THESIS

SOIL ECOLOGICAL INTERACTIONS OF SPOTTED KNAPWEED AND NATIVE PLANT SPECIES

Submitted by
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Graduate Degree Program in Ecology

In partial fulfillment of the requirements
For the Degree of Master of Science
Colorado State University
Fort Collins, Colorado
Spring 2008
COLORADO STATE UNIVERSITY

January 23, 2008

WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR
SUPERVISION BY MATTHEW JEREMIAH SCHULTZ ENTITLED SOIL
ECOLOGICAL INTERACTIONS AMONG SPOTTED KNAPWEED AND NATIVE
PLANT SPECIES BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS
FOR THE DEGREE OF MASTER OF SCIENCE.

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Effective, long-term management methods are lacking for many invasive exotic plants in North America. Planting some native species as “nurse” or “cover” crops may ameliorate adverse conditions, thus improving the performance of other native species. Allelopathy, the production of phytotoxins, has been suggested as a mechanism of invasion for *Centaurea stoeb* (spotted knapweed) in North American grasslands. Some native plants may resist the *Centaurea* allelochemical (±)-catechin by secreting organic acids that detoxify (±)-catechin. My objective was to evaluate whether (±)-catechin-resistant native species can reduce effects of (±)-catechin on (±)-catechin-sensitive native species and thus facilitate their growth in interactions with *Centaurea*. A (±)-catechin-sensitive native species, *Festuca idahoensis*, was grown with or without *Centaurea* or exogenous (±)-catechin, and with or without the (±)-catechin-resistant plants *Gaillardia aristata* and *Lupinus sericeus* or exogenous oxalic acid. Activated carbon, which adsorbs organic compounds, was added to separate effects of resource and interference competition (i.e. allelopathy). However, *Gaillardia*, *Lupinus*, and oxalic acid treatment did not improve *Festuca* biomass when in competition with *Centaurea* or when treated
with (±)-catechin. Several lines of evidence suggest that (±)-catechin did not influence *Centaurea-Festuca* interactions in my experiment. Activated carbon did not improve *Festuca* growth in the presence of *Centaurea*, suggesting that organic compounds from *Centaurea* did not inhibit *Festuca*. Exogenous (±)-catechin appeared to degrade rapidly and had no phytotoxic effect, and *Centaurea* (±)-catechin production was episodic, both of which limited opportunities to observe facilitation. Given the episodic nature of soil (±)-catechin and the conditional nature of (±)-catechin phytotoxicity, the potential benefits for (±)-catechin-sensitive species of planting (±)-catechin-resistant species to detoxify (±)-catechin are unlikely to counterbalance the costs of additional resource competition.

Exotic plant invasion consists of multiple stages each with specific mechanisms for invader success. The mechanisms behind effects of establishment order (i.e. priority effects) on competitive outcomes are unclear. Priority effects could result from interference competition, but this mechanism has never been tested. I conducted a greenhouse experiment to examine effects of establishment order on competition between *Centaurea stoebe*, an allelopathic invasive species, and two native species, *Festuca idahoensis* and *Gaillardia aristata*. Advantages due to prior establishment were analyzed by comparing plant responses to competition when the native species were established before, simultaneously, and after *Centaurea*. Activated carbon amendments were used to separate interference competition from resource competition. Early establishment conferred a strong competitive advantage regardless of species identity. The species that established earlier strongly inhibited the later colonizing species. These results indicate
that *Centaurea* requires competitors to be absent for invasion, and suggest that rapid coloni-
zation of disturbed areas, not superior competitive ability, may contribute to the establish-
ment phase of *Centaurea* invasion. Priority effects were similar for the native spe-
cies and *Centaurea*, perhaps offering an explanation for relictual native species popula-
tions and persistent *Centaurea* infestations. Activated carbon did not alter priority
effects for any species, which suggests that resource competition was more important
than chemical interference competition when the species were established at different
times. In contrast, when the species were established simultaneously there was some
evidence of *Centaurea* allelopathy. These results suggest that allelopathy is not
responsible for *Centaurea* invasion of intact native vegetation, but may play a role during
competition between establishing seedlings of *Centaurea* and some native species.
However, the priority effects in this experiment might have been weaker under field
conditions with more spatial heterogeneity in vegetation or belowground resources.
Understanding priority effects in interactions between native and exotic species may
indicate ways that establishment order can be manipulated to improve community
composition and diversity in restored ecosystems and maximizebiotic resistance to
*Centaurea* invasion.

The rhizosphere dynamics of *Centaurea* (±)-catechin under natural conditions are
poorly understood. (±)-Catechin is often absent from *Centaurea* soil and occurs only
episodically. Pulses in allelochemical concentrations could have phytotoxic effects on
neighboring plants, but measuring episodic (±)-catechin concentrations will require new
sampling approaches that can capture pulses throughout a sampling period. Current
methods for measuring (±)-catechin involve solvent extraction of soil, which is time-consuming, expensive, and might miss a (±)-catechin pulse. My objectives were to determine the ability of adsorbent and sorbent materials to consistently recover (±)-catechin under laboratory conditions and, based on those results, evaluate (±)-catechin degradation after adsorption to gauge applicability in long-term field studies. Three materials, polydimethylsiloxane (PMDS) tubing, nylon bags containing Amberlite™ XAD-7HP resin, and polyester capsules containing 50% IRN-150 resin and 50% Ambersorb® 563 resin, were exposed to (±)-catechin in aqueous solution and later extracted to determine recovery. Of the three materials tested, Amberlite™ XAD-7HP resin bags recovered the greatest amount of (±)-catechin. Amberlite™ XAD-7HP resin bags were then impregnated with (±)-catechin, and placed outside underground for a variable number of weeks to assess the degradation of adsorbed (±)-catechin over time. Even though Amberlite™ XAD-7HP readily adsorbed (±)-catechin, variable losses occurred over time. The reliability of Amberlite™ XAD-7HP for (±)-catechin recovery after one week is questionable. Deploying resin bags for longer than two weeks risks loss of adsorbed catechin as little (±)-catechin recovery was observed after the third week of incubation. When designing future experiments, it may be necessary to replace resin bags every two weeks for continuous sampling of (±)-catechin which involves considerable sampling effort. Amberlite™ XAD-7HP offers promise for recovering (±)-catechin from various experimental designs over limited periods of time, but numerous field conditions could affect adsorption and loss. Given the small number of materials tested, screening of other materials in the future could be beneficial. The use of
adsorbent materials could elucidate allelochemical dynamics and clarify the role of (±)-catechin in *Centaurea* invasion.

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ACKNOWLEDGMENTS

I thank my partner Nora Love for her extreme patience, kindness, and good cheer over the last few years. I would also like to thank my family for their years of support in all my endeavors no matter where they might occur. I am grateful for the support and guidance of my advisor Dr. Mark Paschke. In addition, I greatly appreciate all the ideas and encouragement from my committee members, Drs. Jim Ippolito, Laura Perry, and Jorge Vivanco. And also thanks to Restoration Ecology Lab members, graduate students, and Seth Munson for contributing to a stimulating environment.
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CHAPTER 1

Effects of (±)-catechin-resistant species on (±)-catechin-sensitive species in interactions

with Centaurea stoebe
1.1 INTRODUCTION

Large areas of the United States are infested by exotic plant species that reduce biodiversity and habitat quality (D'Antonio and Meyerson 2002). Effective, long-term management methods are lacking for many of North America’s most destructive exotic plants, including knapweeds (*Centaurea stoebe*, *C. diffusa*, and *A. repens*), which occupy approximately three million hectares in western North America (Duncan et al. 2004). Conventional exotic plant control measures often rely on frequent and expensive herbicide applications, which do not necessarily favor native species establishment and may have adverse environmental effects. Further, eradicated exotic plant populations may have residual effects, such as altered soil chemistry, nutrient cycling, or microbiotic communities, all of which may impede long-term restoration goals (Vitousek 1990, Wolfe and Klironomos 2005).

Planting some native species as “nurse” or “cover” crops may ameliorate adverse conditions, thus improving the performance of other native species (King and Hobbs 2006, Padilla and Pugnaire 2006). Facilitation in plant interactions often occurs through edaphic modification (Bertness and Callaway 1994, Callaway 1995, Callaway and Walker 1997), including alteration of soil moisture (Kennedy and Sousa 2006), nutrients (Pugnaire et al. 1996), structure (Rhoades et al. 1998), oxygenation (Callaway and King 1996), or substrate stabilization (Bruno 2000). Cover crops also have been used to competitively exclude exotic species and favor other native species (Simmons 2005). However, cover crops often non-selectively suppress native species as well as exotic
species (Perry and Galatowitsch 2003, 2006). To reduce exotic species success and improve native species success, cover crops must create conditions that negatively influence exotic species more than native species.

The novel weapons hypothesis for plant invasions argues that some exotic plants may be successful in new ranges because they produce phytotoxins (i.e., allelochemicals) that are novel to resident native species (Callaway and Aschehoug 2000, Callaway and Ridenour 2004). When invasion is mediated by allelopathy, native plants that are relatively tolerant of the allelochemical(s) involved may be useful as cover crops for competing with allelopathic exotic species and favoring other, more sensitive native species. Allelopathy has been suggested as a mechanism of invasion for knapweeds in North American grasslands (Callaway and Aschehoug 2000, Bais et al. 2003, Stermitz et al. 2003, Vivanco et al. 2004, Alford et al. 2007); however, the potency and stability of the allelochemicals under natural conditions is still a matter of debate (Blair et al. 2005, Blair et al. 2006). Native North American plant species vary widely in sensitivity to the *Centaurea stoebe* L. (spotted knapweed) allelochemical (±)-catechin (Perry et al. 2005a). Some native plants, such as *Gaillardia aristata* Pursh (blanketflower) and *Lupinus sericeus* Pursh (silky lupine), may resist (±)-catechin phytotoxicity by secreting organic acids that detoxify (±)-catechin (Weir et al. 2006). Here, I explore whether cover crops that are allelochemical-resistant may be able to act selectively against allelopathic exotic plants. I hypothesized that native plant species that excrete large quantities of organic acids into the rhizosphere and are relatively resistant to (±)-catechin can reduce effects of
(+)-catechin on (+)-catechin-sensitive native species and thus facilitate their establishment in interactions with spotted knapweed.

1.2 METHODS

EXPERIMENTAL DESIGN

A (+)-catechin-sensitive native species, Festuca idahoensis Elmer var. Winchester (Idaho fescue), was grown with or without Centaurea stoebe L. (spotted knapweed), and with or without the (+)-catechin-resistant plants Gaillardia aristata Pursh (blanketflower) and Lupinus sericeus Pursh (silky lupine), to determine whether (+)-catechin-resistant plants that secrete organic acids can increase the success of (+)-catechin-sensitive plants exposed to spotted knapweed. A synthetic organic acid was added to the media of Festuca growing with Centaurea to test for organic acid detoxification of Centaurea allelochemicals. In addition, (+)-catechin (Figure 1.1) was added to the media of Festuca growing with each of the (+)-catechin-resistant species, to test whether the (+)-catechin-resistant species improved Festuca growth by detoxifying (+)-catechin. Activated carbon, which adsorbs organic compounds (Cheremisinoff and Ellerbusch 1978, Mahall and Callaway 1992), was added to the media in half of the pots for each treatment to test for soil chemical effects on Festuca performance. Thus, the experimental design included four organic acid treatments (none, Gaillardia, Lupinus, and pure oxalic acid), three (+)-catechin treatments (none, Centaurea, and pure (+)-catechin), and two activated carbon treatments (present and absent) (Figure 1.2). Each of the 24 treatment combinations was replicated seven times.
Figure 1.1. Chemical structure of (+)-catechin and (-)-catechin.

Figure 1.2. Detailed schematic of the 24 treatments and planting positions within the 24-cm x 2.5-cm, 40-cm deep rectangular pots (hereafter rootboxes). The factorial experimental design included four organic acid treatments (none, *Gaillardia*, *Lupinus*, and pure oxalic acid), three (±)-catechin treatments (none, *Centaurea*, and pure (±)-catechin), and two activated carbon treatments (present and absent) for a total of 24 treatments. The planting positions consisted of *Centaurea* seedlings planted at each end of the rootbox. Two seedlings of the (±)-catechin-resistant species were planted between the *Centaurea* location and the center. One *Festuca* seedling was planted at the center of each rootbox.
PLANTING

The greenhouse experiment was conducted in 24-cm x 2.5-cm, 40-cm deep rectangular pots (hereafter rootboxes; Fort Collins Plastics; Fort Collins, CO), which were angled 45° to promote root growth via gravity to a clear Plexiglas side with a hinged door which allowed experimental access (Figure 1.3). The Plexiglas doors were covered with an opaque vinyl material and secured with Velcro straps (FasTech; Jacksonville, FL) (Figure 1.3). Each rootbox contained a 2.5 cm layer of sterilized pea gravel (Permagreen Products, Arvada, CO) beneath 35 cm of a 1:1:1 mixture (by volume) of sterilized washed sand (US Mix Products Co.; Denver, CO), vermiculite (Therm-o-rock East Inc.; New Eagle, PA), and calcine clay (Schultz Clay Soil Conditioner; Bridgeton, MO) (Figure 1.4). In half of the rootboxes, the sterilized media mixture was amended with 20 mL activated carbon per L of media (Sigma-Aldrich Co.; St. Louis, MO) (Figure 1.4). All media components were sterilized in a Tuttnauer Brinkmann 3870E autoclave (Westbury, NY) for 30 minutes at 121°C.
**Figure 1.3.** The angled 24-cm x 2.5-cm x 40-cm rootbox. The angle promotes root growth via gravity to the clear Plexiglas side covered with vinyl.

_Centaurea_ was planted in 56 (1/3) of the rootboxes in August 2007 and given five weeks to establish before the other species were planted, in order to increase allelopathic effects from _Centaurea_ on the other species. Two _Centaurea_ seedlings were planted in each of the 56 rootboxes, with one plant at each end (Figure 1.2). After five weeks, two seedlings of the (±)-catechin-resistant species were planted in 42 (1/4) of the rootboxes between the _Centaurea_ location and the center (Figures 1.2 and 1.3). Ten weeks after planting _Centaurea_, one _Festuca_ seedling was planted at the center of each rootbox (N=168) (Figure 1.2). The (±)-catechin-resistant species were planted before _Festuca_ to allow them to detoxify (±)-catechin before _Festuca_ exposure. After planting _Festuca_, the experiment was maintained for 25 weeks, until April 2007. _Centaurea_ seeds were
collected from Missoula County, Montana, *Gaillardia* seeds were purchased from Wind River Seed (Manderson, WY), and *Festuca* and *Lupinus* seeds were purchased from Granite Seed Company (Lehi, UT).

**Figure 1.4.** A rootbox containing two *Gaillardia aristata* adults with roots clearly visible. Each rootbox contained a 2.5 cm layer of sterilized pea gravel beneath 35 cm of a 1:1:1 mixture (by volume) of sterilized washed sand, vermiculite, and calcine clay. In half of the rootboxes (including the rootbox shown), the sterilized media mixture was amended with 20 mL activated carbon per L of media.
CHEMICAL ADDITIONS

Beginning one week after the Festuca planting, (±)-catechin and oxalic acid solutions were added bimonthly to specific rootboxes. A 0.8 mg mL\(^{-1}\) (±)-catechin solution (Figure 1.1, Shivambu International; New Delhi, India) was applied to the 42 catechin-treated rootboxes. A 100 µM (~9 µg mL\(^{-1}\)) solution of oxalic acid (Figure 1.5, Sigma-Aldrich Co.; St. Louis, MO) was applied to the 28 oxalic acid-treated rootboxes. A solution of 0.8 mg ml\(^{-1}\) (±)-catechin and 100 µM oxalic acid was applied to the 14 catechin + oxalic acid treated rootboxes. Weir et al. (2006) reported that (±)-catechin and oxalic acid remained stable in co-incubation studies. To solubilize the (±)-catechin, all solutions were made with 0.5% methanol (MeOH) in distilled water. The 84 untreated rootboxes received a control solution of 0.5% MeOH. To add 100 mL of each solution, the rootbox door was opened and 2 mL of solution added to 50 points in a 5 x 10 grid overlaying the top \(\frac{1}{2}\) of each box (Figure 1.6). All chemical concentrations were based on Weir et al. (2006).

![Chemical structure of oxalic acid](image)

**Figure 1.5.** Chemical structure of oxalic acid.
Figure 1.6. (±)-Catechin and oxalic acid solutions were added bimonthly to specific rootboxes. A 0.8 mg mL⁻¹ (±)-catechin solution was applied to the 42 catechin-treated rootboxes. A 100 µM (~9 µg mL⁻¹) solution of oxalic acid was applied to the 28 oxalic acid-treated rootboxes. A solution of 0.8 mg mL⁻¹ (±)-catechin and 100 µM oxalic acid was applied to the 14 catechin + oxalic acid treated rootboxes. To solubilize the (±)-catechin, all solutions were made with 0.5% methanol (MeOH) in distilled water. The 84 untreated rootboxes received a control solution of 0.5% MeOH. To add 100 mL of each solution, the rootbox door was opened and 2 mL of solution added to 50 points in a 5 x 10 grid overlaying the top ½ of each box.

EXPERIMENTAL MAINTENANCE

The experiment was arranged on a greenhouse bench in a randomized complete block design (Figure 1.7). Each block contained one replicate of each treatment randomly placed in three adjacent crates (Eldon SuperCrate-A-File; Oak Brook, IL). Greenhouse temperatures ranged from 21 to 28°C during the day, and from 13 to 21°C at night. High pressure sodium lights were used to supplement natural light for a simulated 16 hour day. Blocks and rootboxes within blocks were rotated bimonthly to avoid environmental gradient effects. Rootboxes were fertilized thrice weekly with a ¼X
strength complete nutrient solution (Hussdanell 1978) with N adjusted to 2X strength. To avoid calcium precipitation, calcium sulfate and calcium chloride were applied in a separate ¼X strength solution thrice weekly alternating with the other solution. I provided N at a high rate to prevent positive effects of *Lupinus* N-fixation from masking positive effects of *Lupinus* organic acid secretion. Eight weeks after planting *Festuca*, the rootbox media was inoculated with soil biota by applying 10 mL of a liquid slurry of 200 cm³ of *Festuca* rhizosphere soil (Waterworks, Montana) in 1000 mL distilled water. The slurry was mechanically shaken for 1 hour, allowed to settle for 15 minutes, and decanted before application. To expose the plants to UV radiation, the rootboxes were taken outdoors thrice weekly for approximately 5 hours during the last month of the experiment.

![Figure 1.7](image.png)

**Figure 1.7.** The complete randomized block design of the rootboxes with each block consisting of one replicate (7 replicates=7 blocks total) of each treatment (N=24 rootboxes) in three plastic crates arranged on a greenhouse bench. Blocks and rootboxes within blocks were rotated bimonthly to avoid environmental gradient effects.
MEDIA CHEMICAL EXTRACTION AND ANALYSIS

Media (±)-catechin concentrations were monitored every four weeks, one week after (±)-catechin additions, using standard techniques (Perry et al. 2005b). To limit the media analysis to a reasonable number, samples were collected from three replicates of each treatment combination avoiding treatments with activated carbon. To collect media samples, the rootbox door was opened and 0.25 cm$^3$ samples were collected from points at 4-cm intervals along rows 5 cm and 25 cm below the media surface, for a total of 10 points (Figure 1.8). The samples from each rootbox were pooled, forming a 2.5 cm$^3$ sample in a 15 mL centrifuge tube. 10 mL of 100% MeOH were immediately added to extract the (±)-catechin. The extracts were then vortex mixed, centrifuged, transferred, concentrated, and resuspended in 0.4 mL MeOH. MeOH negative controls were included during sampling to test for contamination. (±)-Catechin concentrations were determined by high-performance liquid chromatography (HPLC) in comparison to 1 mg mL$^{-1}$ standards and are expressed on a g$^{-1}$ dry media basis as in Perry et al. (2005b). (±)-Catechin recovery efficiency from the artificial soil media with this method is 53 ± 12 [SD] % (Schultz, unpublished data). Samples testing positive for (±)-catechin by HPLC were confirmed with mass spectroscopy (Thermo Finnigan Surveyor MSQ; San Jose, CA). During the final two months, samples were assayed for (±)-catechin using a colorimetric reagent dimethylaminocinnamaldehyde (DMACA) (Broeckling and Vivanco in press). Samples testing positive for (±)-catechin by this method were then processed and analyzed by HPLC.
Figure 1.8. Media (±)-catechin concentrations were monitored every four weeks. To collect media samples, the hinged Plexiglas door was opened and 0.25 cm³ samples were collected from points at 4-cm intervals along rows 5 cm and 25 cm below the media surface, for a total of 10 points (represented by yellow diamonds). The samples from each rootbox were pooled, forming a 2.5 cm³ sample in a 15 mL centrifuge tube, and 10 mL of 100% MeOH were immediately added to extract the (±)-catechin.

PLANT MEASUREMENTS

After 25 weeks, the plants were individually harvested, dried at 60°C to a constant mass, and aboveground biomass determined. Essential nutrients in aboveground Festuca tissue samples treated solely with activated carbon, (±)-catechin, or oxalic acid were analyzed using inductively coupled plasma-atomic emission spectroscopy (Thermo Jarrell Ash IRIS Advantage Dual View High Resolution ICP-AES; Thermo Jarrell Ash Corp.; Franklin, MA; Soltanpour et al. 1996). Tissue nitrogen concentration was measured by elemental analysis on a Leco TruSpec C/N analyzer (LECO Corp.; St. Joseph, MI; Nelson and Sommers 1996).
DATA ANALYSIS

Statistical analyses were performed using SAS Proc GLM (SAS Institute Inc.; Cary, NC). For Festuca biomass and tissue nutrient concentrations, effects of the (±)-catechin, organic acid, and activated carbon treatments were examined using a three-way ANOVA. For Centaurea biomass, effects of the organic acid and activated carbon treatments were examined with a two-way ANOVA. For Gaillardia and Lupinus biomass, effects of the (±)-catechin and activated carbon treatments were compared with two-way ANOVAs. All ANOVAs included block as an additional factor. Significant main effects were examined with Tukey HSD means comparison tests (α=0.05). Festuca, Gaillardia, and Lupinus biomass, as well as Festuca tissue B, Ca, Cu, Fe, Mg, Na, and Zn concentrations, were log-transformed for analysis to correct unequal variances, but the results are presented with the untransformed data.

1.3 RESULTS

FESTUCA

Competition with Centaurea and Gaillardia reduced mean Festuca biomass by 61% and 42%, respectively (Figures 1.9 and 1.10, Tukey HSD, p<0.05, ANOVA, $F_{2,138}=29.49$, p<0.0001 and $F_{3,138}=9.09$, p<0.0001 respectively). Treatment with (±)-catechin, oxalic acid, or Lupinus did not significantly affect Festuca biomass (Tukey HSD, p>0.05). Activated carbon decreased mean Festuca biomass by 51 % (Figure 1.9, ANOVA, $F_{1,138}=41.57$, p<0.0001). No treatment interactions were significant. In particular, Gaillardia, Lupinus, oxalic acid treatment, and activated carbon did not improve Festuca biomass in competition with Centaurea or when treated with (±)-
catechin. Activated carbon, pure (±)-catechin, and pure oxalic acid did not significantly affect nutrient concentrations in *Festuca* aboveground tissue (data not shown).

**Figure 1.9.** *Festuca* aboveground biomass in response to catechin and activated carbon treatments without organic acid treatments. Bars show means ± 1 standard error of the mean. Shared letters indicate no significant difference in Tukey multiple comparison *t*-tests (*α*=0.05). Data were log transformed for analysis but are shown in the original scale.
Figure 1.10. *Festuca* aboveground biomass in response to catechin and organic acid treatments without activated carbon. Bars show means ± 1 standard error of the mean. (*) indicate significant differences between controls and organic acid treatments in each catechin treatment category (Tukey multiple comparison *t*-test, α=0.05). Data were log transformed for analysis but are shown in the original scale.

**CENTAUREA, GAILLARDIA, AND LUPINUS BIOMASS**

Activated carbon increased mean *Centaurea* biomass by 35%, from 45.6 ± 2.2 g to 61.7 ± 1.7 g (ANOVA, $F_{1,42}=28.13$, p<0.0001). The organic acid treatments did not significantly affect *Centaurea* biomass (Figure 1.11). *Centaurea* decreased mean *Gaillardia* and *Lupinus* biomass (Figure 1.11, Tukey HSD, p<0.05, ANOVA, $F_{2,30}=10.87$, p=0.0003 and ANOVA, $F_{2,30}=23.06$, p<0.0001 respectively). Neither (±)-catechin nor activated carbon affected *Gaillardia* or *Lupinus* biomass (data not shown).
Figure 1.11. Mean aboveground biomass in response to competition with *Festuca* (2-species mixture) and *Festuca* with cover crops or *Centaurea* (3-species mixture). Bars show means ± 1 standard error of the mean. (*) indicate significant differences between interspecific competition with a 2-species mixture and a 3-species mixture (Tukey multiple comparison *t*-test, \( \alpha=0.05 \)). Data were log transformed for analysis but are shown in the original scale.

MEDIA (±)-CATECHIN CONCENTRATIONS

On most sampling dates, (±)-catechin was not detected in any of the rootboxes. At the end of the third month of the experiment (January 2007), (±)-catechin occurred in a number of treatments at concentrations ranging from 2.6 to 348 µg g\(^{-1}\) (Table 1.1). However, (±)-catechin concentrations did not differ significantly among treatments.
Table 1.1. Mean, standard error of the mean (SEM), and range of (±)-catechin concentrations detected in the third month of the experiment. N=3.

<table>
<thead>
<tr>
<th>(±)-Catechin</th>
<th>Organic acid</th>
<th>(±)-catechin (µg g⁻¹)</th>
<th>Mean</th>
<th>SEM</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>control</td>
<td>37.80</td>
<td>37.80</td>
<td>0 - 113.40</td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>Gaillardia</td>
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<td>19.06</td>
<td>0 - 60.33</td>
<td></td>
</tr>
<tr>
<td>control</td>
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<td>3.53</td>
<td>0 - 10.59</td>
<td></td>
</tr>
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<td>0 - 4.14</td>
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</table>

1.4 DISCUSSION

The hypothesis that native (±)-catechin-resistant species would facilitate (±)-catechin-sensitive vegetation in the presence of Centaurea and its allelochemical (±)-catechin was not supported. Neither Gaillardia, Lupinus, nor pure oxalic acid increased Festuca aboveground biomass in competition with Centaurea or when treated with pure (±)-catechin (Figure 1.10). Gaillardia and Lupinus had different effects on Festuca in the absence of Centaurea. Lupinus did not affect Festuca biomass, while Gaillardia reduced Festuca biomass similarly to Centaurea (Figure 1.10). These results agree with other observations that native Gaillardia spp. can be relatively strong competitors (Simmons, 2005), but suggest that planting Gaillardia may reduce success of other native species substantially under some conditions.
Several lines of evidence suggest that (±)-catechin did not mediate *Centaurea-Festuca* interactions in my experiment, which may explain why *Gaillardia*, *Lupinus*, and oxalic acid failed to facilitate *Festuca* in the presence of *Centaurea*. First, activated carbon amendments, which were intended to adsorb allelochemicals (Ridenour and Callaway 2001), did not improve *Festuca* growth in the presence of *Centaurea*. Second, (±)-catechin was rarely detected in the rootbox media planted with *Centaurea* or treated with pure (±)-catechin. Third, treatment with relatively high concentrations of pure (±)-catechin did not reduce *Festuca* biomass. Together, these results suggest that effects of *Centaurea* on *Festuca* were mediated by resource competition rather than interference competition (i.e., allelopathy) under my experimental conditions.

Activated carbon amendments did not positively affect *Centaurea-Festuca* interactions, suggesting that *Centaurea* soil allelochemicals did not inhibit *Festuca* in my experiment. This is in contrast to results of Ridenour and Callaway (2001), who used the same activated carbon concentration but a different type of container and soil substrate. Direct effects of activated carbon on *Festuca* and perhaps *Centaurea* in my experiment, however, also may have masked effects of activated carbon on *Centaurea-Festuca* interactions. Activated carbon reduced mean *Festuca* biomass by 51% in the presence and absence of competitors. In contrast, activated carbon increased mean *Centaurea* biomass by 35%, either because of lower *Festuca* competition or because of direct effects of activated carbon. Other studies also have observed direct, species-specific effects of activated carbon (Ridenour and Callaway 2001, Kulmatiski and Beard 2006, Newingham and Callaway 2006, Lau et al. in press). Numerous factors could explain
such effects. Activated carbon can alter soil nutrient availability (Kulmatiski and Beard 2006, Lau et al. in press), water retention (Ridenour and Callaway 2001, Inderjit and Callaway 2003) and pH (Inderjit and Callaway 2003, Lau et al. in press). In this experiment, activated carbon did not affect nutrient levels in *Festuca* tissue, but carbon effects on water retention and pH were not tested. Activated carbon also may alter microbial community composition (Pietikainen et al. 2000) or non-selectively adsorb other critical chemicals in the rhizosphere (Pan and van Staden 1998). The direct effects of activated carbon on *Festuca* and perhaps *Centaurea* in my experiment make it impossible to separate allelopathic effects from resource competition with certainty. Although activated carbon offers a convenient method for potentially separating resource and interference competition, further research is required to minimize activated carbon effects on other soil chemical, hydrological, and microbial characteristics.

The absence of (±)-catechin on most sampling dates in rootboxes planted with *Centaurea* suggests that *Centaurea* (±)-catechin production was episodic rather than consistent. Initial work on *Centaurea* (±)-catechin production suggested that *Centaurea* consistently produced high soil concentrations, with means ranging from 300 to 3,600 µg g\(^{-1}\) (Bais et al. 2003, Perry et al. 2005b, Thelen et al. 2005). More recent research, however, including the present study, suggests that (±)-catechin is most often absent from *Centaurea* bulk soil (Blair et al. 2005, Perry et al. 2007, Schultz unpublished data) and occurs only episodically at mean concentrations ranging from 1 µg g\(^{-1}\) (Blair et al. 2006) to ~600 µg g\(^{-1}\) (Perry et al. 2007). On the one sampling date in which I detected (±)-catechin, it was present at comparable concentrations in rootboxes with and without
Centaurea or pure (±)-catechin (Table 1.1), suggesting that Centaurea and (±)-catechin treatments were not the source of (±)-catechin in at least some rootboxes. Festuca, Gaillardia, Lupinus, and/or soil microbes in the media also may have produced the (±)-catechin. Specific environmental conditions may be necessary for (±)-catechin production or accumulation in soil (Perry et al. 2007), but the factors that may have caused the (±)-catechin episode in my experiment are unclear. Regardless, the absence of (±)-catechin on most sampling dates in rootboxes planted with Centaurea may explain in part why the (±)-catechin-resistant species failed to improve Festuca growth in the presence of Centaurea.

Pure (±)-catechin did not reduce Festuca biomass in my experiment, suggesting that (±)-catechin phytotoxicity also was limited under my experimental conditions. Concentrations required for (±)-catechin phytotoxicity vary widely among studies, with some reporting strong phytotoxic effects of 50 µg mL\(^{-1}\) (±)-catechin on Festuca growth (Weir et al. 2003) and others reporting only weak phytotoxicity of 1000 µg mL\(^{-1}\) on Festuca (Blair et al. 2005). I applied a relatively high concentration, 800 µg mL\(^{-1}\), and observed no effect on Festuca. The absence of (±)-catechin in media treated with pure (±)-catechin on most sampling dates in my experiment suggests that (±)-catechin may have degraded rapidly or become bound to the media in the rootboxes after treatment. Other studies also have reported rapid declines in (±)-catechin concentrations after soil application (Blair et al. 2005, Inderjit et al. in review, Pollock et al in prep). Soil type (Blair et al. 2005, Furubayashi et al. 2007, Inderjit et al. in review), pH (Blair et al. 2005, Furubayashi et al. 2007), moisture (Blair et al. 2006), and metal content (Pollock et al. in prep).
prep) each can influence (±)-catechin degradation or binding to soil constituents. The 33% clay in my media may have increased (±)-catechin adsorption, since clay adsorbs phenolics (Nayak and Singh 2007), but effects of clay on (±)-catechin (a phenolic compound) have not been tested (Furubayashi et al. 2007). Given my ~50% (±)-catechin recovery efficiency, however, I likely would have detected (±)-catechin had it been present. Inderjit et al. (in review) observed phytotoxic effects even after (±)-catechin concentrations had declined in (±)-catechin-amended soil. Under my experimental conditions, however, (±)-catechin that was applied at relatively high concentrations and appeared to degrade rapidly had no phytotoxic effect.

Given the episodic nature of soil (±)-catechin and the conditional nature of (±)-catechin phytotoxicity, the potential benefits for (±)-catechin-sensitive species such of planting (±)-catechin-resistant species to detoxify (±)-catechin might not be expected to counterbalance the costs of additional resource competition. In conceptual models of facilitation, outcomes of species interactions are a function of positive, facilitative effects and negative effects of resource and interference competition (Callaway 1995). In the case of interactions between allelochemical-resistant and allelochemical-sensitive species, the balance between competition and facilitation may depend in part on the duration and concentration of allelochemical exposure. In my experiment, the lack of (±)-catechin exposure may have limited opportunities for facilitation, shifting the net balance towards resource competition. Conditions that increase allelochemical exposure, however, could change the balance between facilitation and resource competition in interactions between allelochemical-resistant and allelochemical-sensitive species.
Continued investigation of the conditions required for allelochemical production, stability, and phytotoxicity may indicate conditions under which allelochemical-resistant species are more likely to improve restoration success.

1.5 REFERENCES


CHAPTER 2

Importance of establishment order in competition between native plant species and

*Centaurea stoebe*
2.1 INTRODUCTION

Exotic plant invasion consists of four stages: introduction, establishment, spread, and impact (Levine et al. 2004). Each stage involves specific mechanisms for invader success (Gurvich et al. 2005, Seastedt and Suding 2007) with competition between native and exotic species varying between stages (Vila and Weiner 2004). Research has focused mainly on the mechanisms responsible for spread and impact (Seastedt and Suding 2007), yet the introduction and establishment stages initiate the invasion process. Some exotic plant species can invade relatively undisturbed native plant communities (King and Grace 2000), which suggests that they are competitively superior to native species even as seedlings competing with established adults. Other exotic species may require disturbance to initiate invasion, but once established persist as an alternative stable state excluding native species establishment (Corbin and D'Antonio 2004, Kulmatiski 2006).

The mechanisms behind effects of establishment order on competitive outcomes (i.e., priority effects, sensu Belyea and Lancaster 1999) are unclear. In some cases, size asymmetry between early and late colonizers may result in resource preemption by the earlier colonizers (Ross and Harper 1972, Miller 1987, Weiner 1990). However, this mechanism may be relevant only in light-limited environments, since competition for belowground resources is not thought to be asymmetric (Wilson 1988, Schwinning and Weiner 1998). In other cases, early colonizers may generate positive feedbacks that favor their own persistence and reduce success of later colonizers, by altering soil processes (Vitousek and Walker 1989, Duda et al. 2003), microbial communities
(Klironomos 2002, Callaway et al. 2004), herbivore populations (Chase 2003), or other factors.

Priority effects could also result from interference competition (i.e., allelopathy), but this mechanism has never been tested. Allelopathy, the production of plant phytotoxins, could lead to priority effects since established plants have the resources to invest in chemical interference. Established root systems could then exude allelochemicals in response to encroachment. Thus, by using allelopathy, species that establish earlier might outcompete species that are superior resource competitors but that arrive later (Amarasekare 2002).

Allelopathy has been suggested as a mechanism of invasion for *Centaurea stoebe* (spotted knapweed) (Callaway and Aschehoug 2000, Ridenour and Callaway 2001, Bais et al. 2003), making *Centaurea* a potential model organism for evaluating the roles of resource and interference competition in *Centaurea* establishment and dominance. In some cases, *Centaurea* spp. are unable to invade established plant communities (Pokorny et al. 2005, Seastedt and Suding 2007). Established plant community productivity is an important constraint to *Centaurea stoebe* invasion (Rinella et al. 2007), but other rhizosphere interactions may have an effect on competitive outcomes depending on the establishment order of species.

I conducted a greenhouse experiment to examine effects of establishment order on competition between *Centaurea* and two native species, *Festuca idahoensis* (Idaho
fescue) and *Gaillardia aristata* (common blanketflower). Advantages due to prior establishment were analyzed by comparing plant responses to competition when the native species were established before, simultaneously, and after *Centaurea*. Activated carbon amendments, which adsorb large organic compounds, were used to separate interference competition from resource competition. I hypothesized (1) that *Centaurea* would require competitors to be absent in order to establish and (2) that once established *Centaurea* would rely on allelopathy to exclude native species.

### 2.2 METHODS

**EXPERIMENTAL DESIGN**

Two native species, *Gaillardia aristata* Pursh (blanketflower), and *Festuca idahoensis* Elmer var. Winchester (Idaho fescue), were established before, simultaneously, and after the invasive exotic, *Centaurea stoebe* L. (spotted knapweed). Activated carbon, which adsorbs organic compounds (Cheremisinoff and Ellerbusch 1978) including allelochemicals (Mahall and Callaway 1992), was added to test for allelopathic effects on competitive outcomes. Thus, the experimental design included two species combinations (*Centaurea:* *Festuca* and *Centaurea:* *Gaillardia*), three orders of establishment (native species first, simultaneously, and after *Centaurea*), and two activated carbon treatments (present and absent). Monoculture controls for each species were included at the three establishment times with and without activated carbon. The 12 experimental treatments and 18 control combinations were replicated 10 times for a total of 300 units in a complete randomized design.
PLANTING

The greenhouse experiment was conducted in 6.4 cm diameter x 36.0 cm deep Deepots™ (D60; Stuewe and Sons; Corvallis, OR). Each Deepot™ contained fiberglass screen (Ace Hardware; Fort Collins, CO) and 2.5 cm of sterilized pea gravel (Permagreen Products; Arvada, CO) beneath 31 cm of a 1:1 mixture (by volume) of sterilized washed sand (US Mix Products Co.; Denver, CO) and calcine clay (Schultz Clay Soil Conditioner; Bridgeton, MO). In half the Deepots™, the media mixture was amended with 20 mL L⁻¹ activated carbon (Sigma-Aldrich Co., St. Louis, MO) which is the same concentration used in previous experiments examining allelopathic effects of spotted knapweed (Ridenour and Callaway 2001). All media components were sterilized in a Tuttnauer Brinkmann 3870E autoclave (Westbury, NY) for 30 minutes at 121°C.

In November 2006, *Centaurea*, *Festuca*, and *Gaillardia* were planted for the initial and simultaneous establishment times, with one individual per Deepot™ in the initial establishment time treatments and one individual per species in the simultaneous establishment time treatments. Monoculture controls for the initial and simultaneous establishment time treatments also were planted, with one individual per Deepot™. The species were randomly assigned to the west or east side of each Deepot™. The plants were allowed to establish for 12 weeks. In February 2007, the colonizing species were planted on the opposite side of the established plants, and additional monoculture controls were planted for the colonizing establishment time treatments. The experiment was maintained for another 15 weeks until May 2007. *Centaurea* seeds were collected from Missoula County, Montana, while *Gaillardia* and *Festuca* seeds were purchased
from Wind River Seed (Manderson, WY) and Granite Seed Company (Lehi, UT) respectively.

EXPERIMENTAL MAINTENANCE

The experiment was arranged on a greenhouse bench in a complete randomized design at Colorado State University. Greenhouse temperatures ranged from 21 to 28°C during the day, and from 13 to 21°C at night. High pressure sodium lights were used to supplement natural light for a simulated 16 hour day. Deepots™ were fertilized with Scott’s Champion (The Scotts Company; Marysville, OH) 13-2-13 fertilizer at a 50 ppm nitrogen (N) amount four times weekly, and a corresponding rate of Peter’s S.T.E.M. (The Scotts Company; Marysville, OH) micronutrient solution once weekly. Supplemental water was provided as needed. To reduce aboveground light competition, a 10 cm X 8 cm plastic divider (One Step Ahead; Lake Bluff, IL) was installed above the media surface along the north-south axis of every container to separate the species (Figure 2.1).
Figure 2.1. *Gaillardia aristata* individual (left) established 12 weeks after *Centaurea stoebe* individual (right) in a Deepot™ with a plastic divider separating the aboveground biomass.

PLANT MEASUREMENTS

In May 2007, the plants were individually harvested and aboveground biomass determined after drying at 60°C to a constant mass.

DATA ANALYSIS

All statistical analyses were performed using SAS 9.1 Proc GLM (SAS Institute Inc.; Cary, NC). For each native species, the effects of competition (monoculture vs. mixed), establishment order (native species first, simultaneously, and after *Centaurea*), and activated carbon (present vs. absent) were examined with a three-way MANOVA.
with native species and *Centaurea* biomass as dependent variables (SAS Institute 1989). *Centaurea* responses to *Gaillardia* and *Festuca* were examined with an ANOVA with establishment order, native species (*Gaillardia, Festuca*, or none), and activated carbon as factors. Post-hoc MANOVAs and ANOVAs with Bonferroni corrected values for \( \alpha \) were used to unravel significant treatment interactions. Aboveground biomass was log transformed to correct unequal variances, but the results are presented with the untransformed data.

### 2.3 RESULTS

Establishment order influenced the competitive outcomes between both *Centaurea* and *Gaillardia* (order x species x competition, \( F_{2,107}=218.26 \; p<0.0001 \)) and *Centaurea* and *Festuca* (order x species x competition, \( F_{2,106}=317.37 \; p<0.0001 \)) (Figures 2.2 and 2.3; Table 2.1). When the native species were established first, the native species reduced *Centaurea* biomass by 98%, while *Centaurea* did not affect the native species (species x competition, \( p<0.0001 \)). Likewise, when *Centaurea* was established first, *Centaurea* reduced native species biomass by 93%, while the native species did not affect *Centaurea* (species x competition, \( p<0.0001 \)). When the competitors were established simultaneously, *Centaurea* reduced *Gaillardia* biomass by 78%, while *Gaillardia* did not affect *Centaurea* biomass (species x competition, \( p<0.0001 \)). In contrast, *Festuca* and *Centaurea* reduced one another similarly, by 45% and 34%, respectively, when established simultaneously (competition, \( p<0.0001 \), species x competition, \( p>0.05 \)).
Figure 2.2. *Gaillardia* and *Centaurea* aboveground biomass in monocultures and species mixtures in the three establishment orders, without activated carbon. Bars show means ± 1 standard error of the mean. An asterisk with a line (*) indicates a significant species × competition interaction (p<0.0001) within each establishment order, where competition decreased the biomass of one species but not the other. Data were log transformed for analysis but are shown in the original scale.
Figure 2.3. *Festuca* and *Centaurea* aboveground biomass in monocultures and species mixtures in the three establishment orders, without activated carbon. Bars show means ± 1 standard error of the mean. An asterisk with a line (*) indicates a significant species x competition interaction (p<0.0001) within each establishment order, where competition decreased the biomass of one species but not the other. In the simultaneous establishment treatment, the asterisk with an extended line (**) indicates that competition decreased both *Festuca* and *Centaurea* biomass (p<0.0001). Data were log transformed for analysis but are shown in the original scale.

Activated carbon influenced competitive interactions between *Centaurea* and *Gaillardia* differently with the different establishment orders (species x competition x order x AC, $F_{2,107}=4.69$ p=0.0112) (Figure 2.4). When *Gaillardia* was established first, activated carbon had no effect. However, when *Gaillardia* and *Centaurea* were established simultaneously, activated carbon increased *Gaillardia* biomass in
monoculture and in mixture with *Centaurea* (AC, $F_{1,36}=8.98$, p=0.0049). Activated carbon increased *Gaillardia* biomass more in competition with *Centaurea* than in monoculture (by 205% compared to 30%), but the competition by activated carbon interaction was only marginally significant ($F_{1,36}=2.94$ p=0.0950). Finally, when *Centaurea* was established first, activated carbon reduced *Gaillardia* biomass in competition with *Centaurea*, but did not affect *Gaillardia* biomass in monoculture (competition x AC, $F_{1,36}=4.40$ p=0.0431; species x competition x AC; $F_{1,36}=5.59$ p=0.0236). Activated carbon reduced *Festuca* biomass by 29% when competing with *Centaurea* regardless of establishment order (species x competition x AC, $F_{2,106}=6.14$ p=0.0147; competition x AC $F_{1,106}=5.21$ p=0.0244) (Figure 2.5). Activated carbon did not affect *Centaurea* biomass (data not shown).
Figure 2.4. *Gaillardia* aboveground biomass in monoculture and species mixtures in the three establishment orders, with and without activated carbon (AC). Bars show means ± 1 standard error of the mean. The asterisk with an extended line (\(*\)) indicates a significant effect of activated carbon on *Gaillardia* biomass across competition treatments (p=0.0049) in the simultaneous establishment treatment. The asterisk with a line (\(\ast\)) indicates a significant competition x AC interaction (p=0.0431), where activated carbon negatively affected *Gaillardia* only when competing with *Centaurea* when *Centaurea* was established first. Data were log transformed for analysis but are shown in the original scale.
Figure 2.5. *Festuca* aboveground biomass in monocultures and species mixtures in the three establishment orders, with and without activated carbon (AC). Bars show means ± 1 standard error of the mean. Activated carbon reduced *Festuca* biomass when competing with *Centaurea* regardless of establishment order (species x competition x AC, p=0.0147; competition x AC p=0.0244). Data were log transformed for analysis but are shown in the original scale.
### Table 2.1. MANOVA results for *Centaurea:Festuca* and *Centaurea:Gaillardia* combinations.

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ns=non-significant; AC=activated carbon

#### 2.4 DISCUSSION

Early establishment conferred a strong competitive advantage regardless of species identity. The species that established earlier strongly inhibited the later colonizing species (Figures 2.2 and 2.3). These experimental results support the hypothesis that *Centaurea* requires competitors to be absent for invasion, and suggest that rapid colonization of disturbed areas, not superior competitive ability, may contribute to the introduction and establishment phases of *Centaurea* invasion. Invasive species success often may be due to increased resource availability and altered disturbance
regimes rather than superior competitive ability (Daehler, 2003, but see Vila and Weiner 2004). Priority effects were similar for the native species and *Centaurea*, perhaps offering an explanation for relictual native species populations and persistent *Centaurea* infestations. Resource competition from established native vegetation is often an important constraint to invasion (Levine et al., 2004).

Because my results stem from a relatively short-term greenhouse experiment, however, the long-term consequences of establishment order for *Centaurea* invasion are not clear. *Centaurea* might have been able to invade the established native species treatments, or vice versa, given more time. Longer-term experiments have demonstrated that initial effects of establishment order on outcomes of competition can disappear over time (Wedin and Tilman 1993). In addition, I examined only two native species in interactions with *Centaurea*. Other native species might have had smaller priority effects on colonizing *Centaurea* or greater success colonizing next to established *Centaurea*.

The priority effects in this experiment appear to be resource-based. Activated carbon did not alter priority effects for any species, which suggests that resource competition was more important than chemical interference competition when the species were established at different times. Resource competition often influences invasion; high resource availability due to removal of competitors facilitates invasion (Hobbs and Huenneke 1992, Burke and Grime 1996, Beckstead and Augspurger 2004), while low resource availability and intense resource competition limit invasion (Hobbs and Atkins 1988, Huenneke et al. 1990, Stohlgren et al. 1999). Since I minimized light competition
in this experiment by separating aboveground biomass, preemption of belowground resources (i.e. nutrients, water, or space) is more likely to explain the observed priority effects. For example, established plants with dense roots at the soil surface may have prevented colonizing plants from accessing surface-applied water or fertilizer. Thus, the priority effects in this experiment might have been weaker under field conditions with more spatial heterogeneity in vegetation (Peart 1989) or belowground resource supply.

Allelopathy has been implicated in the spread and impact of *Centaurea stoebe* across western North America (Ridenour and Callaway 2001, Bais et al. 2003). Therefore, it was surprising that interference competition was not a significant factor in *Centaurea* dominance in my experiment. When the species were established at different times, strong priority effects on resource competition may have swamped potential allelopathic effects. In contrast, when the species were established simultaneously there was some evidence of *Centaurea* allelopathy. Activated carbon amendments did not improve *Festuca* biomass (Figure 2.5) but did marginally improve *Gaillardia* success in competition with *Centaurea* (species x competition, p=0.095; Figure 2.4), suggesting that *Centaurea* soil allelochemicals may have inhibited *Gaillardia* seedling growth in competition between the evenly-sized forb seedlings. This effect might have been significant if activated carbon had not also directly increased *Gaillardia* biomass in the simultaneous establishment period (Figure 2.4). These results suggest that allelopathy is not responsible for *Centaurea* invasion of intact native vegetation, but may play a role during competition between establishing seedlings of *Centaurea* and some native species. Which native species are likely to be influenced by *Centaurea* allelopathy during
simultaneous establishment may be difficult to predict, however. Previous studies have suggested that *Centaurea* allelochemicals inhibit *Festuca* (Ridenour and Callaway 2001) and that *Gaillardia* is relatively resistant to *Centaurea*’s allelochemical (±)-catechin while *Festuca* is more (±)-catechin-sensitive (Perry et al. 2005), but I found more evidence for allelopathic effects of *Centaurea* on *Gaillardia* than *Festuca*.

Direct effects of activated carbon on plant growth, such as those I observed for *Gaillardia* in the simultaneous establishment period, make it difficult to draw firm conclusions about allelopathy from activated carbon experiments. Other studies also have observed direct, species-specific and experiment-specific effects of activated carbon (Ridenour and Callaway 2001, Kulmatiski and Beard 2006, Newingham and Callaway 2006, Lau et al. in press, Schultz et al. in prep), perhaps because of altered soil nutrient availability (Kulmatiski and Beard 2006, Lau et al. in press), water retention (Ridenour and Callaway 2001, Inderjit and Callaway 2003) or pH (Inderjit and Callaway 2003, Lau et al. in press). Fortunately, in the present experiment, direct effects of activated carbon did not interfere with testing for allelopathic effects of *Centaurea* on *Festuca*, or on *Gaillardia* when the species established at different times.

When the competitors were established simultaneously, *Festuca* appeared to be a stronger competitor with *Centaurea* than *Gaillardia* both in terms of responses to *Centaurea* competition and effects on *Centaurea* biomass. This difference could reflect greater *Gaillardia* sensitivity to *Centaurea* allelochemicals or greater *Festuca* competitive ability for resources, or both. Resource competition with *Centaurea* may
have inhibited *Gaillardia* more than *Festuca* because *Centaurea* and *Gaillardia* are both tap-rooted forbs and competition can be more intense between plants from the same functional group (Fargione et al. 2003). By the same logic, however, one would expect *Gaillardia* competition to inhibit *Centaurea* more than *Festuca* competition. *Festuca* and *Centaurea* may have shared resource requirements and resource pools despite being from different functional groups (Bengtsson et al. 1994). Alternatively, *Festuca* may have been able to capture more nutrients and water from overhead fertilization and irrigation if its fibrous root system allowed it to produce more roots at the soil surface than *Centaurea* or *Gaillardia*. *Festuca* also may have used available resources more effectively since *Festuca* uses water more efficiently than *Centaurea* (Blicker et al. 2003).

Understanding priority effects in interactions between native and exotic species may indicate ways to manipulate establishment order to improve community composition and diversity in restored ecosystems (Lockwood et al. 1997, Palmer et al. 1997, Young et al. 2001). Poor *Centaurea* performance in competition with established native species in my experiment highlights the importance of protecting extant native communities from disturbance to prevent *Centaurea* invasion. In addition, my results suggest that restoration efforts that remove *Centaurea* and rapidly establish native cover may be effective at preventing *Centaurea* reinvasion (Bakker and Wilson 2004). However, biotic resistance due to competition is unlikely to completely exclude invasion (Levine et al. 2004). Future experimental questions might examine the environmental and biotic conditions that maximize priority effects of native species and thus maximize biotic resistance to *Centaurea* invasion.
2.5 REFERENCES


CHAPTER 3

Adsorbent material recovery of (±)-catechin

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3.1 INTRODUCTION

Numerous mechanisms have been proposed for invasive plant success (Mitchell et al. 2006). Allelopathy, the secretion of plant phytotoxins, has emerged as a possible mechanism for the success of some exotic plants based on the novel weapons hypothesis (Callaway and Aschehoug 2000, Callaway and Ridenour 2004). In particular, the root-secreted allelochemical (±)-catechin has been implicated as a factor for spotted knapweed (Centaurea stoebe L.) invasion (Bais et al. 2002, Bais et al. 2003, Weir et al. 2003). However, the rhizosphere dynamics of (±)-catechin under natural conditions are poorly understood (Perry et al. 2007). (±)-Catechin is unstable, degrades rapidly, and interacts with soil cations (Blair et al. 2005, Pollock et al. in prep). Moreover, (±)-catechin concentrations in Centaurea soils exhibit substantial temporal and spatial variation (Blair et al. 2006, Perry et al. 2007).

Initial reports suggested that Centaurea consistently produced high soil (±)-catechin concentrations, with means ranging from 300 to 3,600 µg g\(^{-1}\) (Bais et al. 2003, Perry et al. 2005, Thelen et al. 2005). However, recent research suggests that (±)-catechin is often absent from Centaurea soil (Blair et al. 2005, Perry et al. 2007, Schultz unpublished data) and occurs only episodically at mean concentrations ranging from 1 µg g\(^{-1}\) (Blair et al. 2006) to ~600 µg g\(^{-1}\) (Perry et al. 2007). Fluctuations in allelochemical concentrations may be due to microbial degradation, oxidative degradation, leaching, root exudate elicitors, plant uptake, changes in abiotic conditions, insect activity, or other processes affecting production, stability, or detection (Perry et al. 2007, Weidenhamer
Pulses in allelochemical concentrations could have phytotoxic effects on neighboring plants (Loi et al. in press), but evaluating the potential effects of soil (±)-catechin pulses will require a greater understanding of how often and why they occur.

Measuring episodic (±)-catechin concentrations will require new sampling approaches that can capture pulses throughout a sampling period. Current methods for measuring (±)-catechin involve solvent extraction of soil (Bais et al. 2003, Blair et al. 2005), which is time-consuming, expensive, and might miss a (±)-catechin pulse unless it occurs at or near the time of sampling. Moreover, chemical extractions result in static concentrations that do not provide information on exudation kinetics (Qian and Schoenau 2002, Weidenhamer 2005). New colorimetric methods for detecting soil (±)-catechin (Broeckling and Vivanco in press) are faster and less expensive but still provide only point-in-time measurements.

Alternatively, adsorbent and sorbent materials have the potential to measure allelochemical dynamics over time (Weidenhamer 2005, 2007). Adsorbent and sorbent materials have been used to monitor soil nutrients and environmental pollutants (Binkley and Matson 1983, Cornelissen et al. 1997, Doong et al. 2000, Qian and Schoenau 2002), and can be modified to detect root-exuded organic compounds (Skogley 1992, Szmigielska et al. 1996, Weidenhamer 2005). Depending on specific chemical affinities, adsorbent materials (e.g., resin) adsorb chemicals to the surface of the material, while sorbent materials (e.g., PDMS) absorb chemicals into the material (Weidenhamer 2005). The chemical of interest is later extracted from the material and analyzed with standard
techniques such as gas chromatography or high-performance liquid chromatography (HPLC) (Weidenhamer 2005). Adsorbents allow cost effective, non-destructive monitoring of rhizosphere root exudates in situ (Morse et al. 2000). These materials act as “sinks” taking up compounds from the surrounding environment somewhat comparably to plant roots as a relative measure of chemical bioavailability (Skogley 1992, Weidenhamer 2005).

My goal was to assess the potential of adsorbent and sorbent materials to detect (+)-catechin in situ. Here I report on preliminary studies aimed at identifying appropriate techniques. My objectives were to (1) determine the ability of various materials to consistently recover (+)-catechin under laboratory conditions and, based on those results, (2) evaluate (+)-catechin degradation after adsorption by Amberlite™ XAD-7HP resin to gauge its potential in long-term field studies.

3.2 METHODS

EXPERIMENT 1: ADSORBENT AND SORBENT MATERIAL RECOVERY OF (+)-CATECHIN

EXPERIMENTAL DESIGN

Three materials, polydimethylsiloxane (PMDS) tubing, nylon bags containing Amberlite™ XAD-7HP resin (hereafter resin bags), and polyester capsules containing 50% IRN-150 resin and 50% Ambersorb® 563 resin, were exposed to (+)-catechin in aqueous solution and later extracted to determine recovery. The materials were chosen
for their general organic compound adsorption and sorption capabilities. Each material had five treatment replicates and one control replicate for 18 experimental units total.

MATERIAL PREPARATION

The three materials were prepared and tested in July 2006. Silastic® PDMS tubing (9.53 mm outer diameter X 4.78 mm inner diameter; Dow Corning; Midland, MI) was cut into 4-cm segments. Resin bags consisted of 2 tsp of Amberlite™ XAD-7HP organic adsorbing resin (Acros Organics; Fairlawn, NJ) contained in washed fine nylon mesh (Sara Lee Corporation; Winton-Salem, NC). Polyester capsules (2 cm diameter; Unibest Inc.; Bozeman, MT) were preassembled with 50% IRN-150 ionic adsorbing resin (Rohm and Haas Co.; Philadelphia, PA) and 50% Ambersorb® 563 organic adsorbing resin (Rohm and Haas Co.; Philadelphia, PA). Preservative salts were removed from the Amberlite™ XAD-7HP resin by rinsing the resin bags in deionized distilled water six times. To remove any contaminants, all three materials were extracted with 100% methanol (MeOH) for 30 minutes and then rinsed with deionized distilled water five times.

To test (±)-catechin recovery of the materials, 25 mL of a 1 mg mL⁻¹ (±)-catechin solution (Shivambu International; New Delhi, India) or deionized distilled water were added to 50-mL Fisherbrand centrifuge tubes (Fisher Scientific; Pittsburgh, PA). To solubilize the (±)-catechin, all solutions were made with 1% MeOH in deionized distilled water. One material was added to each centrifuge tube, completely immersing it in
solution. The tubes were capped and placed in a dark Fisher Scientific Isotemp Incubator (Pittsburgh, PA) set to 25°C.

After 5 days, the materials were removed with forceps and each placed in a 50-mL centrifuge tube containing 15 mL 100% MeOH to extract the (±)-catechin. These tubes were stored at 4°C. After one day, the liquid extracts were transferred to 15-mL Fisherbrand centrifuge tubes (Fisher Scientific; Pittsburgh, PA). The samples were centrifuged (Sorvall Super T21; Newtown, CT; relative centrifugal force=5,867 X g) at 7,000 rpm for 5 minutes at 4°C, and the supernatants transferred to new 15-mL centrifuge tubes. The samples were concentrated by evaporating the MeOH with nitrogen gas blown through a Pierce Reacti-Vap III evaporator (Rockford, IL) equipped with Pierce Reacti-Vap Teflon coated needles (4” X 19 gauge; Rockford, IL). Once dry, two consecutive 0.75-mL MeOH rinses were added, vortexed, and transferred to a 1.5-mL Eppendorf tube (Westbury, NY). The samples were centrifuged (VWR Galaxy 16; Batavia, IL; relative centrifugal force=15,996 x g) at 14,000 rpm for 5 minutes, and the supernatants transferred to HPLC vials (Sun-SRI; Rockwood, TN) and stored at 0°C until HPLC analysis.

(±)-Catechin amounts recovered were determined by HPLC in comparison to 1 mg mL⁻¹ standards. HPLC separations were conducted using mobile phase solutions of (A) 0.1% acetic acid in distilled water and (B) 0.1% acetic acid in absolute methanol, with a multistep gradient of 0–3 min, 10% B; 3–43 min, increase to 90% B; 43–51 min, 90% B. The column was a reverse phase, 5 µm C18 (4.6×150 mm) (Dionex Corp.,
Sunnyvale, CA, USA). The flow rate was 0.7 mL min\(^{-1}\), the sample injection volume was 5 µL, and visible absorbance was measured at 280 nm with a Dionex Summit System (Sunnyvale, CA).

DATA ANALYSIS

Statistical analyses were performed using SAS Proc GLM (SAS Institute Inc.; Cary, NC). (±)-Catechin recovery (µg) of the materials was compared with a one-way ANOVA followed by Tukey HSD means comparison tests (\(\alpha=0.05\)). (±)-Catechin recovery (µg) was log-transformed for analysis to correct unequal variances, but the results are presented with the untransformed data.

EXPERIMENT 2: (±)-CATECHIN STABILITY WHILE ADSORBED TO AMBERLITE™ RESIN

EXPERIMENTAL DESIGN

Since the Amberlite™ XAD-7HP resin bags had the highest (±)-catechin recovery in Experiment 1 (see Results), this adsorbent material was impregnated with (±)-catechin, and placed outside underground for a variable number of weeks to assess the degradation of adsorbed (±)-catechin over time. Each weekly time period (0-5; \(n=6\)) had three treatment replicates and one control replicate for 24 experimental units total.

MATERIAL PREPARATION AND IMPREGNATION

In April 2007, resin bags containing Amberlite™ XAD-7HP were constructed, pre-extracted, and rinsed as in Experiment 1. 25 mL of a 1.0 mg mL\(^{-1}\) (±)-catechin
solution or deionized distilled water were added to 50-mL centrifuge tubes. To solubilize
the (±)-catechin, all solutions were made with 1.0% MeOH in deionized distilled water.
One resin bag was added to each centrifuge tube, completely immersing it in solution.
The centrifuge tubes were placed in a dark Fisher Scientific Isotemp incubator set to
25°C.

INSTALLATION

After three days exposure, the solution was drained from the tubes. Week 0 resin
bags served as a baseline and were immediately extracted with 15 mL of 100% MeOH in
a 50-mL centrifuge tube. The Kentucky bluegrass (*Poa pratensis* L.) lawn north of the
Natural Resources building on the Colorado State University campus (Fort Collins, CO)
served as the incubation area. Holes in a linear transect were dug approximately 10 cm
deep and 30 cm in diameter and spaced 2 meters apart. Time periods (in weeks) 1
through 5 were randomly assigned to each hole. Three (±)-catechin treated resin bags
plus one control resin bag for each time period were placed into each hole spacing the
resin bags to be completely separated. The holes were backfilled with original soil and
sod.

RECOVERY AND EXTRACTION

The appropriate resin bags were retrieved weekly, vigorously shaken to rid any
adhered soil, and any impaled roots removed. The resin bags were placed in separate 50-
mL centrifuge tubes, and immediately extracted with 15 mL of 100% MeOH. After
extraction, the tubes were capped and stored at 4°C. After five weeks all the resin bags were recovered.

HPLC ANALYSIS

In September 2007, the liquid extracts from 50-mL centrifuge tubes were vortex mixed, centrifuged, transferred, concentrated, and resuspended in 1.5 mL MeOH as in Experiment 1. (±)-Catechin amounts recovered were determined by HPLC in comparison to 1 mg mL$^{-1}$ standards. HPLC separations were conducted using mobile phase solutions of double distilled water with 0.1% acetic acid (A) and absolute methanol (B), with a multistep gradient of 0–5 min, 5% B; 5–15 min, increase to 20% B; 15–20 min, 20% B; 20–40 min, increase to 80% B; 40–60 min, increase to 100% B; 60–70 min, 100% B; and 70–80 min, 5% B. The column was a reverse phase, 5 µm C18 (25 X 0.46 cm) (Supelco Co.; Bellefonte, PA). The flow rate was 1 mL min$^{-1}$, the sample injection volume was 20 µL, and visible absorbance was measured at 280 nm with a Dionex Summit System (Sunnyvale, CA).

DATA ANALYSIS

Statistical analyses were performed using SAS Proc GLM (SAS Institute Inc.; Cary, NC). (±)-Catechin recovery (µg) over the variable number of weeks was compared with a one-way ANOVA followed by Dunnett’s means comparison tests to the week 0 baseline ($\alpha=0.05$).

3.3 RESULTS
The Amberlite™ XAD-7HP resin bags recovered significantly more (±)-catechin than either the PDMS tubing or the IRN-50/Ambersorb® 563 resin capsules (material, \( F_{2,12}=225.89 \), \( p<0.0001 \); Tukey HSD, \( p<0.0001 \)) (Figure 3.1). No (±)-catechin was detected in any of the controls.

**Figure 3.1.** (±)-Catechin recovered (µg) from adsorbent and sorbent materials after exposure to a solution of 1 mg mL\(^{-1}\) of (±)-catechin for 5 days at 25°C in a dark laboratory incubator. Bars show means ± 1 standard error of the mean. Shared letters indicate no significant difference (Tukey HSD, \( \alpha=0.05 \)). Data were log transformed for analysis; untransformed data are shown here on a log scale.
Figure 3.2. (±)-Catechin recovered (µg) from Amberlite™ XAD-7HP resin bags exposed to a 1 mg mL⁻¹ (±)-catechin solution for 3 days at 25°C in a dark laboratory incubator, and then incubated outside underground for 0-5 weeks. Points show means ± 1 standard error of the mean. An asterisk indicates a significant difference (Dunnett’s, α=0.05) from week 0 (±)-catechin recovery.

(±)-Catechin recovered (µg) from Amberlite™ XAD-7HP resin bags buried outside underground for 0 to 5 weeks varied somewhat erratically with time (week, $F_{5,12}=10.89$ p=0.0004) (Figure 3.2). After the first week of incubation, recovered (±)-catechin decreased 60% (p=0.0298) from baseline levels (week 0), but returned to baseline levels after the second week. Little (±)-catechin recovery (78-97% decreases;
p<0.01) was observed after the third week of incubation. No (±)-catechin was detected in any of the control resin bags.

### 3.4 DISCUSSION

These results demonstrate that adsorbent materials can be used to detect (±)-catechin over a limited period of time. Of the three materials tested, Amberlite™ XAD-7HP resin bags recovered the greatest amount of (±)-catechin. Because the surface area and volume of the materials differed, conclusions cannot be drawn on (±)-catechin adsorption efficiency. Different results may have been obtained with other volumes, but I used the most convenient amount for field work and analysis. Given the small number of materials tested, screening of other resins in the future could be beneficial.

Materials possess different recovery capabilities depending on the compound (Morse et al. 2000). Amberlite™ XAD-7HP resin adsorbed 89% of a catechin solution while desorbing 85% (Morse et al. 2000). Meanwhile Ambersorb® 563 adsorbed 92% while only desorbing 18% (Morse et al. 2000). The lower (±)-catechin recovery by PDMS might be explained by polarity. PDMS is a non-polar material with higher affinities for non-polar compounds (Baltussen et al. 1999), including the non-polar allelochemical sorgoleone from sorghum (*Sorghum bicolor*) (Weidenhamer 2005), while (±)-catechin is neither polar nor non-polar.

Given that adsorbent and sorbent materials extract an unknown volume of medium, the recovered amounts reflect relative quantities of soluble chemicals, and
cannot be transformed into concentrations or recovery percentages (Weidenhamer 2005). Since the controversial aspects of (±)-catechin are the actual soil concentrations and phytotoxicity thresholds, relative measures may not determine the importance of (±)-catechin to *Centaurea* ecology conclusively, but might offer insight into (±)-catechin dynamics for more thorough testing.

Even though Amberlite™ XAD-7HP readily adsorbed (±)-catechin, variable losses occurred over time. Most notably, a decline in recovered (±)-catechin was observed during week 1 compared to weeks 0 and 2. More replicates could have diminished this variability, but the reliability of Amberlite™ XAD-7HP for (±)-catechin recovery after one week is questionable. Deploying resin bags for longer than two weeks risks partial loss of adsorbed catechin. When designing future experiments, it may be necessary to replace resin bags every two weeks for continuous sampling of (±)-catechin, which limits the ability of this method to capture (±)-catechin pulses without considerable sampling effort.

While Amberlite™ XAD-7HP is a promising candidate for studying *Centaurea* soil (±)-catechin levels, numerous field conditions could affect adsorption and loss. The material should be tested over a range of environmental gradients and (±)-catechin concentrations. To my knowledge, Amberlite™ XAD-7HP has yet to measure (±)-catechin exuded from *Centaurea* roots and migrated through rhizosphere soil. My experiments used a relatively high concentration of (±)-catechin, and resin adsorption may behave differently at lower concentrations especially in a complex soil environment.
As with ion exchange resins, adsorption will likely depend on soil moisture content (Schaff and Skogley 1982) and soil temperature (Yang et al. 1991). Adsorbents depend on diffusion so placement near *Centaurea* roots would be necessary. However, minimizing root disturbance is also critical since installation disturbance might elicit allelochemical exudation (Paavolainen et al. 1998).

Amberlite™ XAD-7HP offers promise for recovering (±)-catechin from various experimental designs. This resin is relatively inexpensive and could be used in large-scale studies over limited periods of time. Field studies of Amberlite™ XAD-7HP (±)-catechin recovery still are needed to incorporate critical soil environment complexities, including nutrient limitation, water stress, temperature fluctuations, microbial interactions, plant root interference, and other factors (Morse et al. 2000). With additional testing, the use of adsorbent materials could elucidate allelochemical dynamics and clarify the role of (±)-catechin in *Centaurea* invasion.

### 3.5 REFERENCES


