

Concentrations of the Allelochemical (\pm)-Catechin IN *Centaurea maculosa* Soils

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Abstract The phytotoxin (\pm)-catechin has been proposed to mediate invasion and autoinhibition by the Eurasian plant *Centaurea maculosa* (spotted knapweed). The importance of (\pm)-catechin to *C. maculosa* ecology depends in part on whether sufficient catechin concentrations occur at appropriate times and locations within *C. maculosa* soil to influence neighboring plants. Previous research on catechin in *C. maculosa* soils has yielded conflicting results, with some studies finding high soil catechin concentrations and other, more recent studies finding little or no catechin in field soils. Here, we report the most extensive study of soil catechin concentrations to date. We examined soil catechin concentrations in 402 samples from 11 *C. maculosa* sites in North America sampled in consecutive months over 1 yr, excluding winter months. One site was sampled on seven dates, another was sampled twice, and the remaining nine sites were each sampled once on a range of sampling dates. Methods used were similar to those with which we previously measured high soil catechin concentrations. We detected catechin only in the site that was sampled on seven dates and only on one sampling date in that site (May 16 2006), but in all samples collected on that date. The mean soil catechin concentration on that date was 0.65 ± 0.45 (SD) mg g⁻¹, comparable to previously reported high concentrations. There are a number of possible explanations for the infrequency with which we detected soil catechin in this work compared to previous studies. Differences in results could reflect spatial and temporal variation in catechin exudation or degradation, as we examined different sites in a

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different year from most previous studies. Also, large quantities of catechin were detected in blanks for two sampling periods in the present study, leading us to discard those data. This contamination suggests that previous reports of high catechin concentrations that did not include blanks should be viewed with caution. Our results suggest that pure catechin is only rarely present in *C. maculosa* bulk soils. Thus, although catechin may play a role in *C. maculosa* invasion, the infrequency of soil catechin that we determined in this study suggests that we cannot be as certain of its role as previous reports of high soil catechin concentrations suggested.

Keywords Allelopathy · Catechin · *Centaurea maculosa* · *Centaurea stoebe* · Invasion · Novel weapons · Root exudate · Spotted knapweed

Introduction

Centaurea maculosa Lam. (spotted knapweed, recently suggested to be *C. stoebe* L. [USDA, NRCS 2007]) is a Eurasian species in the Asteraceae that invades North American grasslands. It reduces native biodiversity and rangeland forage quality (Watson and Renney 1974; Tyser and Key 1988). A possible role of allelopathy in *C. maculosa* invasion has received considerable attention. Allelopathy has been proposed to mediate some plant invasions because allelopathic exotic plants may produce chemicals to which native species have not yet evolved resistance (i.e., the novel weapons hypothesis) (Callaway and Aschehoug 2000; Callaway and Ridenour 2004). Initial studies of *C. maculosa* allelopathy indicated phytotoxins in *C. maculosa* shoot tissue (Fletcher and Renney 1963; Kelsey and Locken 1987). More recently, an experiment that used activated carbon, which adsorbs organic compounds, suggested that phytotoxins in *C. maculosa* root exudates may inhibit native species (Ridenour and Callaway 2001).

Efforts to identify phytotoxins in *C. maculosa* root exudates revealed a racemic mixture of (\pm)-catechin (hereafter catechin) (Bais et al. 2002). Since then, several studies have reported high catechin concentrations in *C. maculosa* soils (Bais et al. 2002, 2003; Perry et al. 2005b; Thelen et al. 2005; Weir et al. 2006). Catechin also has been reported to inhibit growth and/or reduce survival of susceptible North American plants *in vitro* (Bais et al. 2002, 2003; Weir et al. 2003; Perry et al. 2005a), in soil in controlled environments (Bais et al. 2003), and in soil in the field (Thorpe 2006). (+)-Catechin alone also has been reported to inhibit growth of several plant species *in vitro* (Buta and Lusby 1986; Iqbal et al. 2003; Furubayashi et al. 2007; K. Simoes, unpublished data), but not in one experiment in soil (Furubayashi et al. 2007). (–)-Catechin alone has been reported to inhibit green algae (D’Abrosca et al. 2006). However, catechin effects vary considerably among experimental conditions and species within and among studies (Bais et al. 2003; Weir et al. 2003; Blair et al. 2005; Perry et al. 2005a; Thorpe 2006). Minimum concentrations reported for catechin phytotoxicity range from 0.0016–0.0032 mg g⁻¹ (Thorpe 2006) to 0.04 mg g⁻¹ (J. Pollock, unpublished data), 0.05 mg ml⁻¹ (Weir et al. 2003), 0.25 mg ml⁻¹ (Perry et al. 2005a), and >1.0 mg ml⁻¹ (Blair et al. 2005). Catechin at high concentrations has been reported to inhibit *C. maculosa* growth and germination, acting as an autoinhibitor (Perry et al. 2005a, b). Finally, catechin has been reported to inhibit the North American species displaced by *C. maculosa* more than European species that coexist with *C. maculosa*, thus providing support for the novel weapons hypothesis (Bais et al. 2003; Thorpe 2006; W. He and Y. Feng, unpublished data).

Other recent studies have reported much lower soil catechin concentrations or have failed to find catechin in *C. maculosa* soil (Blair et al. 2005, 2006). Furthermore, some

studies have reported weak or no effects of high catechin concentrations on the same species for which other studies have reported strong effects (Bais et al. 2003; Weir et al. 2003; Blair et al. 2005; Perry et al. 2005a; Thorpe 2006; W. He and Y. Feng unpublished data; S. Duke personal communication). These results have raised doubts about the role of catechin in *C. maculosa* invasion, and suggested a need for additional research to resolve differences among studies. Therefore, we conducted extensive measurements of soil catechin concentrations. Most previous studies have sampled *C. maculosa* soil at only one time point and at only one or a few sites (Bais et al. 2002, 2003; Blair et al. 2005; Perry et al. 2005b; Thelen et al. 2005; Weir et al. 2006), with the exception of Blair et al. (2006), who sampled *C. maculosa* soil from July to October. Here, we examined soil catechin in many sites and over a 1-yr time course at one site to better assess spatial and temporal variation in catechin concentrations in *C. maculosa* soils.

Methods and Materials

Sites Soil catechin concentrations were examined at 11 North American sites where *C. maculosa* was abundant (>20% cover; G. Thelen, pers. obs.). Samples were collected during the 2005 and 2006 growing seasons (Table 1). Most sites were sampled only once, but one site (Nelson Gulch 1) was sampled twice and another (Mt. Sentinel) was sampled seven times.

Collection of Soils At each site, 10 1.5-cm diameter×5-cm deep cores were collected from within 5 cm of each of 7–20 randomly selected *C. maculosa* plants with root crown diameters greater than 5 mm. Some sampled plants at the Nelson Gulch site were smaller because *C. maculosa* plants there were generally smaller than at other sites. The cores from around each plant were pooled immediately after collection, sieved through a 2-mm screen, and homogenized by stirring. Two 3-cm³ subsamples from each homogenized sample were mixed thoroughly with 10 ml of methanol in 15-ml centrifuge tubes. Most samples were placed in methanol immediately in the field. However, samples from Mt. Sentinel on June 14 and July 2, 2005 were placed in methanol 1–2 hr after collection, and samples from Big Creek were placed in methanol 3–4 hr after collection. All samples were stored in methanol at 4°C until processed.

Sample Extraction In preparation for analysis, samples were centrifuged for 5 min at 7,000 rpm (Sorvall SL 50T; relative centrifugal force=5,867×g). Supernatants were transferred to new 15-ml tubes, concentrated under blown N₂, resuspended in two rinses of 0.75 ml methanol, transferred to 2.0-ml centrifuge tubes, and centrifuged for 5 min at 14,000 rpm (VWR Galaxy 16; relative centrifugal force=15,996×g). The resulting supernatants were transferred to new 2.0-ml tubes, concentrated under blown N₂, resuspended in 0.4 ml methanol, and stored at 0°C until analysis. Extracted soil samples were dried to a constant weight in a 65°C forced-air drying oven and weighed. Soil weights were corrected for 0.06% hydrostatic water determined from weights of a subset of samples dried in a 105°C forced-air drying oven. Catechin concentrations were calculated on a per gram dry soil basis.

Determination of Catechin Concentrations We used methanol to extract soil catechin because we wanted to use methods similar to those with which we had previously measured high catechin concentrations (Bais et al. 2003; Perry et al. 2005b; Thelen et al. 2005; Weir

Table 1 Locations, sampling dates, sample sizes, and soil catechin concentrations for 11 *Centaurea maculosa* sites sampled during the 2005 and 2006 growing seasons

Site	Location	Soil Type ^a	Date ^b	N	Soil Catechin (mg g ⁻¹)		n ^c
					Mean±SD	Range	
Skalkaho Pass MT, USA	46.17521°N, 114.04364°W		6/20/2005	20	0	0	0
Clearwater MT, USA	47.00726°N, 113.37319°W	Perma gravelly loam	6/21/2005	40	0	0	0
Blue Mountain MT, USA	46.82703°N, 114.09897°W	Bigarm gravelly loam	6/30/2005	40	0	0	0
Nelson Gulch 1 MT, USA	46.57040°N, 112.14981°W	Windham-Whitecow- Lap channery loams	7/5/2005 8/4/2005	10 ^d 14	0 0	0 0	0 0
Petty Mountain MT, USA	46.97157°N, 114.38408°W		7/7/2005	20	0	0	0
Kooskia ID, USA	46.20869°N, 116.00644°W	Nicodemus loam	7/25/2005	18	0	0	0
Big Creek MT, USA	46.45580°N, 114.18236°W	Dumps, mine	7/29/2005	40	0	0	0
Nelson Gulch 2 MT, USA	46.57047°N, 112.14991°W	Windham-Whitecow- Lap channery loams	8/4/2005	20	0	0	0
Elko BC, CAN	49.29157°N, 115.12134°W		8/6/2005	18	0	0	0
Lee Metcalf MT, USA	46.58737°N, 114.05169°W	Riverside-Tiechute- Curlew complex	8/8/2005	20	0	0	0
Mt. Sentinel MT, USA	46.84102°N, 113.98251°W	Bigarm gravelly loam	6/14/2005 7/2/2005 7/29/2005 9/2/2005 4/6/2006 5/16/2006 6/23/2006	20 20 20 22 20 20 20	0 0 0 0 0 0.65±0.45 0	0 0 0 0 0 0.14–2.15 20	0 0 0 0 0 0 0

^a Soil information was obtained from the USDA-NRCS web soil survey (<http://websoilsurvey.nrcs.usda.gov/app/>). Soil information was unavailable for some sites.

^b The Nelson Gulch 1 site was sampled twice and the Mt. Sentinel site was sampled seven times. All other sites were sampled once.

^c Number of samples in which catechin was detected.

^d One subsample, rather than two, was collected from the pooled cores from each plant for this site and date.

et al. 2006). However, methanol extraction results in poor recovery efficiency (<17%) for pure catechin added to soil at known concentrations (Blair et al. 2005; L. Perry, unpublished data). Poor catechin recovery with our method may have caused us to underestimate catechin concentrations, or fail to detect catechin at concentrations lower than our detection limit (0.025 mg g⁻¹).

Catechin concentrations were determined by HPLC in comparison to 1 mg ml⁻¹ (±) catechin standards (Shivambu International, Himachal Pradesh, India). HPLC separations used mobile phase solutions of (A) 1% acetic acid in distilled water and (B) absolute methanol, with a multistep gradient of 0–5 min, 5% B; 5–15 min, increase to 20% B; 15–20 min, 20% B; 20–

40 min, increase to 100% B; 40–50 min, 100% B; 50–55 min, 5% B. The column was a reverse phase, 5 μm C_{18} (4.6 \times 150 mm) (Dionex Corp., Sunnyvale, CA, USA), the flow rate was 1 ml min^{-1} , the sample injection volume was 20 μl , and absorbance was measured at 280 nm. For samples in which catechin was detected by HPLC, the presence of catechin was confirmed by LCMS. LCMS analyses that used 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 C_{18} (4.6 \times 150 mm) (Dionex Corp., Sunnyvale, CA, USA), the flow rate was 0.7 ml min^{-1} , and absorbance was measured at 280 nm. Ionization for MS analysis was performed in both positive and negative ion modes by using electrospray ionization with a nitrogen flow of 80 psi, cone voltage of 70 V, needle voltage of 3 kV, and cone temperature of 600°C. Mass data were collected over the range of the gradient program at a rate of one scan per 1.5 sec.

Results and Discussion

In the most extensive study of soil catechin to date, we examined catechin in 402 samples from 11 *Centaurea maculosa* sites. We detected catechin in 20 samples, all collected on one date (May 16, 2006) at one site (Mt. Sentinel, Missoula, MT) (Table 1). No other sites were sampled on that date. The infrequency with which we detected catechin differs from many previous studies of catechin in *C. maculosa* soils in which catechin was detected in most samples regardless of site or date (Bais et al. 2002, 2003; Perry et al. 2005b; Thelen et al. 2005; Weir et al. 2006) but is similar to results from Blair et al. (2005, 2006), who reported no catechin or only trace amounts (<0.0001 mg g^{-1}) on eight of 10 dates. The catechin concentrations on May 16, 2006 at Mt. Sentinel (Table 1), however, are comparable to previous reports of high catechin concentrations, which include means of $\sim 2.2\pm 0.2$ (Bais et al., 2003), 1.6 ± 0.1 (Perry et al. 2005b), 0.7 ± 0.02 (Weir et al. 2006), and 0.3 ± 0.02 to 3.6 ± 0.1 (\pm SE) mg g^{-1} (Thelen et al. 2005), and are much higher than those Blair et al. (2006) reported (0.001 ± 0.0004 mg g^{-1}).

There are several potential explanations for the infrequency with which we detected catechin compared to previous studies. Variation in results could be caused by temporal or spatial variation in soil catechin. We detected catechin in every sample from one date at one site, suggesting that specific environmental conditions may be necessary for catechin production, stability, or detection. Conditions could have been poor for catechin in 2005, when little or none was found (Table 1; Blair et al. 2006), compared to 2004 and 2006 when results were mixed (Table 1; Blair et al. 2005; Perry et al. 2005b; Weir et al. 2006), and 2001 when high concentrations were found (Bais et al. 2002, 2003). In the one site studied in multiple years (Mt. Sentinel), high concentrations were reported in 2004 (Perry et al. 2005b), no catechin was detected in 2005, and results were mixed in 2006 (Table 1). Temperature and precipitation did not differ substantially among these years at Mt. Sentinel (Fig. 1), but other factors were not measured. The data from Mt. Sentinel seem to suggest that catechin is present in soil mainly in spring, but other reports from summer and autumn (Bais et al. 2003—June, August; Blair et al. 2006—August; Thelen et al. 2005—November; Weir et al. 2006—July) suggest that it is not predictable with seasons. Thus, temporal variation in conditions such as climate, soil chemistry, microbes, insects, or vegetation might be responsible for variation in soil catechin, but the particular factors that might be important are uncertain. The infrequency with which we detected catechin also

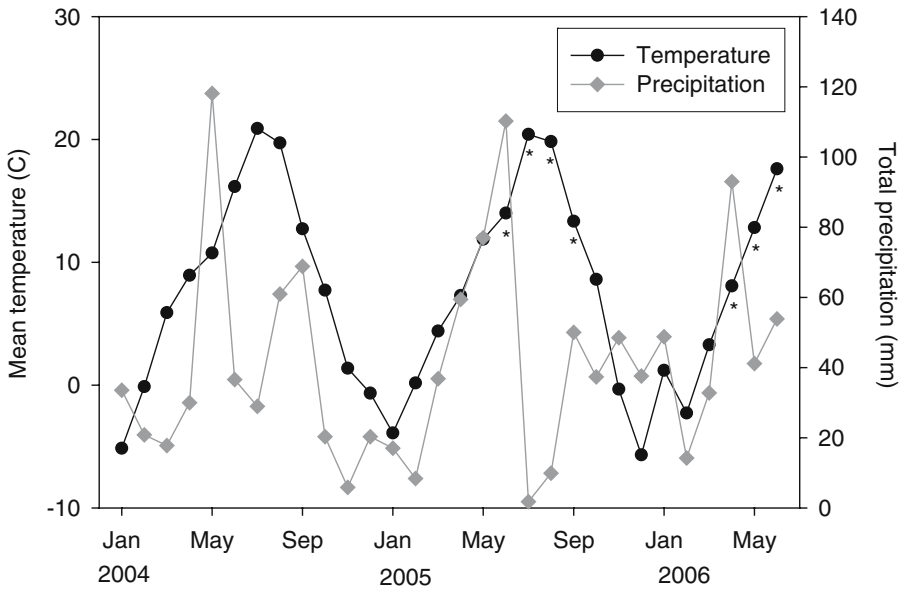


Fig. 1 Climate data for Missoula, Montana from January 2004 to May 2006. Data were obtained from the Western Regional Climate Center (<http://www.wrcc.dri.edu>). Asterisk indicates months in which samples were collected from Mt. Sentinel, Missoula, MT for the present study (Table 1)

could be explained by spatial variation, with unfavorable conditions in the sites we sampled this time; however, it seems unlikely that 10 of our 11 sites contained unfavorable conditions for catechin, whereas most previous studies sampled favorable sites.

The high catechin concentrations reported previously also could have been caused by undetected catechin contamination. Throughout the present study, we included blanks that contained only methanol as negative controls with our samples during extraction. In one set of samples, we found relatively high catechin concentrations (0.35 ± 0.41 [SD] mg g^{-1}), but also found large quantities of catechin (1.4 ± 0.6 [SD] mg) in all six blanks. In another set of samples, we found catechin in only one sample (0.03 mg g^{-1}), but again found it (0.3 mg) in one of three blanks. Several tests failed to identify the source of the contamination but indicated that it was not an artifact of the HPLC protocol. Because catechin was present in the blanks, we discarded the data for these dates; they are not included in Table 1. In subsequent tests, no quantifiable catechin was found in blanks. However, blanks were not included during sample extraction for most previous reports of high catechin concentrations (Bais et al. 2002, 2003; Weir et al. 2003, 2006; Perry et al. 2005b; Thelen et al. 2005). An exception is Fig. 1C of Thelen et al. (2005), which had one blank. Given the potential for catechin contamination demonstrated by the current study, previous results not including blanks should be regarded with caution.

Differences in our methods between this and previous studies are unlikely to account for our infrequent detection of catechin here. In previous studies, we stored intact soil cores for weeks or months before extracting 0.5-cm^3 subsamples with 1 ml of methanol (Bais et al. 2002, 2003; Perry et al. 2005b; Thelen et al. 2005; Weir et al. 2006). In the current study, we sieved and homogenized the soil and extracted 3-cm^3 subsamples with 10 ml of methanol immediately upon collection. Such changes in protocol seem unlikely to have reduced recovery efficiency, particularly to such an extent that we could no longer detect

catechin in most samples. Furthermore, a preliminary experiment in which we compared the two methods suggested that the changes in methods improved catechin detection (data not shown).

The absence of catechin on most dates in the present study and studies by Blair et al. (2005, 2006) suggests that pure catechin (i.e., not complexed with other compounds) may occur only rarely at sufficient concentrations in *C. maculosa* bulk soils to inhibit other plants. Catechin concentrations required to inhibit other plants are uncertain, as minimum concentrations for inhibition vary among studies from 0.0016–0.0032 mg g⁻¹ (Thorpe 2006) to 0.04 mg g⁻¹ (J. Pollock, unpublished data), 0.05 mg ml⁻¹ (Weir et al. 2003), 0.25 mg ml⁻¹ (Perry et al. 2005a), 0.27 mg g⁻¹ (Inderjit, unpublished data), and weak effects of 1.0 mg ml⁻¹ (Blair et al. 2005). Here, our detection limit was 0.025 mg g⁻¹. The detection limits of Blair et al. (2005, 2006) were even lower, 0.00002 mg g⁻¹ or trace amounts (<0.0001 mg g⁻¹), much lower even than those Thorpe (2006) reported to be inhibitory. Thus, if pure, stable catechin in bulk soil is an important driver of competitive effects, these results suggest that those effects would have to occur sporadically.

Nevertheless, our results do not disprove a role of catechin or other allelochemicals in *C. maculosa* competitive effects. Studies that have used activated carbon soil amendments, which adsorb organic compounds, indicated that *C. maculosa* root exudates may inhibit growth of neighboring plants (Ridenour and Callaway 2001; Callaway et al. 2005; Perry et al. 2005b). Although catechin does not appear to influence *C. maculosa* ecology via continuously high concentrations of pure catechin in bulk soil, it could play a role through episodic effects on competitors, by producing phytotoxic soil concentrations more frequently, either of phytotoxic catechin-metal complexes (J. Pollock and B. Holben, unpublished data) or phytotoxic degradation products, by occurring more frequently in the rhizosphere or near competing roots than in bulk soil, or by producing frequent but transient catechin pulses (Appel 1993). *C. maculosa* also may produce other phytotoxins with ecological effects. However, none of these hypotheses has been tested.

Although previous consistent reports of high soil catechin concentrations provided circumstantial evidence for a role of catechin in *C. maculosa* invasion (Bais et al. 2002, 2003; Perry et al. 2005b; Thelen et al. 2005; Weir et al. 2006), this more extensive study, together with those of Blair et al. (2005, 2006), suggests that high catechin concentrations rarely occur in *C. maculosa* soil. More confidence can be placed in the current results. Thus, the infrequency of soil catechin weakens the hypothesis that it plays a role in *C. maculosa* invasions.

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