	0.182	2.080	0.0011	0.0111
Non-polar phase								
17.167	Phosphoric Acid	13.386	0.620	16.579	1.132	0.807	0.0074	0.0094
29.172	glycerophosphate	0.143	0.013	0.203	0.022	0.706	<.0001	0.0032
37.49	Phytol	0.037	0.003	0.050	0.002	0.743	0.0053	0.0005
38.215	Linoleic acid	4.647	0.313	5.849	0.384	0.794	0.0003	0.0119
49.39	Hexacosanol	13.546	0.975	16.826	1.173	0.805	<.0001	0.0057
50.714	Hexacosanoic acid	0.197	0.019	0.267	0.024	0.738	<.0001	0.0035
52.367	Octacosanol	4.575	0.365	5.521	0.329	0.829	<.0001	0.0064

These include polar metabolites such as phosphate, ethanol amine, and glycerol. Non-polar metabolites including phosphate and glycerophosphate, which are hydrolytic products of membrane lipids, were found at decreased levels in high-density samples. Linoleic acid is an 18-carbon unsaturated fatty acid component of membrane lipids and was found at decreased levels under high density conditions. Wax components such as hexacosanol, octacosanol, and hexacosanoic acid were found at diminished levels under high density conditions (Table 4.1).

Nearly all of the identified metabolites are primary metabolites that were found at lower concentrations in plants growing at high-densities. However, a few metabolites demonstrated increased abundance under high density conditions. These include the two polar fraction compounds that share mass spectral similarity to inositol (retention times 33.257 and 33.649 minutes), the primary metabolites galactonic acid and galactose, and the phenolic secondary metabolites quinic and chlorogenic acid.

In addition to the identified metabolites, several unidentified polar metabolites were found at increased abundance in high density plants. Most of these metabolites eluted from the chromatographic column at longer retention times (higher oven temperatures). To further examine this trend, the average peak area for high density (H) and low density (L) treatments for each peak in the polar fraction were calculated, and the ratio of high density to low density average peak area (H/L) plotted against the retention time of that peak. A linear regression through this scatterplot demonstrated that compounds with

longer retention times (which generally have higher molecular weights) tend to increase in abundance under high density growth conditions (Figure 4.2). Retention time explains ~50% of the variation in the high density to low density ratio and results in a highly significant ANOVA ($p < 0.001$).

UPLC-MS metabolome analysis

GC-MS indicated that some secondary metabolites accumulated at higher levels in the leaves of high density plants than in the leaves of low density plants. To significantly expand the view of secondary metabolism beyond the capabilities of GC-MS, the same tissue samples were analyzed with ultra high pressure liquid chromatography coupled to high resolution mass spectrometry (UPLC-MS). UPLC samples were prepared via aqueous methanol extraction, as is commonly used for phenolics, flavonoids, and tepenoids (Huhman et al., 2005; Naoumkina et al., 2007). Analysis of these samples by UPLC-MS followed by multivariate data analysis demonstrates that high density and low density samples were distinguished (Figure 4.3). Univariate ANOVA revealed approximately 100 mass spectral signals that were significantly affected by plant density at $p < 0.01$ (Table 4.2). Included on this list was the sesquiterpene lactone, cnicin, which was identified by comparison of retention time and MS fragmentation pattern with an authentic cnicin standard (Phytoplan, Heidelberg, Germany). Cnicin accumulated at slightly higher levels in high density samples as compared to low density samples ([M+H]+ m/z 379.18 at 10.78 minutes, $p = 0.0048$). Several other unidentified metabolites demonstrate altered accumulation patterns, some of which decreased in abundance in high density plants (Table 4.2).

Figure 4.2. Higher molecular weight compounds increase in abundance in high density leaf samples.

The retention time (Rt – in minutes) of all polar metabolites showing significant ANOVA density effects is plotted against the ratio of mean high density vs low density (H/L) abundance of that compound. Longer retention times are generally indicative of lower volatility and therefore higher molecular weights in gas chromatography. Longer retention times positively correlate with higher ratios, indicating that high molecular weight compounds tend to increase in abundance in high density samples. $r^2 = 0.492$. Regression line indicates best fit linear regression model, and the linear model ANOVA was highly significant ($p < 0.001$).

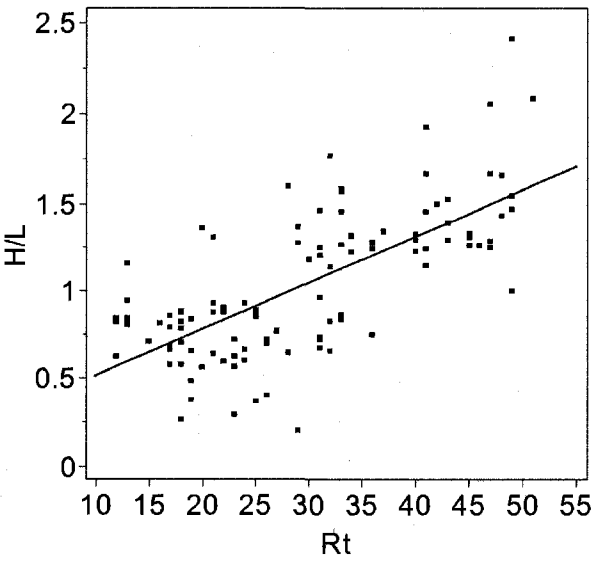


Figure 4.3. UPLC-MS analysis allowed distinction of low and high density samples. Samples were processed and analyzed as described in materials and methods. The resulting dataset was analyzed by principle component analysis followed by partial least squares – discriminant analysis. The resulting plot demonstrated that high and low density samples are metabolically differentiated. Open circles indicate low density samples and filled squares indicate high density samples. The x- and y-axes represent the first and second OPLS components, respectively. Ellipse represents the 95% confidence interval. Randomization of the density variable eliminated predictive power of the PLS-DA model.

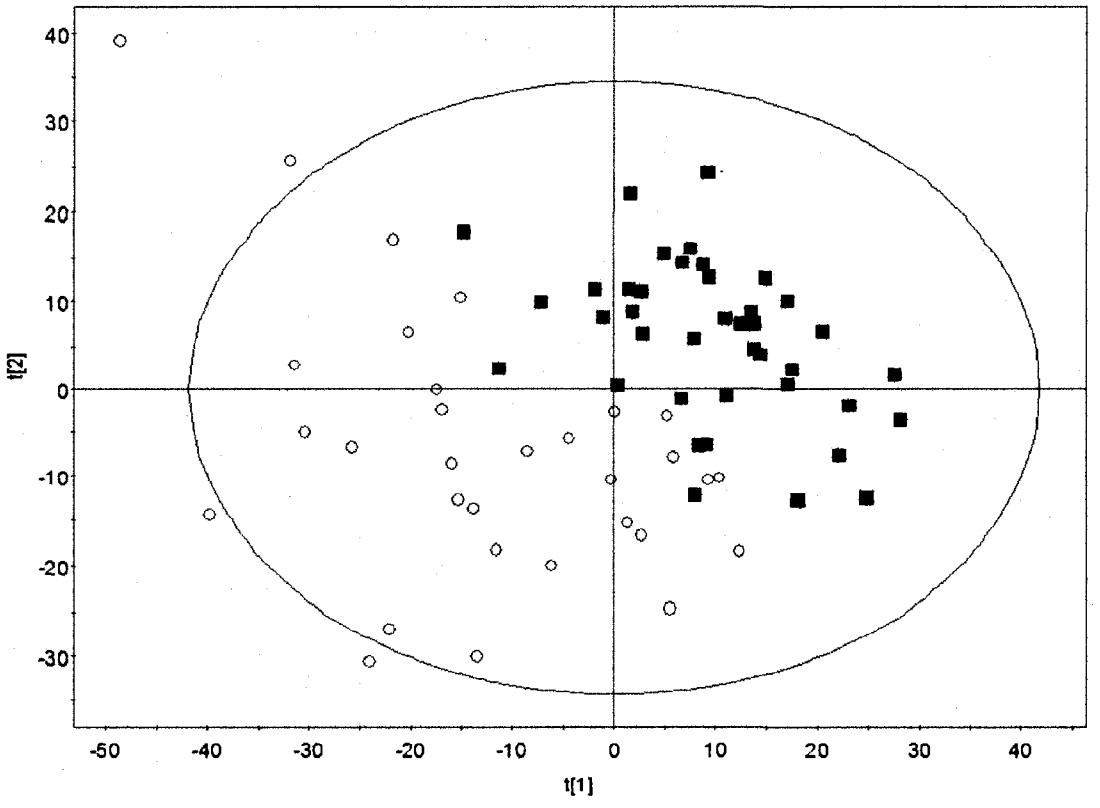


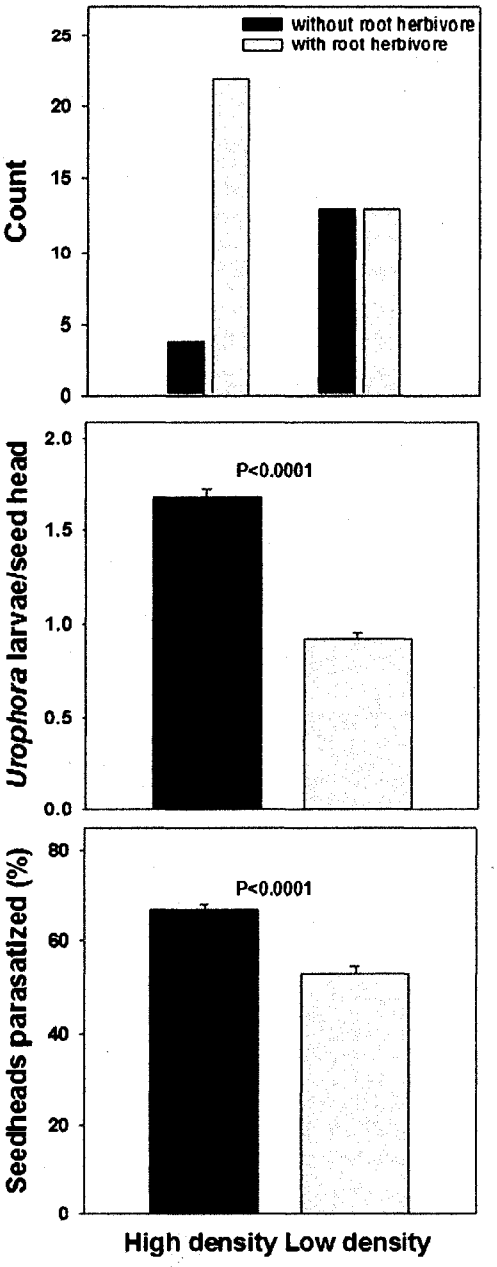
Table 4.2. UPLC features that are density responsive. See table 4.1 for description.

			High density		Low density		fold change	ANOVA results	
Feature (Rt_m/z)	Rt	m/z	mean	std err	mean	std err	(H/L)	p(ANOVA)	p(Density)
0.44_638.8851	0.44	638.8851	0.089	0.012	0.142	0.018	0.626	0.0001	0.0054
0.44_640.8818	0.44	640.8818	0.126	0.015	0.177	0.017	0.710	0.0001	0.008
0.44_412.9185	0.44	412.9185	0.111	0.011	0.154	0.012	0.724	0.0001	0.0032
0.49_504.7753	0.49	504.7753	0.448	0.025	0.358	0.021	1.249	0.0036	0.0085
0.50_222.9944	0.5	222.9944	0.219	0.021	0.383	0.039	0.572	0.0023	0.0005
0.52_191.9830	0.52	191.983	0.058	0.013	0.134	0.020	0.431	0.0047	0.0018
0.53_210.9498	0.53	210.9498	0.035	0.009	0.110	0.019	0.317	0.0086	0.0007
0.54_70.0669	0.54	70.0669	0.651	0.101	1.368	0.182	0.476	0.0049	0.001
0.54_116.0703	0.54	116.0703	0.714	0.086	1.289	0.185	0.554	0.0093	0.0029
0.54_555.1175	0.54	555.1175	0.033	0.011	0.000	0.000	inf	0.0041	0.0032
0.55_797.1394	0.55	797.1394	0.078	0.014	0.127	0.015	0.601	0.0006	0.0082
0.55_130.0514	0.55	130.0514	0.897	0.046	1.090	0.055	0.823	0.0016	0.006
0.55_263.0463	0.55	263.0463	0.660	0.026	0.538	0.035	1.226	0.0026	0.0051
0.59_272.0871	0.59	272.0871	0.560	0.033	0.445	0.029	1.256	0.0033	0.0088
0.61_83.0506	0.61	83.0506	0.899	0.028	0.717	0.028	1.254	<.0001	<.0001
0.61_95.0506	0.61	95.0506	0.332	0.013	0.261	0.013	1.270	0.0008	0.0003
0.62_69.0347	0.62	69.0347	0.898	0.020	0.779	0.025	1.154	0.0019	0.0004
0.63_193.0714	0.63	193.0714	0.312	0.016	0.247	0.017	1.263	0.0036	0.0056
0.63_215.0444	0.63	215.0444	0.879	0.036	0.658	0.049	1.337	0.0082	0.0007
0.83_247.0499	0.63	247.0499	0.534	0.045	0.350	0.052	1.527	0.0032	0.0073
5.20_153.0914	5.2	153.0914	1.651	0.039	1.364	0.066	1.211	0.005	0.0005
5.21_125.0950	5.21	125.095	0.918	0.022	0.779	0.032	1.179	0.0091	0.0008
5.28_395.1379	5.28	395.1379	0.179	0.022	0.086	0.019	2.077	0.0064	0.0021
6.08_107.0504	6.08	107.0504	0.510	0.025	0.359	0.031	1.422	0.0049	0.0004
6.30_334.1997	6.3	334.1997	0.020	0.006	0.080	0.018	0.254	0.0009	0.0017
6.30_128.0715	6.3	128.0715	0.043	0.010	0.136	0.030	0.317	0.0024	0.0037
6.31_402.1938	6.31	402.1938	0.131	0.037	0.427	0.098	0.307	0.0022	0.004
6.31_380.2078	6.31	380.2078	0.892	0.195	2.812	0.622	0.317	0.0025	0.0034
6.31_312.1489	6.31	312.1489	0.069	0.013	0.164	0.031	0.421	0.0015	0.0039
6.48_195.0675	6.48	195.0675	0.689	0.048	0.525	0.041	1.311	0.0068	0.0097
6.87_561.1958	6.87	561.1958	3.499	0.154	2.911	0.159	1.202	0.0011	0.0062
6.87_161.0823	6.87	161.0823	0.672	0.033	0.512	0.040	1.313	0.001	0.0019
6.88_131.0502	6.88	131.0502	0.779	0.041	0.619	0.041	1.257	0.0012	0.005
7.01_591.2090	7.01	591.209	0.680	0.045	0.532	0.033	1.277	0.0019	0.0071
7.26_475.1279	7.26	475.1279	3.228	0.130	2.558	0.151	1.262	0.0008	0.0009
7.26_595.1598	7.26	595.1598	17.509	0.785	13.784	0.850	1.270	0.0023	0.0016
7.26_457.1139	7.26	457.1139	6.938	0.289	5.433	0.323	1.277	0.0017	0.0008
7.26_559.1461	7.26	559.1461	3.058	0.131	2.390	0.142	1.278	0.0009	0.0007
7.27_617.1496	7.27	617.1496	3.193	0.064	2.820	0.107	1.132	0.0016	0.0027
7.27_355.0842	7.27	355.0842	0.427	0.017	0.349	0.021	1.222	0.0065	0.0051
7.27_523.1262	7.27	523.1262	0.703	0.027	0.561	0.033	1.253	0.0024	0.0012
7.27_577.1522	7.27	577.1522	9.779	0.416	7.752	0.453	1.261	0.0014	0.0012
7.27_541.1402	7.27	541.1402	1.160	0.046	0.912	0.053	1.271	0.0025	0.0007
7.27_489.1264	7.27	489.1264	1.124	0.047	0.865	0.050	1.300	0.0005	0.0003
7.27_421.0962	7.27	421.0962	0.261	0.011	0.194	0.014	1.345	0.0035	0.0005
7.27_439.1107	7.27	439.1107	0.476	0.019	0.353	0.025	1.349	0.0001	0.0001
7.45_311.0804	7.45	311.0804	0.201	0.023	0.115	0.014	1.746	0.0008	0.0015
7.57_263.1259	7.57	263.1259	0.225	0.016	0.149	0.015	1.504	0.0024	0.0009
7.58_516.2240	7.58	516.224	0.634	0.137	1.379	0.204	0.460	0.0016	0.0025
7.58_362.1994	7.58	362.1994	0.136	0.026	0.291	0.041	0.467	0.0014	0.0018
7.58_494.2358	7.58	494.2358	4.756	0.903	9.381	1.315	0.506	0.0019	0.0036
7.64_525.2464	7.64	525.2464	0.113	0.014	0.245	0.033	0.463	0.0012	0.0004
7.64_181.0987	7.64	181.0987	0.380	0.017	0.289	0.019	1.312	0.0089	0.0009
7.64_227.1120	7.64	227.112	0.612	0.027	0.442	0.032	1.384	0.0013	0.0002
7.72_565.1553	7.72	565.1553	8.608	0.329	6.469	0.444	1.331	0.0032	0.0003
7.72_427.1048	7.72	427.1048	4.277	0.149	3.131	0.242	1.366	0.0008	0.0002
7.90_499.1269	7.9	499.1269	1.787	0.063	1.485	0.056	1.204	0.006	0.0008
7.90_427.1036	7.9	427.1038	6.000	0.194	4.927	0.198	1.218	0.0033	0.0003
7.90_529.1341	7.9	529.1341	4.968	0.155	4.071	0.209	1.220	0.0079	0.0011
7.90_547.1447	7.9	547.1447	7.406	0.233	6.067	0.247	1.221	0.0022	0.0002
7.90_565.1473	7.9	565.1473	22.164	0.790	18.126	0.752	1.223	0.0037	0.0005
7.98_417.1090	7.98	417.109	0.515	0.047	0.328	0.045	1.578	0.0025	0.0037
8.49_619.1301	8.49	619.1301	0.123	0.037	0.019	0.013	8.391	0.0071	0.0094
8.54_627.1585	8.54	627.1585	1.504	0.242	0.789	0.108	1.907	0.0038	0.0088
8.57_413.2182	8.57	413.2182	0.032	0.025	0.207	0.059	0.155	0.002	0.0055
8.82_337.0947	8.82	337.0947	0.000	0.000	0.061	0.028	0.000	0.0005	0.0028
9.71_536.2560	9.71	536.256	0.209	0.040	0.520	0.089	0.401	0.0016	0.0017
9.72_558.2415	9.72	558.2415	0.015	0.005	0.054	0.013	0.283	0.0084	0.0047
10.03_227.1113	10.03	227.1113	0.246	0.018	0.171	0.017	1.438	0.008	0.0019
10.25_536.2517	10.25	536.2517	0.262	0.055	0.560	0.088	0.468	0.0017	0.0037
10.72_437.0843	10.72	437.0643	0.327	0.031	0.173	0.029	1.892	0.0071	0.0007
10.77_229.1084	10.77	229.1084	129.564	0.974	124.908	1.156	1.037	0.0008	0.0019
10.77_361.1676	10.77	361.1676	1.140	0.018	1.042	0.022	1.095	0.004	0.0009
10.77_261.1519	10.77	261.1519	2.860	0.043	2.533	0.128	1.137	0.0082	0.0093
10.78_379.1772	10.78	379.1772	7.191	0.102	6.776	0.101	1.081	0.0098	0.0048
10.78_217.1245	10.78	217.1245	4.946	0.066	4.635	0.077	1.067	0.0062	0.0028
10.78_343.1578	10.78	343.1578	0.687	0.011	0.637	0.013	1.079	0.0091	0.0034
10.78_147.1008	10.78	147.1008	1.459	0.022	1.326	0.026	1.100	0.0033	0.0003
10.78_160.1045	10.78	160.1045	0.479	0.011	0.425	0.017	1.127	0.0074	0.0077
11.00_181.1217	11	181.1217	0.708	0.054	1.039	0.057	0.681	0.0002	<.0001
16.16_307.1154	16.16	307.1154	0.011	0.005	0.041	0.011	0.279	0.0007	0.0083
16.23_553.3014	16.23	553.3014	0.488	0.060	0.225	0.046	2.171	0.002	0.0008
17.56_79.0582	17.56	79.0562	0.017	0.011	0.180	0.063	0.095	0.0034	0.0093
17.56_329.1300	17.56	329.13	0.076	0.025	0.247	0.045	0.308	0.0001	0.0005
17.57_372.1511	17.57	372.1511	0.040	0.008	0.080	0.013	0.504	0.0036	0.0059
18.11_329.2113	18.11	329.2113	0.043	0.030	0.220	0.050	0.193	0.0007	0.0021
18.52_670.3864	18.52	670.3864	0.125	0.016	0.198	0.024	0.634	0.0009	0.0097
18.52_133.1012	18.52	133.1012	0.725	0.085	0.972	0.081	0.746	0.0022	0.0052
18.53_339.0956	18.53	339.0958	0.193	0.015	0.259	0.012	0.746	0.0051	0.0011
19.05_177.1422	19.05	177.1422	0.000	0.000	0.133	0.051	0.000	0.0025	0.0081
19.05_346.1723	19.05	346.1723	0.071	0.009	0.127	0.015	0.558	0.0001	0.0011
19.43_159.0111	19.43	159.0111	0.000	0.000	0.023	0.009	0.000	0.01	0.0096
19.98_691.3984	19.98	691.3984	0.076	0.012	0.123	0.012	0.620	0.0004	0.0052
20.01_205.1629	20.01	205.1629	0.239	0.030	0.122	0.031	1.956	0.0059	0.0089
20.44_489.3108	20.44	489.3106	0.182	0.024	0.097	0.018	1.872	0.0087	0.0051
20.51_407.3518	20.51	407.3518	0.142	0.010	0.095	0.014	1.494	0.0002	0.0039
20.63_261.1424	20.63	261.1424	0.033	0.016	0.114	0.026	0.287	0.0034	0.0066
20.63_265.1517	20.63	265.1517	0.129	0.011	0.197	0.016	0.654	0.0021	0.0009
20.63_577.3692	20.63	577.3692	0.258	0.020	0.152	0.024	1.692	0.0015	0.001
20.70_545.3501	20.7	545.3501	0.685	0.049	0.438	0.048	1.564	0.0026	0.0005
20.70_507.3711	20.7	507.3711	0.375	0.034	0.231	0.027	1.623	0.0028	0.0014
20.70_547.3620	20.7	547.362	1.630	0.130	0.957	0.114	1.702	0.0001	0.0001
20.70_489.3590	20.7	489.359	0.371	0.041	0.209	0.033	1.774	0.0022	0.0024
20.70_273.2545	20.7	273.2545	0.199	0.019	0.110	0.018	1.808	0.0028	0.0008
20.71_578.2936	20.71	578.2936	0.146	0.016	0.092	0.013	1.588	0.0033	0.0082
20.96_199.0990	20.96	199.099	0.434	0.036	0.321	0.024	1.352	0.0078	0.0091
20.96_501.3230	20.96	501.323	0.826	0.053	0.586	0.032	1.409	0.0006	0.0002
20.96_503.3377	20.96	503.3377	1.757	0.165	1.146	0.073	1.530	0.0008	0.0008
20.96_463.3457	20.96	463.3457	0.327	0.036	0.194	0.022	1.686	0.0043	0.0023
21.29_441.3731	21.29	441.3731	0.379	0.021	0.303	0.020	1.254	0.0051	0.0084
21.33_207.1792	21.33	207.1792	0.216	0.057	0.428	0.060	0.505	0.0012	0.0084
21.33_322.2223	21.33	322.2223	0.150	0.014	0.213	0.017	0.703	0.0048	0.0044
21.63_531.3686	21.63	531.3688	0.262	0.027	0.169	0.021	1.547	0.0001	0.0026
21.75_403.2603	21.75	403.2603	0.476	0.008	0.441	0.009	1.080	0.0009	0.0035

Herbivores and plant density

Centaurea maculosa plants growing at high densities accumulated lower levels of many primary metabolites and higher levels of many secondary metabolites, including the *Centaurea*-specific defense chemical cnicin. Many secondary metabolites provide defense against insect herbivores suggesting that herbivores may contribute to the observed density dependence of the metabolome. However, there was no effect of observed leaf damage on metabolite accumulation, as revealed by ANOVA (see Materials and Methods). To expand our view, an independent analysis of specialist herbivore density was performed at different times/sites than that for metabolome analysis. Specialist root and seed head herbivores occurred more frequently at high *C. maculosa* densities. No evidence of *C. achaetes* was observed at the North Hills site, but the frequency of the root herbivore *A. zoegana* and root damage was higher in high density patches of *C. maculosa* (Figure 4.4a). Flower head herbivore (*Urophora spp.*) abundance was also higher in high density stands of *C. maculosa* (Figure 4.4b) and a higher percentage of seed heads were parasitized under high density conditions (Figure 4.4c).

Figure 4.4. High density plants are exposed to increased specialist herbivory. *a.* Numbers of *C. maculosa* plants with and without the root herbivore, *Agapeta zoegana*, or evidence of damage from root herbivores in high density and low density stands of *C. maculosa* in Montana. For each density level n=26 plants. Chi-square = 7.08, $p = 0.008$. *b. Urophora spp.* are found at increased numbers in high density stands of *C. maculosa*. *c.* A higher percentage of seed heads are parasitized by *Urophora spp.* under high density *C. maculosa* growth conditions.



Cumulative distribution function (CDF) plots were used to visualize the distribution of metabolite data as a function of plant density. CDF plots of the GC-MS data for some secondary metabolites indicate that the minimum, maximum, or both minimum and maximum values were quite different in plants growing at high versus low density (Figure 4.5b). For example, quinic acid demonstrates considerably higher minimum and maximum values, and at all times the distribution curve for high density samples is greater than that for low density samples. For chlorogenic acid, the minimum values are the same, but the maximum value is much higher for high density samples. A similar but opposite trend is observed for three representative primary metabolites and a wax component (Figure 4.5c). Soil physiochemical properties may contribute to altered plant physiology, so soil was analyzed from high and low density patches at each site. There was no statistically significant difference detected using several parameters (Table 4.3).

Figure 4.5. Distribution of the metabolite data suggests factors other than herbivory are important. For all panels, dark lines on the cumulative distribution function (CDF) plot represent high density samples and light lines represent low density samples. The x-axis represent relative metabolite levels. *a.* theoretical data representing plots observed if herbivory was the only factor driving observed metabolite changes. *b.* CDF plots of two secondary metabolites and *c.* three primary metabolites and a wax component demonstrate distributions inconsistent with an individual plant response to herbivory.

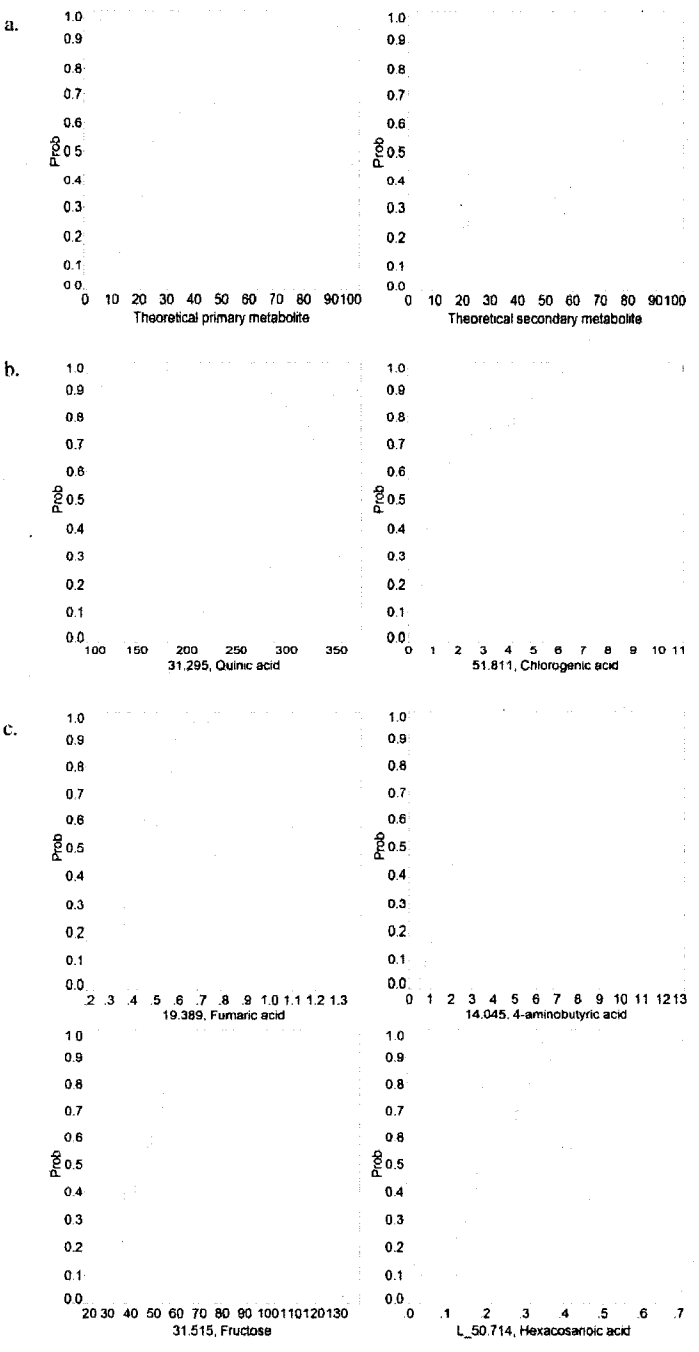


Table 4.3: Soil physiochemical properties in high and low density patches of *C. maculosa* stands in Montana. NO3-N, P, K, Zn, Fe, Mn, and Cu values are presented in units of parts per million. Three replicates were collected for each site and density, and values in table represent the mean (stardard deviation). There was no effect of density on any of the measured soil characteristics at $p < 0.01$.

Site	Density		pH	OM (%)	NO_3-N	P	K	Zn	Fe	Mn	Cu
	A	L	5.8 (0.4)	14.5 (1.4)	4.7 (3.8)	13.5 (5.3)	400.5 (85.2)	12.3 (3.0)	64.1 (33.0)	3.7 (3.8)	8.3 (1.9)
		H	5.5 (0.4)	12.9 (4.7)	1.4 (1.2)	13.9 (3.5)	352.8 (159.4)	11.9 (2.4)	84.2 (40.6)	7.7 (8.1)	7.7 (1.1)
	B	L	6.0 (0.2)	21.6 (13.9)	5.4 (3.3)	13.1 (1.9)	250.5 (54.9)	11.9 (3.9)	33.9 (6.6)	1.7 (0.3)	6.4 (2.0)
		H	6.3 (0.1)	21.4 (0.9)	17.2 (13.7)	14.1 (3.1)	403.6 (55.7)	11.5 (1.9)	46.7 (6.1)	2.4 (1.3)	7.1 (1.6)

Discussion

Technological advances have allowed biological researchers to simultaneously measure relative abundance of hundreds to thousands of individual metabolites, transcripts, or proteins. Despite this recent emphasis on more inclusive measurement tools such as transcriptome and metabolome analyses, most studies using 'omic' tools use experimental designs involving highly controlled conditions to minimize variation and maximize sensitivity of the analysis. This high level of control allows for highly reproducible results that may not translate to more natural conditions. This study applied metabolome analysis to field grown plants and found substantial differences in the metabolome profiles of individual *C. maculosa* plants growing at either high or low population densities. These results demonstrate that the biochemistry and physiology of plants is dependent on their immediate environment, and that this can be measured under field conditions using metabolome analyses.

GC-MS based analysis of high and low density *C. maculosa* leaf tissue indicated reduced accumulation of many small primary metabolites of glycolysis, the tricarboxylic acid cycle, lipid metabolism, and amino acids under high density growth conditions. These metabolites are critical to growth of the plant and suggest decreased energy metabolism, or other central metabolic processes. In contrast, some secondary metabolites, inositol-like compounds, and galactose demonstrated increased accumulation. These compounds are frequently associated with biotic or abiotic stresses. An additional trend was also observed in which higher molecular weight metabolites tended to increase in abundance under high density growth conditions, suggesting a shift in metabolism toward higher

oligomers with spectra indicative of di- and trisaccharides that might be involved in cell wall biosynthesis. UPLC-MS analysis of secondary metabolites also detected metabolic changes between high and low density plants. Together, these results indicate that an effect of density is apparent at the level of both primary and secondary metabolism. A similar result was demonstrated recently under controlled growth conditions in the model species, *Arabidopsis thaliana* (Wentzell and Kliebenstein, 2008), where the authors report that the developmental trajectory of glucosinolate accumulation was dependent on plant density. Together, these studies demonstrate that physiology is either directly or indirectly impacted by plant density.

Many mechanisms could account for such an effect, including altered light quality under high density conditions, increased competition for light, space, or soil nutrients, or through changes in effects of herbivores or pathogens. For example, sulfur deprivation reduces many measures of metabolism, including total protein, chlorophyll, and mRNA, as well as both increasing and decreasing many individual primary metabolites in *Arabidopsis thaliana* (Nikiforova et al., 2005). However, measurement of soil nitrate, pH, conductivity, phosphorus, potassium, zinc, iron, manganese, and copper revealed no consistent differences between high and low density *C. maculosa* patches (Table 4.3). This suggests that soil nutrient content may not contribute a great deal to the density-dependent patterns in the metabolome, but it is possible that nutrients other than those measured influence physiology.

Far red light quantity is recognized by plants as an indicator of competition, and in tomato and potato plants, increased far red light results in decreased phenolic defense compounds (Izaguirre et al., 2006). In *C. maculosa* the opposite effect was observed, with increased chlorogenic and quinic acids (both of which are phenolic metabolites) under high density conditions (Table 4.1), indicating that the metabolic changes in *C. maculosa* are inconsistent with previously characterized responses to far red light.

Competition frequently affects the growth habits of plants (taller growth for shade avoidance, root to shoot ratio, etc.), and changes in competition can affect susceptibility of a plant to herbivores (Cipollini and Bergelson, 2002; Stamp et al., 2004; Tiainen et al., 2006; Schadler et al., 2007). Plants defend themselves through both physical (trichomes, fiber content, leaf toughness, lignin, etc.) and chemical mechanisms (plant secondary metabolites, proteinase inhibitors, etc.). Regardless of the mechanism of defense, changes in defensive status of the plant are likely to affect gene expression (Kant et al., 2004; Guimil et al., 2005; de Torres-Zabala et al., 2007), protein (Jones et al., 2006; Devouge et al., 2007), and/or metabolite accumulation patterns (Broeckling et al., 2005; Hamzehzarghani et al., 2005; Allwood et al., 2006; Ormeno et al., 2007).

The increase in secondary metabolite accumulation suggested that herbivory could provide a link between the observed metabolic effects and density. Leaf herbivory damage data collected at both sites while sampling did not improve ANOVA model fit, thus it is unlikely that visible leaf herbivore damage was responsible for the observed

metabolic changes in leaf tissue. However, higher population density of both root and flower head herbivores (collected independently of tissue samples for metabolome analysis) correlated with high levels of defensive metabolites in high- density *C. maculosa* patches (Figure 4.2, Table 4.1, Table 4.2). Population density or patch size can have strong effects on herbivore abundance and impact (Root, 1973; Bach, 1980; Shea et al., 2000; Sholes, 2008) and herbivores can have strong effects on host chemistry by inducing defenses (Karban and Baldwin, 1997) or initiating growth responses (Paige, 1999). Root herbivores can have strong effects on allocation and the production of hormones and secondary chemicals (Blossey and Hunt-Joshi, 2003), and *A. zoegana*, the root herbivore most commonly found in our study, can stimulate growth, competitive ability, and reproductive overcompensation in *C. maculosa* (Müller-Schärer, 1991; Ridenour and Callaway, 2003; Thelen et al., 2005) and translocation of nitrogen from the root to the shoot (Newingham et al., 2007).

Steinger and Müller-Schärer (1992) also found that root herbivores reduced total soluble carbohydrates and increased starch in the leaves of *C. maculosa*, and one of the two root herbivores tested reduced leaf nitrogen content (Steinger and Müller-Schärer, 1992). The reduced total soluble carbohydrates and nitrogen correspond with our observed decrease in many primary metabolites observed in the GC-MS data (Table 4-I). The increase in starch observed by Steinger and Müller-Schärer (1992) supports the trend observed in the current study, in which higher molecular weight metabolites tended to increase under high density growth conditions (Figure 4-3). Many of these unidentified late eluting metabolites possessed mass spectra that included prominent ions at m/z = 204, 217, and

361 (data not shown), similar to spectra of di- to trisaccharides such as sucrose and raffinose.

Correlations between secondary metabolites and herbivore effects suggest that increased herbivore pressure under high density growth conditions may induce defense responses by *C. maculosa*. If the increase in some secondary metabolites was due solely to the increased frequency of herbivores, the metabolites should have roughly a binomial distribution. For instance, individual insect-infested plants would have high levels of defenses whereas uninfested plants would have low levels of defenses, regardless of the *C. maculosa* patch density. Since more plants are infested in high density patches, more plants would be in the induced state, and mean metabolite levels would increase under high density growth conditions. If this was the case the minimum and maximum values of the secondary metabolite should be approximately equal when comparing plants from low and high density patches, but the frequency of plants in the induced state should change, as depicted in a cumulative distribution function (CDF) plot of theoretical data (Figure 4.5a). This theoretical pattern was not observed under field conditions for several primary and secondary metabolites (Fig 4.5b,c), indicating that the observed metabolic changes are not solely due to individual plants responding to herbivores. However, because metabolomics was applied to field collected tissue, only correlations between density-dependent physiology and herbivory can be drawn; the effects of density cannot be separated from patch size, the effects of conspecifics or interspecific neighbors, unmeasured site conditions, or other ecological variables.

This study demonstrates that metabolome analysis can be used as a tool to explore ecological phenomena in natural field conditions. Modern metabolomic tools provide higher resolution than some traditional ecological measures of physiology which should contribute to a more detailed understanding of plant biotic and abiotic stress physiology and the ecological implications of these responses. The metabolome data presented here indicate that under field conditions plant density influences individual plant physiology, though the mechanism by which this occurs has yet to be determined. Though density-dependent herbivory correlates well with metabolome data, additional ecological factors are likely involved, and will be the focus of future studies. Our results demonstrate that the physiology of an individual plant in the field is highly variable but reflects the composition of the plant community in which it grows.

5. **Root exudates regulate soil fungal community composition and diversity**

Summary

Plants are in constant contact with a community of soil biota that contains fungi ranging from pathogenic to symbiotic. A few studies have demonstrated a critical role of chemical communication in establishing highly specialized relationships, but the general role for root exudates in structuring the soil fungal community is poorly described. This study demonstrates that two model plant species (*Arabidopsis thaliana* and *Medicago truncatula*) are able to maintain resident soil fungal populations, but unable to maintain non-resident soil fungal populations. This is mediated largely through root exudates: the effects of adding *in-vitro*-generated root exudates to the soil fungal community were qualitatively and quantitatively similar to the results observed for plants grown in those same soils. This effect is observed for total fungal biomass, phylotype diversity, and overall community similarity to the starting community. Non-resident plants and root exudates influenced the fungal community by both positively and negatively impacting relative abundance of individual phylotypes. A net increase in fungal biomass was observed when non-resident root exudates were added to resident plant treatments, suggesting that increases in specific carbon substrates and/or signaling compounds support an increased soil fungal population load. This study establishes root exudates as a mechanism through which a plant is able to regulate soil fungal community composition.

Introduction

Interactions between plants and soil microbes are highly dynamic in nature and based on co-evolutionary pressures (Hamilton and Frank, 2001; Klironomos, 2002; Dobbelaere et al., 2003; Klironomos, 2003; Duffy et al., 2004; Morrissey et al., 2004; Morgan et al., 2005; Reinhart and Callaway, 2006). Thus, it is not surprising that rhizosphere microbial communities differ between plant species (Westover et al., 1997; Priha et al., 1999; Innes et al., 2004; Batten et al., 2006), between genotypes within species (Kowalchuk et al., 2006), and between different developmental stages of a given plant (Mougel et al., 2006; Weisskopf et al., 2006). At a community scale, microbial diversity in the soil has been linked to plant diversity, though it is unclear whether this is through increased habitat heterogeneity, increased plant biomass commonly observed with highly diverse plant communities, or through increased diversity of carbon substrates and signaling compounds provided by the plants (Zak et al., 2003).

Fungi are heterotrophic organisms, depending on exogenous carbon for growth, and different clades of fungi utilize different carbon substrates. Plant root exudates contain simple carbon substrates including primary metabolites such as sugars, amino acids, and organic acids in addition to a diverse array of secondary metabolites that are released into the rhizosphere and surrounding soil (Jones et al., 2004).

Previous research suggests that secondary metabolites in root exudates are critical in specialized associations between plants and individual species of soil microbes ranging from mutualistic to pathogenic. For example, *Rhizobium spp.* are bacterial symbionts of legumes responsible for nitrogen fixation, and communication between the two organisms is mediated in part through root-secreted flavones (Redmond et al., 1986). Saponin ginsenosides from American ginseng stimulate the growth of two specialist soil-

borne fungal pathogens, although these same compounds are identified as possessing general anti-fungal properties (Nicol et al., 2003). A sesquiterpene from *Lotus japonicus* induces hyphal branching in an arbuscular mycorrhizal fungus, which is thought to be essential for establishment of a symbiotic relationship between the two species (Akiyama et al., 2005). Many studies on the role of plant secondary metabolites or root exudates analyze only a specific group of fungal species (often mycorrhizal fungi or pathogenic fungi), making interpretation of the results challenging in the context of soil fungal biodiversity. A detailed analysis of the effects of novel plant species and the role of their root exudates on the soil fungal community is a necessary step in the investigation of how plants regulate biological diversity in the soil, and in the nature of the interactions between plants and intact fungal communities.

The purposes of this study were to: 1. examine the effect of a novel plant species on an existing soil fungal community and 2. determine the relative importance of root exudates in structuring the soil fungal community. The results of these experiments demonstrate that root exudates play an important role in shaping the soil fungal community.

Materials and methods

Experiment 1

This experiment compared the effect of resident and non-resident plants, or their root exudates, on the soil fungal communities from various natural soils. The experiment

consisted of a factorial design (n = 10) with two plant species (*Arabidopsis thaliana* Col-0 and *Medicago truncatula* Jemalong) and three natural soils obtained from Illinois, Texas, or Oregon. This design results in two conditions in which a soil supports its resident plant species or its root exudates (*Arabidopsis thaliana* in Illinois soil and *Medicago truncatula* in Texas soil), four conditions in which a soil supports a non-resident plant species or its root exudates (*A. thaliana* in Texas or Oregon soil and *M. truncatula* in Illinois or Oregon soil), and three control conditions (no plants or exudates in Illinois, Texas, or Oregon soil (Fig. 5.1). The use of soils from diverse locations increases confidence in the results, as *Medicago truncatula* has very likely not grown recently in the *Arabidopsis thaliana* soil. Further, the properties of the soils are quite different from each other (see below for descriptions), demonstrating that the results observed are a general phenomenon.

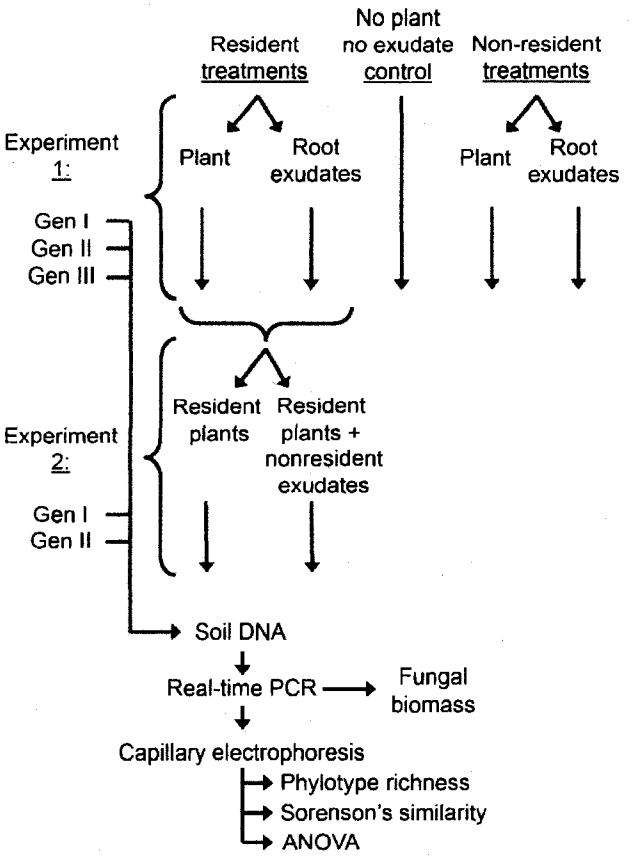


Fig 5.1. Schematic representation of the experimental design. Two experiments were performed; experiment 1 consisted of four treatments (resident plant or exudates and non-resident plant or exudates) and one control, which received neither plants nor exudates. Experiment 1 was conducted for three generations (Gen). Resident treatments included *A. thaliana* in IL soil and *M. truncatula* in TX soil. Non-resident treatments included *M. truncatula* in IL soil, *A. thaliana* in TX soil, or either plant species in OR soil. Experiment 2 was conducted on a subset of the pots from experiment 1 following the completion of experiment 1. Those pots in experiment 1 that received resident plant or resident exudate treatments were treated for an additional two generations with resident plants or resident plants supplemented with non-resident exudates. Soil samples were taken after each generation and DNA isolated from soil. DNA was subjected to real-time PCR with ITS primers to quantify fungal biomass, and amplified products were subjected to length heterogeneity analysis by capillary electrophoresis to estimate differences in phlyotype richness, community similarity, and differences in relative abundance via ANOVA.

The three field-collected soils (see below) were shipped to Fort Collins, CO, air dried, cleaned of plant debris and thoroughly homogenized. Six (5 g) sub-samples were randomly selected from each soil type to establish the pre-treatment (generation 0) fungal community composition. The remaining soil was transferred to 9-cm x 9-cm x 12-cm pots, the bottoms of which were lined with Whatman 3 mm Chr paper to prevent soil particle loss. Pots were then well watered as necessary (two to three times weekly) for three to four weeks, during which time the soil's existing seed bank seedlings were continuously removed. After the bulk of seedling emergence had passed, each treatment was applied to ten replicate pots. Pots were maintained in a greenhouse under ambient conditions and watered as needed. At the conclusion of each generation (approximately ten weeks after germination, or the time at which *M. truncatula* plants began flowering), soil from each pot was sampled and analyzed for fungal community composition and total C and N contents, as described below. Any plants (particularly *A. thaliana*) that had begun flowering prior to the 10 week termination point were clipped of their inflorescence to extend the generation time. The aerial portions of the plant were harvested at the end of each generation, tissue was dried for three days at 70°C, and dry weight biomass was recorded. Plant biomass values are not reported here, as seasonal changes confound interpretation of generational or fungal community effects. A two- to three-week dormancy period (no watering) was applied between each generation to allow the root systems of previous plants to die. The first generation was seeded in July 2005 and harvested October 2005, the second seeded in late October 2005 and harvested in January 2006, and the third seeded in February 2006 and harvested in June 2006.

Experiment 2

To examine whether the observed changes in soil fungal communities in experiment 1 were due to the loss of the resident plants/exudates or the addition of the non-resident plants/exudates, a second experiment was conducted (Fig. 5.1). For this experiment, only those pots that had been treated with resident plants or exudates were used. Resident plants (n = 10) were grown in either IL or TX soil and amended with the root exudates of the non-resident plant. Controls (n = 10) consisted of resident plants only. All other conditions were the same as experiment 1. The experiment was carried out for a total of two generations. The first generation was seeded in July 2006 and harvested October 2006, the second seeded in late November 2006 and harvested in February 2007.

Description of soils

Texas soil was collected in 2005 from under *M. truncatula* plants that were seeded at that location in 2000. Loose litter and plant material was removed from the site, and the top four inches of soil were collected and shipped to Fort Collins, CO. The soil was collected in 2005 at 28° 36' 31.59" N, 97°, 39' 21.43 W, elevation 322 feet. Other common species growing nearby included burr medic (*Medicago polymorpha*), bermudagrass (*Cynodon sp.*), huisache (*Acacia sp.*), mesquite (*Prosopis sp.*), and live oak (*Quercus virginiana*) (William Ocumpaugh, Texas Agricultural Experiment Station Beeville, TX 78102 – personal communication). Texas soil contained 3.38% total carbon and 0.32% total nitrogen before treatment.

Illinois soil was collected from under *A. thaliana* plants. The soil was collected in 2005 at 42° 05' 34" N, 86°, 21' 19" W, elevation 630 feet. Common co-occurring species included chickweed (*Stellaria media*), mouse-ear chickweed (*Cerastium vulgatum*), vetch spp. (*Vicia spp.*), sand bittercress (*Cardamine parviflora*), shepherd's purse (*Capsella bursa-pastoris*), houstonia (*Houstonia spp.*), purple dead nettle (*Lamium purpureum*), whitlow grass (*Draba verna*), small-flowered cranesbill (*Geranium pusillum*), dandelion (*Taraxacum officinale*), asters (*Aster spp.*), curly dock (*Rumex crispus*), field peppergrass (*Lepidium campestre*), bedstraw (*Galium spp.*), hop clover (*Trifolium agrarium*), and field violet (*Viola arvensis*) (Dr. Joy Bergelson, Dr. Joel Kniskern, and Megan Dunning, University of Chicago, IL, USA, pers comm). Illinois soil contained 2.21% total carbon and 0.19% total nitrogen before treatment.

Oregon soil was collected in 2005 from Trask Mountain, at 45° 26' 29" N, 123°, 20' 39" W, elevation 2000 ft. Co-occurring species included an over story of coastal Douglas-fir (*Pseudotsuga menziesii*) and western hemlock (*Tsuga heterophylla*) and an understory consisting of salal (*Gaultheria shallon*), oregon grape (*Mahonia aquifolium*), and sword fern (*Polystichum munitum*) (Dr. Rick Kelsey, USDA – Forest Service, pers comm). Oregon soil contained 5.13 % total carbon and 0.21% total nitrogen before treatment.

Plant material

Arabidopsis thaliana Col-0 and *Medicago truncatula* Jemalong were used for this study as resident or non-resident plant species. *A. thaliana* seeds were surface sterilized for

five minutes in 20% bleach solution, rinsed 4X with sterile deionized water, and germinated directly in pre-moistened experimental soils. *M. truncatula* seeds were scarified in sulfuric acid for five minutes, rinsed until the rinsate measured pH > 5.5, surface-sterilized for ten minutes in 20% bleach solution, rinsed 4X with sterile deionized water, and directly seeded to pre-moistened experimental soils.

Root exudate preparation

Plant root exudates were generated under sterile conditions *in vitro*. *A. thaliana* and *M. truncatula* seeds were surface-sterilized as above, plated on MS agar plates (0.8% agar, 1% sucrose), and the seedlings transferred to liquid MS media (MS, 3% sucrose, pH 5.8) and allowed to grow for two weeks. After two weeks, MS media was removed, plants were rinsed with Hoagland's media (a carbon-free and low nitrogen media, pH 5.8) and grown for three days in Hoagland's media. After three days, the Hoagland's media containing the exudates from each species was collected and held at -20°C until application. This media was replaced with MS media, in which plants were grown for four days. After this time, the procedure was repeated, replacing MS with Hoagland's for exudate collection. This method was used to allow for rapid growth under laboratory conditions while preventing the application of excess carbon/nitrogen to the soil when using root exudates as treatments. Though exudate profiles of soil grown plants are likely to be different than *in-vitro* grown plants, attempts were made to minimize this effect by using an equivalent number of plants for *in-vitro* exudate generation as was used under greenhouse conditions. The exudate solution was then divided equally

(typically 2.0-3.0 mL) among the replicates. Exudates were applied weekly, and watering was scheduled such that water was not applied to the pots within 24 hours of exudate application, to prevent immediate exudate leaching through the soil.

Soil sampling

The top 2.0 cm of soil within a 0.7 cm radius around the crown of the plant was collected using a cork borer sterilized with 20% bleach and rinsed with deionized water. This sampling procedure was used to allow for multi-generation sampling without great soil disturbance between generations. Soils were transferred to scintillation vials and stored at -20°C until processing.

Fungal Community Analysis

Soil samples (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. Soil DNA was quantified using a Nanodrop spectrophotometer (Nanodrop technologies, Wilmington DE) and diluted with distilled water to a concentration of 20 ng μl^{-1}.

Fungal DNA in the soil samples was amplified using fungal-specific primers (ITS1F and ITS4) previously described (White et al., 1990; Gardes and Bruns, 1993; Manter and Vivanco). Quantification of total fungal DNA in the sample was determined by SYBR

green fluorescence (iCycler iQ, Biorad, Hercules, CA) using an external standard curve generated from serial dilutions of fungal DNA obtained from five different fungal species (*Fusarium equiseti*, *Alternaria solani*, *Verticillium* sp., *Rhizoctonia solani*, and *Scletorina sclerotium*). All PCR contained 5 µl (100 ng) soil DNA, 10 µL 2X jumpstart reaction mix (Sigma, St. Louis, MO), 2.4 µL 25 mM $MgCl_2$, 0.2 µl 1 µM fluorescein, 0.4 µl 10 µM forward and reverse primers, and were brought to 20 µl with distilled water. PCR were amplified for 32 cycles (at 95°C – 30 sec, 55°C – 30 sec, 72°C – 60 sec).

Length heterogeneity analysis of PCR amplicons was achieved by capillary electrophoresis (ABI Prism 310, Applied Biosystems) by means of a 5' 6-carboxyfluorescein (FAM)-labeled ITS1-F primer. Briefly, PCR samples from the previously described amplification reactions were diluted with 80 µl of distilled water and 2 µl was added to 13 µl of a loading buffer (12 µl formamide, 0.5 µl 0.3 M NaOH, and 0.5 µl Genescan 2500 (TAMRA) size standard). Analysis conditions were as follows: genescan POP4 polymer, 15 sec 15 kV injection, and 60 min 10 kV electrophoresis. Scoring of amplicons into unique 2 bp bins (or fungal phylotypes) was performed using Genemapper software (vers 4). For each sample, phylotype richness was determined as the number of unique fragments. Phylotype abundance was determined by capillary electrophoretic peak heights of individual phylotypes. Fragments with a peak height below 50 relative fluorescent units were not included in the analysis.

The PCR step of this method selectively amplifies ITS sequences from ascomycetes, basidiomycetes, and zygomycetes. It does not amplify from oomycetes (Manter and Vivanco, 2007), bacteria or plants (Manter, unpublished data). Template heterogeneity prevents its use as an absolutely quantitative measure, but it is reliable in determining relative abundance of phylotypes, as amplicon quantity reflects the abundance of template in the original sample (Manter and Vivanco, 2007). During analysis by electrophoresis, one electrophoretic peak may represent one or more phylotypes. Fragment length heterogeneity analysis likely underestimates phylotype diversity (Manter and Vivanco, 2007).

Soil carbon and nitrogen analysis

Remaining soil samples not used for DNA extraction were dried at 70°C and samples for C:N analysis were generated by pooling two-three replicates for each treatment and generation to provide three replicates for C:N analysis. Dried soil (200 mg) was analyzed for total carbon and nitrogen using a LECO CHN1000 analyzer.

Statistical analysis

All analyses were conducted in SAS 9.1 using the mixed model procedure. Significant changes in the measured fungal community characteristics over time (generations) were analyzed using a repeated measures ANOVA, with soil a fixed effect, generation a repeated measure, and individual pots serving as the subjects. Since pre-treatment

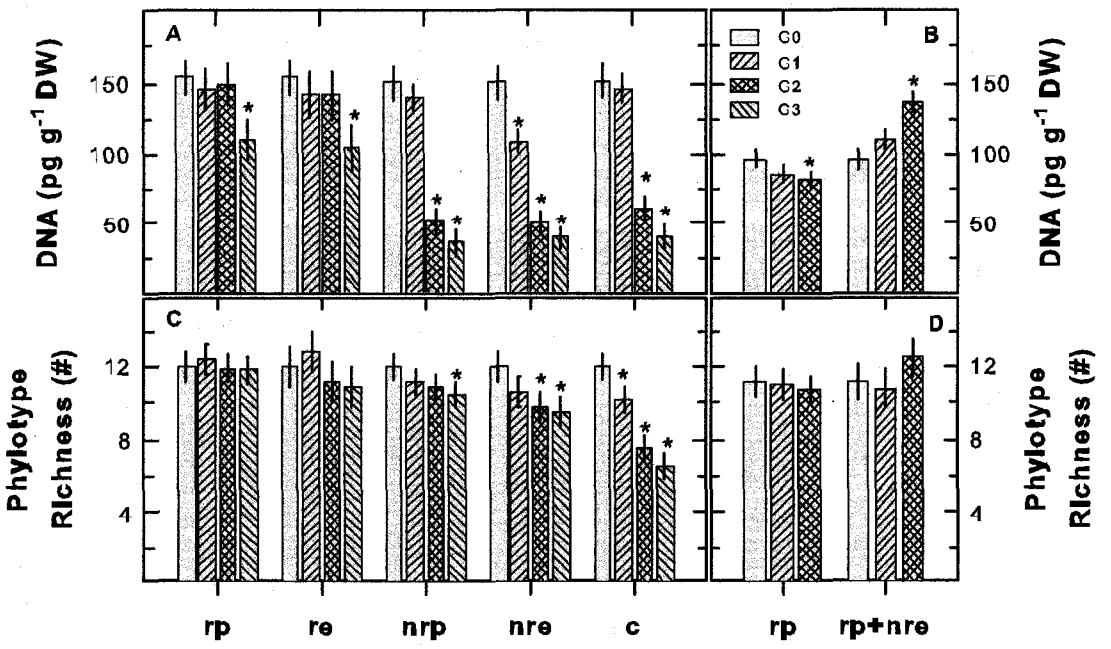
(generation 0) were not available for all pots the average pre-treatment value of the six pre-treatment sub-samples was used. Values of $p < 0.05$ are considered significant for all tests. Community characteristics tested included: total fungal abundance (qPCR), phylotype richness (i.e., number of fragments), individual phylotype abundance (peak heights for each unique fragment), and Sorenson's similarity indices. Sorenson similarity indices were calculated for each individual pot in PC-ORD 5.0.

Results

Experiments were designed to determine how the introduction of novel plant species to a soil would influence the soil fungal community previously established under the influence of other plant species. They also examined the role of root exudates in this process. To test these effects, a greenhouse experiment was designed using two non-invasive 'model' plant species in a variety of natural soils to monitor the dynamics of change in the soil fungal population. Natural soil originally supporting *Arabidopsis thaliana* (Illinois soil) and *Medicago truncatula* (Texas soil) communities for several generations was collected along with a third soil (Oregon forest soil) that had not experienced the growth of either *A. thaliana* or *M. truncatula.* Either *A. thaliana* or *M. truncatula* were grown in (or their exudates applied to) these soils for several plant generations. In this design, *A. thaliana* in Illinois soil and *M. truncatula* in Texas soil are considered 'resident' treatments, while *A. thaliana* in Texas soil, *M. truncatula* in Illinois soil, or either species in Oregon soil are 'non-resident' treatments (Fig. 5.1). Control pots receive neither plants nor exudates.

Real-time PCR analysis of soil DNA demonstrated that fungal biomass levels remain elevated through three generations when a resident plant species is present, and that fungal biomass levels fall rapidly when a non-resident plant species or no plant is present (Fig. 5.2a). These effects are most apparent after two or more generations and are consistent for all three soil types: fungal biomass remains elevated only when a plant species that is a resident of that soil is present. However, even in resident treatments, fungal biomass decreases by the third generation.

Fig 5.2. Change in soil fungal community characteristics in response to *A. thaliana* and *M. truncatula* plants or their exudates. Total fungal DNA estimated from qPCR for (a) experiment 1 and (b) experiment 2. Average phylotype richness for (c) experiment 1 and (d) experiment 2. Treatment codes: no plant/exudate control (c); resident plants (rp) and resident exudates (re) - *A. thaliana* in IL soil and *M. truncatula* in TX soil; non-resident plants (nrp) and non-resident exudates (nre) - *A. thaliana* in IL or OR soil and *M. truncatula* in TX or OR soil; pre-treatment (G0) and post-treatment generations 1-3 (G1, G2, G3). Phylotype richness is based on peak counts following length heterogeneity analysis. * - indicates a significant difference ($p < 0.05$) from the pre-treatment value. Error bars represent standard error of the mean.



Many plant secondary metabolites are known to possess anti-fungal activity, and fungal species have been demonstrated to respond to different plant primary and secondary metabolites that may function as carbon substrates and/or growth-modifying signals. Root exudates may serve as a selective agent through which a plant is able to regulate the fungal community in the surrounding rhizosphere. To test the role of root exudates in structuring the soil fungal community, *in-vitro* grown root exudates from *A. thaliana* and *M. truncatula* were applied to each of the three soils described above. Real-time PCR analysis of the soil fungal community revealed results similar to those obtained from whole plants: non-resident plant root exudates failed to support the native soil fungal community to the extent observed for resident plant root exudates (Fig. 5.2a).

These results suggest two non-exclusive scenarios: 1) there is a net positive effect of resident plants or exudates on the biomass of soil fungi or 2) there is a net negative effect of non-resident plants or exudates on the biomass of soil fungi. To distinguish between the two, a second experiment was initiated (see Fig. 5.1). This experiment was performed using soils from experiment 1 that were treated with resident plants or exudates, and followed them for two additional generations. In this experiment resident plants were grown in all pots and half of the pots were supplemented with non-resident exudates. Using this design, one might expect to observe one of three alternative results: 1) fungal biomass would be expected to accelerate its rate of decline if fungal-growth-inhibiting substances are present in the non-native root exudates, 2) fungal biomass would maintain a comparable rate of decrease if the sole factor driving results in Fig 5.2a was the presence or absence of resident plants and their exudates, or 3) fungal biomass

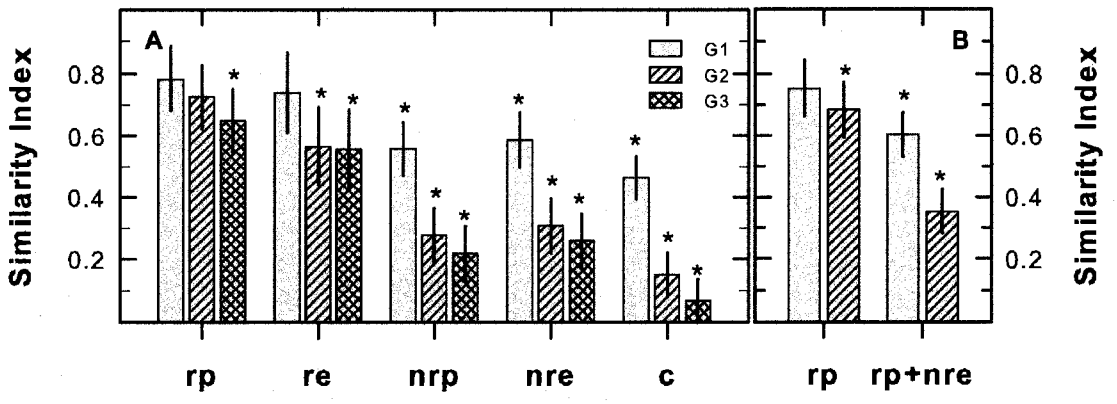
would be expected to increase if carbon substrate (metabolite) diversity is shaping the community. Resident plant treatments continued the trend as observed for the first three generations, with slightly decreasing fungal biomass over time (Fig. 5.2b). However, when resident plants were grown and supplemented with non-resident root exudates, soil fungal biomass increased significantly (Fig. 5.2b).

Biomass is only one measure of the soil fungal community, and not necessarily representative of community complexity. To gain further insight into the community structure, amplified PCR products from biomass measures were analyzed via capillary electrophoresis to gauge community diversity, as measured by phylotype richness. On average, the pre-treatment soils had a phylotype richness of 17, 14, and 11 (phylotypes OR, IL, and TX, respectively). Non-resident plants and root exudates failed to support the diversity of the fungal community as much as resident plants and exudates (Fig. 5.2c). Examination of phylotype diversity in experiment 2, in which pots were treated with resident plants or resident plants supplemented with non-resident exudates, reveals that fungal phylotype richness tended to increase, though not significantly so (Fig 5.2d), when non-resident exudates are applied.

Phylotype richness reports the number of taxa in the soil sample, but provides no information on the quantitative trends for individual taxa. To better understand how the community changed quantitatively in response to treatments, Sorensen's similarity index was calculated, comparing each sample to generation zero. This index takes into account

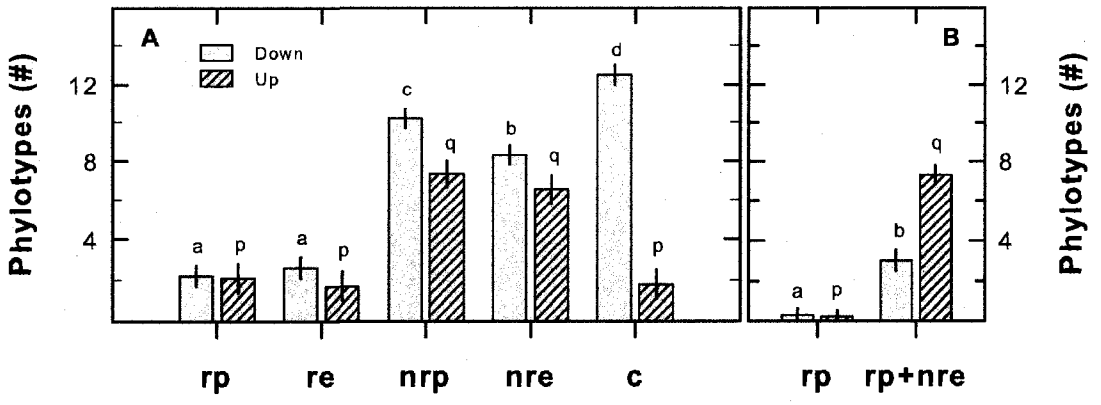
not only phylotype presence/absence, but changes in relative abundance, as measured by capillary electrophoretic peak height, and can respond to both increases and decreases of individual phylotypes. Resident plants and exudates maintained a more similar fungal community than did non-resident plants and exudates or controls (Fig. 5.3a). The second experiment revealed that the community similarity rapidly decreased if non-resident exudates were applied in addition to resident plants (Fig. 5.3b), indicating that the addition of non-resident exudates has a dramatic effect on fungal community structure.

Fig 5.3. Similarity (Sorenson's similarity index) of soil fungal community exposed to *A. thaliana* and *M. truncatula* plants or their exudates as compared to the pre-treatment community. (a) experiment 1 and (b) experiment 2. Treatment codes are the same as Fig. 5.1. * - indicates a significant difference ($p < 0.05$) from the pre-treatment value. Error bars represent standard error of the mean.



Community measures mask the response of individual fungal phylotypes to the treatments. To dissect these treatment effects for individual phylotypes, each phylotype was analyzed with ANOVA and the number of significant increases or decreases is plotted in Fig. 5.4. Treatment with resident plants or exudates resulted in, on average, two phylotypes which decrease in abundance and two phylotypes which increase in abundance between generations zero and three (Fig. 5.4a). In contrast, treatment with non-resident plants reduces abundance of ~10 phylotypes while increasing seven. Likewise, non-resident exudates reduced abundance of eight phylotypes while increasing six (Fig. 5.4a). Supplementing resident plants with non-resident exudates resulted in three phylotypes that decreased in abundance and seven phylotypes that increased in abundance (Fig. 5.4b).

Fig. 5.4. Number of phylotypes significantly decreasing or increasing between pre- and post-treatment (generation 3) for (a) experiment 1 and (b) experiment 2. Treatment codes are the same as Fig. 5.1. Means with different letters are significantly different ($p < 0.05$).



Soil fungi are heterotrophic, thus dependent on soil or plant-derived carbon and/or nitrogen for growth. To ensure that the observed effects were not simply due to altered soil carbon or nitrogen, soil total carbon and nitrogen was quantified on soil remaining from soil samples collected for DNA extraction. The results of this analysis indicate that there was no effect of any treatment on total soil carbon, total soil nitrogen, or the soil carbon to nitrogen ratio (data not shown) indicating that the plant species' specific effects on the soil fungal population are due to the specific composition of roots and their exudates. HPLC-MS profiling revealed dramatic qualitative differences between components of *A. thaliana* and *M. truncatula* root exudates (data not shown).

Discussion

Plants are highly dependent on the soil microbial community and the relationships between plants and microbes are often highly specific and mediated through chemical communication, as demonstrated for legume/rhizobial symbioses (Voegeli and Howard, 1970; Peters et al., 1986; Redmond et al., 1986; Zuanazzi et al., 1998; Bringhurst et al., 2001). Further, microbes are highly dependent on plants for carbon substrates for growth. This interdependence suggests that selective forces would favor tight regulation of the relationship between them, and numerous examples of fungal species responding to chemical signals from a particular plant species are documented (Redman et al., 1999; Nicol et al., 2003; Akiyama et al., 2005). At the community level, the response of the fungal community to a plant is more poorly documented, with many studies targeting specific taxa or functional categories of fungi, such as mycorrhizae. Likewise, root

exudates are often implicated in regulating fungal community structure and abundance (Innes et al., 2004), but convincing scientific data detailing the role of root exudates is sparse (Jones et al., 2004).

In an effort to clarify the role of plants and root exudates in structuring the soil fungal community, multiple soils were treated with either resident plants or their root exudates, non-resident plants or their root exudates, or no-plant/no-exudate controls. This experiment demonstrates that resident plants, or their root exudates in isolation, are capable of maintaining the soil fungal community to a much greater extent than non-resident plants over three generations, and that the effect of plant species was apparent by the second plant generation. These data establish that root exudates of diverse plant species (an arbuscular mycorrhizal and nodulating legume and a non-mycorrhizal Brassicaceae) can influence the composition of soil fungal communities in diverse soil types in the absence of intact root biomass. However, even when resident plants or exudates were applied, soil fungal biomass declined slightly by the third generation. This could be due to depletion of nutrients in the soil other than nitrogen and carbon over multiple generations (Innes et al., 2004) or the reduction of plant species diversity influencing the fungal community. The observed changes in the soil fungal community may be a transient response toward a new stable community driven by the novel plant species. A similar effect has been observed under field conditions with invasive plant species (Batten et al., 2006; Reinhart and Callaway, 2006).

The first experiment displaced resident plant species for non-resident plant species, and therefore confounding variables (loss of one species and gain of a novel species) limit interpretation of the results. Experiment 2 was designed to distinguish between positive growth regulation by resident plants/exudates and negative growth regulation by non-resident plants/exudates. The data from this experiment indicate that plant root exudates regulate the soil fungal community by two mechanisms that act on specific fungal phylotypes. One mechanism serves to reduce relative abundance, through either an antifungal effect of the exudates or a chemical signal that negatively impacts growth, while a second mechanism positively regulates relative abundance, either through growth inducing chemical signals or providing a supply of an appropriate carbon substrate.

Root exudates are estimated to total between two and ten percent of the total fixed carbon for a plant (Jones et al., 2004). The relatively minor absolute abundance of root exudates compared to total root biomass belies the importance of root exudates in structuring the soil fungal community. Recent evidence suggests that root exudation is an active, ATP-dependent process in *A. thaliana* (Loyola-Vargas et al., 2007) and soybean (Sugiyama et al., 2007). The active nature of root exudation coupled with our data suggest that root exudates do not represent a passive loss of carbon to the rhizosphere, as is commonly suggested, but that they play an active role in shaping the soil fungal community. *A. thaliana* is known to accumulate the ubiquitous phenylpropanoids, as well as more phylogenetically restricted glucosinolates in its roots (von Roepenack-Lahaye et al., 2004; Bednarek et al., 2005) and many of these compounds and others are found in root exudates (Narasimhan et al., 2003). *M. truncatula* is known to secrete flavonoids as root

exudates (Peters et al., 1986; Redmond et al., 1986), and accumulates numerous triterpene saponins, flavonoids, and isoflavonoids in its root tissue and as exudates from root-derived cell cultures (Huhman and Sumner, 2002; Huhman et al., 2005; Broeckling et al., 2006; Farag et al., 2007). This wealth of secondary metabolites offers ample opportunity for specificity in the response of fungi to plant root exudates, and our HPLC-MS profiling (data not shown) documents the qualitative differences between *A. thaliana* and *M. truncatula* exudates. Future studies should focus on identifying both the fungal taxa that are selectively responding and the exudate and/or root components that are responsible for this selection.

The data presented in this chapter indicate that plants are able to regulate the proximal soil microbial community. Coupled to research indicating that the soil microbial community influences plant growth (Klironomos, 2002; Klironomos, 2003), the proposition that exotic plant invasion is likely to influence the native soil biota, and that exotic plants will respond to native soil biota seems highly likely. Field sampling of the soil fungal community indicated that *Centaurea maculosa* has a dramatic negative influence on the native North American soil fungal community (Broz et al., 2007). It remains to be seen how much of this effect is derived through root exudation.

Summary and Conclusions

C. maculosa is thought to utilize allelopathy as a mechanism to invade native plant communities in North America. This allelopathic effect has been reported to be through the biosynthesis and secretion of racemic catechin as a root exudate, with catechin accumulation to levels considered toxic to neighboring plants. However, many questions remain regarding the relative importance of catechin in invasion biology, and some studies report extremely low levels of catechin in soil that are unlikely to cause toxicity to competing plant species. The cause of these discrepancies remains uncharacterized, but could include genotypic effects of *C. maculosa*, environmental effects, methodological inconsistencies, or any combination thereof. A limiting factor in understanding patterns in catechin secretion is the measurement of catechin in soil surrounding field grown plants. A rapid and simple assay would provide researchers a tool with which to interrogate the various factors influencing catechin accumulation in a more thorough manner than has been accomplished in the past. An assay using DMACA was developed that is fast, simple, and reliable, which can be used to this end.

In the second portion of this dissertation, the cloning and biochemical and molecular characterization of *C. maculosa* dihydroflavonol reductase, or DFR, is described. This is a gene whose gene product is critical to the biosynthesis of catechin. The results clearly demonstrate the presence of the gene in *C. maculosa*, reveal that the gene product is active when expressed in *E. coli* cells, and clarify the expression profile of this gene in greenhouse grown plants. The gene is expressed at undetectable levels in root tissue, at relatively low levels in rosette leaves, and at the highest levels in inflorescence tissue and

flowers. This pattern of expression calls into question my hypothesis that the biosynthesis of catechin occurs in root tissue, at least in plants grown under greenhouse growth conditions. It remains feasible that under field conditions, catechin is synthesized in the roots or that catechin is synthesized in other tissues and transported to the roots for secretion to the rhizosphere. However, the later alternative is unlikely, as previously published reports indicate that catechin is not found to accumulate in root tissue, as one might expect if transport through the root system were necessary for root exudation. Questions still remain as to the role of DFR in catechin as a root exudate, but characterization of this gene will allow for more detailed gene expression studies as well as potentially developing *C. maculosa* plants deficient in the ability to synthesize and secrete catechin.

Allelopathy remains an ecologically relevant topic of study in *C. maculosa*, but is unlikely to fully explain the invasive behavior of this species. In an effort to provide alternative avenues of research into invasion biology, two other projects were pursued. The first examined the density-dependence of the metabolome of *C. maculosa* rosette leaves from field collected plant material. The results of this study demonstrate that the physiology of plants growing in stands that support high *C. maculosa* density is demonstrably different than plants grown under relatively low density conditions. These results suggest that plants that are growing under high density conditions are more heavily defended by secondary metabolites than low density plants. A supporting study suggests that both specialist root herbivores and seed parasitoids are more abundant under high density growth conditions, which might explain the increased levels of defensive

compounds. It remains unclear whether the herbivores are the cause of the altered physiology, an effect of it, whether two phenomenon are actually independent, or are both products of an unrealized third variable. These can be targets for future study.

Finally, two model plant species were used to examine the roles of root exudates, in isolation, in the structuring of the soil fungal community. This series of experiments revealed that root exudates in the absence of the root tissue, are capable of altering the soil fungal community in a manner that is plant species specific. *A. thaliana* and *M. truncatula* exudates demonstrated differential effects on the soil community, but the root exudates of each species produced results similar to intact plants of the same species. This indicates that a large portion of the ability of a plant to influence soil fungal community structure is through the activity of root exudates. While many have viewed root exudates solely as a carbon source, more recent studies have revealed the critical importance of plant secondary metabolites as signaling compounds through which microbes regulate their growth. Future studies can be developed to target this distinction: is the dramatic activity of root exudates due to specific carbon substrates that favor growth of particular fungal species, antifungal activity specific to particular fungal taxa, or signaling activity that promotes growth of specific taxa? Likely all three potential mechanisms are relevant at some level. These data also suggest that invasive plant species are likely to affect the native soil biota. This effect has been demonstrated with *C. maculosa* (Broz et al., 2007), though it remains to be seen how much of this effect occurs through root exudation.

In conclusion, *C. maculosa* has been extensively studied for its allelopathic behavior, and two tools were developed that will allow for more rigorous study of this behavior. In addition, alternative variables were explored that might be important in the invasive behavior of plants, including density dependent feedback through altered physiology and regulation of the soil fungal community. These results serve as interesting studies in their own right, but also open numerous doors for future studies.

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