

# Dual role for an allelochemical: ( $\pm$ )-catechin from *Centaurea maculosa* root exudates regulates conspecific seedling establishment

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## Summary

**1** Plant allelopathic compounds may have other roles than interspecific interference. We investigated whether ( $\pm$ )-catechin, a phytotoxin exuded from the roots of the exotic invader *Centaurea maculosa* (spotted knapweed), is also one of the factors that regulates *C. maculosa* recruitment.

**2** Adding activated carbon, which adsorbs organic compounds, to soil around *C. maculosa* adults in the field increased seedling density by 78% 25 days after sowing, and by 34% 32 days after sowing, suggesting that soil-borne compounds inhibited or delayed recruitment.

**3** Analysis of field soils near mature *C. maculosa* revealed that they can contain exceptionally high ( $\pm$ )-catechin concentrations (mean = 1.55 mg g<sup>-1</sup> dry soil, with 60% of samples containing  $\geq$  1.0 mg ( $\pm$ )-catechin g<sup>-1</sup>).

**4** In laboratory experiments, treatment with  $\geq$  1.0 mg ( $\pm$ )-catechin mL<sup>-1</sup> reduced seedling root elongation by > 50%, indicating that field concentrations are sufficient to inhibit *C. maculosa* recruitment. Provided that 10% methanol was used to maintain ( $\pm$ )-catechin in solution for > 1 day, treatment with  $\geq$  1.0 mg mL<sup>-1</sup> also reduced *C. maculosa* germination by > 70%.

**5** ( $\pm$ )-Catechin maintained in solution with methanol did not significantly reduce *C. maculosa* seed survival, suggesting that inhibition of germination was due, at least in part, to delayed germination rather than to seed mortality.

**6** Depending on the solubility of ( $\pm$ )-catechin in soil and on the duration of its effects on recruitment, *C. maculosa* may avoid intraspecific competition or regulate the timing of seedling establishment by reducing seedling growth or postponing germination in response to its own phytotoxin.

**7** Chemical regulation of *C. maculosa* recruitment, as demonstrated here, suggests a dual role of ( $\pm$ )-catechin as an allelochemical and an autoinhibitor. The potential for a single plant root exudate to influence both inter- and intraspecific interactions emphasizes the complex effects that plant secondary metabolites may have on plant population and community structure.

*Key-words:* allelopathy, autoinhibition, autotoxicity, chemical interference, intraspecific competition, invasive species, population density, seedling recruitment, spotted knapweed

*Journal of Ecology* (2005) **93**, 1126–1135

doi: 10.1111/j.1365-2745.2005.01044.x

## Introduction

Allelopathy, or chemically mediated interference among species, is receiving renewed attention because of the advent of molecular and biochemical techniques that allow for detailed examination of the plant compounds and modes of action involved (Czarnota *et al.* 2001; Bais *et al.* 2003; Baldwin 2003). In particular, recent studies suggest that allelopathy may mediate some biological invasions, because the allelochemicals produced by exotic plants are novel in the invaded range and more effective against naïve native species than against the species with which the exotic plants naturally co-occur (Callaway & Aschehoug 2000; Bais *et al.* 2003; Callaway & Ridenour 2004; Vivanco *et al.* 2004).

Allelopathy research has focused on direct and indirect mechanisms of interspecific interference (Wardle *et al.* 1990; Siemens *et al.* 2002; Weir *et al.* 2004b), such as phytotoxicity, reduced nitrogen availability due to inhibition of nitrifying bacteria (Rice & Pancholy 1973; Paavolainen *et al.* 1998; Kraus *et al.* 2004) and selective inhibition of mycorrhizal fungi that benefit competing plants (Nilsson *et al.* 1993). However, plant allelochemicals may have other functions, including regulation of intraspecific interactions, perhaps as a form of signalling. Over 30 years ago, McCormick (1968) suggested that 'autotoxicity may result in inhibition of seedlings in the vicinity of mature plants of the parent species and, thus, may play a major role in population density control.' Autoinhibition (*sensu* Whittaker & Feeny 1971) may be a better term to allow inclusion of non-toxic chemical-mediated intraspecific interactions, including self-detection and delayed germination (Mahall & Callaway 1991; Falik *et al.* 2003; Holzapfel & Alpert 2003). Autotoxicity and autoinhibition have been examined in numerous crops (Singh *et al.* 1999), but remain largely unexplored in natural systems.

Fluctuations in recruitment can have powerful effects on plant populations and community composition (Warner & Chesson 1985). Variation in the abiotic environment affects recruitment, but plants may also regulate recruitment by producing compounds that inhibit the establishment or growth of their offspring. Such regulation may function as a form of territoriality, reducing the intensity of intraspecific competition and maximizing the fitness of the dominant members of a population (Schenck *et al.* 1999). Alternatively, seeds and seedlings can improve their chances of success by releasing germination inhibitors that prevent recruitment of their siblings (e.g. Dyer 2004). In addition, some species use germination inhibitors from other species as cues to postpone germination in areas with intense interspecific competition (e.g. Preston & Baldwin 1999). In cases of autoinhibition, chemicals produced by adults might serve to postpone germination of offspring near their parents (Picman & Picman 1984). Although many studies have suggested the occurrence of chemical-mediated intraspecific interference (Webb *et al.* 1967; McNaughton 1968; Picman & Picman 1984;

Mahall & Callaway 1992, 1996; Singh *et al.* 1999; Dyer 2004), to our knowledge none has examined the effects of identified biochemicals in the absence of confounding effects from plant residues or soils, and linked the biochemicals to their effects in the field. Furthermore, it has been suggested that allelochemicals might also regulate intraspecific interactions (McCormick 1968), but to our knowledge no study has demonstrated the potential for plant compounds to serve dual roles as allelochemicals and autoinhibitors under natural conditions. In this report, we examine intraspecific effects of a phytotoxin produced by *Centaurea maculosa* Lam. (spotted knapweed), an exotic invasive plant in North America.

The allelochemistry of *C. maculosa* has been well studied (Ridenour & Callaway 2001; Bais *et al.* 2002, 2003; Weir *et al.* 2003). *Centaurea maculosa* roots exude racemic ( $\pm$ )-catechin. Both catechin enantiomers are phytotoxic (Bais *et al.* 2002, 2003; Iqbal *et al.* 2003), but (–)-catechin is much more potent; 50–100% higher concentrations of (+)-catechin are required to have the same effect as (–)-catechin on the model plant *Arabidopsis thaliana* (Veluri *et al.* 2004). (–)-Catechin induces cell death in the roots of North American plant species and, in combination with (+)-catechin, probably facilitates *C. maculosa* invasion (Bais *et al.* 2003). European species that coexist with *C. maculosa* in its native range, however, tend to be relatively insensitive to (–)-catechin (Bais *et al.* 2003), leading to uncertainty about the role of ( $\pm$ )-catechin in the native range of *C. maculosa*. Previous experiments have indicated that high concentrations of (–)-catechin (0.4 mg mL<sup>-1</sup>) inhibit *C. maculosa* germination *in vitro* (Weir *et al.* 2003), suggesting a potential for autoinhibition by ( $\pm$ )-catechin.

To determine whether ( $\pm$ )-catechin regulates *C. maculosa* seedling establishment, we: (i) tested whether organic compounds including ( $\pm$ )-catechin present in the soil in established North American *C. maculosa* populations inhibit *C. maculosa* seedling establishment, (ii) explored whether soil ( $\pm$ )-catechin concentrations in North American *C. maculosa* populations are sufficient to inhibit *C. maculosa* seedling establishment and (iii) examined effects of ( $\pm$ )-catechin on *C. maculosa* germination and seedling growth in incubator experiments. Our principal objective was to evaluate whether ( $\pm$ )-catechin, an allelochemical known to have strong interspecific effects, also regulates intraspecific interactions in *C. maculosa*.

## Methods

### STUDY SPECIES LIFE HISTORY

*Centaurea maculosa* is a short-lived perennial that forms a basal rosette in its first year and flowers annually beginning in its first, second or third year (Story *et al.* 2001). Some plants produce additional rosettes asexually, but most spread occurs via seed. Annual seed production can exceed 10 000 seeds m<sup>-2</sup> (Schirman 1981), and 60–80% of *C. maculosa* seeds are viable (Jacobs & Sheley

1998). Seed production appears to be reduced by low precipitation (Schirman 1981) and the presence of seed-feeding biological control agents (Muller-Schärer & Schroeder 1993).

#### C. MACULOSA POPULATION DENSITIES AND SPATIAL DISTRIBUTIONS

In April 2004, we examined densities of *C. maculosa* adults and seedlings in 30 randomly located, 30-cm-radius plots, each centred on an adult *C. maculosa*, in a population on the west-facing slope of Mt Sentinel at ~1000 m a.s.l. in Missoula, MT (46°84' N, 113°97' W). The ~350-m<sup>2</sup> area sampled was visually dominated by *C. maculosa* rosettes, but < 2-cm-tall seedlings of *Bromus tectorum* L. (downy brome) and *Poa* spp. (*Poa pratensis* L. or *Poa compressa* L.) were also abundant, and *Sisymbrium altissimum* L. (tumble mustard) was present. Other species may occupy the site but were not apparent in April 2004. *Centaurea maculosa* had been present in the site for at least 10 years (R. Callaway, personal observation). The soil at the site is a Bigarm gravelly loam (loamy-skeletal, mixed, superactive, frigid Typic Haploxeroll) on slopes with a gradient of > 30% (National Cooperative Soil Survey 1995). Mean daily maximum and minimum temperatures in Missoula in April 2004 were 15.9 °C and 0.6 °C, and total monthly precipitation was 2.57 cm, similar to 50-year means for Missoula (Western Regional Climate Center; <http://www.wrcc.dri.edu>).

Root diameters of all *C. maculosa* plants in each plot were measured 2 cm below the root crown with calipers. Seedlings were defined as plants with root diameters < 1.0 mm and adults as plants with root diameters > 4.5 mm (Story *et al.* 2001): intermediate-sized plants were considered juveniles. Nearest-neighbour distances for different size classes were determined by measuring the distances between a target adult at the centre of each plot and all other *C. maculosa* in the plot. The number of *C. maculosa* plants in each plot ranged from 8 to 47 (mean = 22). Effects of distance from the target adult were examined by dividing the plots into concentric 5-cm-wide rings and using ANOVA to compare *C. maculosa* densities among rings. All statistical analyses were performed using Proc GLM in SAS (SAS Institute, Inc., Cary, NC, USA, version 9.1).

#### ACTIVATED CARBON EXPERIMENT

To test whether organic compounds (i.e. allelochemicals) in the soil inhibit *C. maculosa* seedling establishment in field populations, we conducted an experiment at the field site described above. Three 10 cm × 10 cm plots were established within 5 cm of each of five randomly selected *C. maculosa* plants in each of three blocks in April 2004. Ten millilitres of activated carbon (Carbochem, Inc., Ardmore, PA, USA), which adsorbs organic compounds (Mahall & Callaway 1992), was gently incorporated to a depth of 5 cm with a hand-rake into one plot of each set, the second was disturbed with the

hand-rake, but no carbon was added and the third was not disturbed. All plots were sown with 100 *C. maculosa* seeds (10 000 seeds m<sup>-2</sup>) to swamp existing differences in the seed bank and were given a one-time addition of 50 mL (0.5 cm) of water to mimic typical weekly precipitation in April in Missoula (Western Regional Climate Center; <http://www.wrcc.dri.edu>). Plant litter was removed prior to and replaced after carbon, seed and water addition. *C. maculosa* seedling densities were measured 19, 25, 32, 41, 54 and 68 days after sowing. Cube-root-transformed seedling density was compared among treatments with repeated-measures ANOVA, with block and plant nested within block as additional factors.

#### SOIL (±)-CATECHIN CONCENTRATIONS

(±)-Catechin concentrations were estimated in 112 soil samples collected from the field site. Soil cores, 1 cm in diameter by 5 cm deep, were collected at 5-cm intervals along 11 transects between nearest-neighbour pairs of adult *C. maculosa*; transects varied in length from 35 cm to 60 cm and did not overlap the 30-cm-radius plots described above. Soil samples were transported to the laboratory in sealed plastic bags in a cooler and stored at 4 °C in the dark for up to 8 weeks. (±)-Catechin in a 0.5-mL subsample from each core was extracted in 1 mL absolute methanol. Care was taken to avoid gravel and large organic matter particles when subsampling. Because (±)-catechin has not been found in *C. maculosa* leaf, root or stem tissue (H.P. Bais & J.M. Vivanco, unpublished data), small organic matter particles were not removed from the soil subsamples. Extracts were vortex mixed, centrifuged, transferred, concentrated, resuspended in 0.4 mL methanol and stored at -50 °C. (±)-Catechin concentrations were then determined by high-performance liquid chromatography (HPLC) in comparison to 1 mg mL<sup>-1</sup> standards and are expressed on a per g dry soil basis (note that these estimates are conservative because the extraction methods are unlikely to recover all (±)-catechin present).

HPLC separations were conducted using mobile phase solutions of double distilled water (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 × 0.46 cm) (Supelco Co., Bellefonte, PA). The flow rate was 1 mL min<sup>-1</sup>, the sample injection volume was 5 µL; visible absorbance was measured at 280 nm. The catechin fractions eluted by HPLC from two randomly selected samples were analysed by mass spectrometry in the Colorado State University Chemistry Department Laboratory, to confirm that the fractions comprised mainly (±)-catechin. Effects of distance from the *C. maculosa* adults were evaluated with ANOVA, dividing each transect in half and assigning data to the adult at the nearer end. 'Transect half' was included as a factor, nested within transect, in the analysis.

BIOACTIVITY OF SOIL ( $\pm$ )-CATECHIN

To test whether the ( $\pm$ )-catechin in *C. maculosa* soils had similar phytotoxic activity to ( $\pm$ )-catechin from commercial sources, we examined the effects of relatively low concentrations of soil ( $\pm$ )-catechin on the model plant *Arabidopsis thaliana*, for which the physiological effects and minimum inhibitory concentrations of ( $\pm$ )-catechin are well understood (Bais *et al.* 2003). We used methanol to extract ( $\pm$ )-catechin from one randomly selected soil sample from the *C. maculosa* field site. Purified ( $\pm$ )-catechin fractions eluted by HPLC were concentrated, generating no more than 25  $\mu\text{g}$  of material, and re-suspended in 200  $\mu\text{L}$  methanol. In addition, a 1  $\text{mg mL}^{-1}$  solution of ( $\pm$ )-catechin from Sigma Co. (St Louis, MO, USA) was prepared in methanol. *Arabidopsis thaliana* plants grown for 5 days in sterile culture in liquid MS media (Murashige & Skoog 1962) were treated with 100  $\mu\text{L}$  of the extracted ( $\pm$ )-catechin solution per mL of liquid media, 100  $\mu\text{L mL}^{-1}$  of the Sigma ( $\pm$ )-catechin solution, or 100  $\mu\text{L mL}^{-1}$  of methanol. Survival was compared among treatments after 7 days.

EFFECTS OF ( $\pm$ )-CATECHIN ON *C. MACULOSA* GERMINATION AND ROOT ELONGATION

We conducted incubator experiments to evaluate effects of ( $\pm$ )-catechin on *C. maculosa* seedling establishment. ( $\pm$ )-Catechin was applied at seven concentrations (0, 0.125, 0.25, 0.5, 1, 2 and 4  $\text{mg mL}^{-1}$ ) to 50 *C. maculosa* seeds on Whatman #41 ashless filter paper in 60-mm Petri dishes. *Centaurea maculosa* seeds were collected in 1999 from Missoula County. Seeds were surface-sterilized in 10% bleach for 10 min and rinsed with distilled water prior to treatment. Treatments were applied using ( $\pm$ )-catechin (Shivambu International, New Delhi, India) dissolved in absolute methanol and diluted with distilled water to make a 10% methanol, 5  $\text{mg mL}^{-1}$  ( $\pm$ )-catechin solution. A 10% methanol solution was added so that all treatments, including controls, received the same volume of methanol in a total of 2 mL of liquid. Water-only controls were also included. Each treatment was replicated four times. Dishes were sealed with parafilm and placed in darkness at 25 °C. After 2 days, in response to delayed germination in all but the water-only controls, the dishes were opened for 2 h to allow the methanol to evaporate. ( $\pm$ )-Catechin fell out of solution as the methanol evaporated, leaving visible crystals in the higher ( $\pm$ )-catechin concentrations. The evaporated liquid was replaced with water and the dishes were resealed. A similar experiment was also conducted in which the methanol was allowed to evaporate immediately after ( $\pm$ )-catechin treatment. Germination (radicle emergence) and root lengths were measured 10 days after treatment. Percentage germination data were arcsine, square-root transformed for analysis (Sokal & Rohlf 1995).

To test the effects of methanol on the effects of ( $\pm$ )-catechin on *C. maculosa* germination, we conducted a

similar experiment to those described above, except that ( $\pm$ )-catechin was applied at only two concentrations (0 and 4.0  $\text{mg mL}^{-1}$ ) and the dishes were sealed for 0, 1, 2, 3 or 6 days before allowing the methanol to evaporate. Each methanol  $\times$  catechin treatment was replicated five times. To examine effects of ( $\pm$ )-catechin on *C. maculosa* seed viability, we used tetrazolium analyses (Grabe 1970) to evaluate the viability of ungerminated seeds after 10 days of ( $\pm$ )-catechin treatment, with 2 days in 10% methanol and 8 days in water. Tetrazolium staining can be difficult to interpret, but efforts were made to employ consistent criteria for judging viability, under the direction of trained staff at the Colorado Seed Laboratory (Fort Collins, CO, USA). Embryos that were stained uniformly dark pink were identified as alive, whereas partially dark pink, pale pink and white embryos were considered dead.

## Results

*C. MACULOSA* POPULATION DENSITIES AND SPATIAL DISTRIBUTIONS

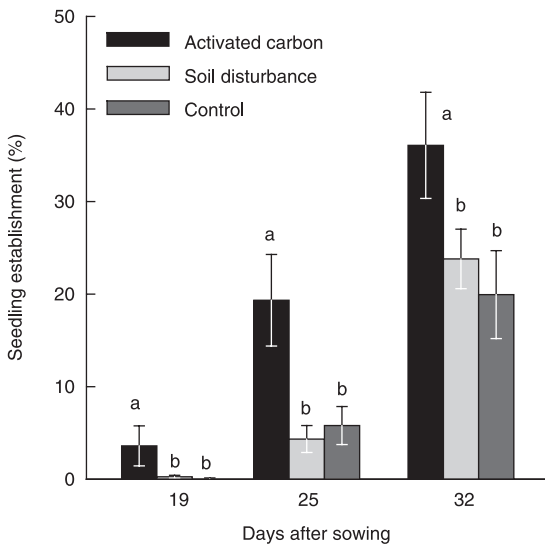
Seedlings, juveniles and adults made up nearly equal proportions of the *C. maculosa* populations in the 30-cm-radius plots at the time of our measurements (Table 1). These proportions probably vary seasonally and annually and, in some cases, *C. maculosa* seedlings can be far more abundant (R.M. Callaway, personal observation). Seedlings were less consistently present than older plants; 20% of plots contained no *C. maculosa* seedlings, whereas only 7% contained no juveniles and only 3% contained no adults (other than the target adult at the centre of each plot). *Centaurea maculosa* plants were often separated by unoccupied space, and nearest-neighbour distances between the adult at the centre of each plot and seedlings, juveniles and other adults were similar (Table 1). Seedling and juvenile densities were relatively low within 5 cm of the target adult, and were greatest between 5 cm and 10 cm of the target adult (Table 1). Mean densities of all three age classes declined with distances > 10 cm from the target adult, but these trends were not statistically significant. The other adults in the plots probably also influenced seedling and juvenile densities and distributions at all distances from the target adult.

## ACTIVATED CARBON EXPERIMENT

*Centaurea maculosa* seedling density was greater in plots with activated carbon added to the soil than in plots with soil disturbance alone and control plots in all measurement periods (Fig. 1). The positive effect of activated carbon on *C. maculosa* seedling density was most pronounced in our earliest observations; mean seedling density was 93% greater in soil with activated carbon than in the controls 19 days after sowing. However, 32 days after sowing, the mean seedling density in soil with activated carbon was still 34% greater than

**Table 1** Mean population density and distance between *C. maculosa* neighbours in a population in Missoula, MT. Densities were determined in circular plots (radius = 30 cm) centred around a ‘target’ adult. Densities are presented for whole plots and for 5-cm-wide concentric rings around the centre of each plot. Nearest-neighbour distances were also determined for the target adult in each plot. Densities and nearest-neighbour distances (mean ± 1 SE,  $n = 30$ ) are given for three age classes: seedlings (root diameter < 1 mm), juveniles (root diameter 1–4.5 mm) and adults (root diameter ≥ 4.5 mm)

	<i>C. maculosa</i> population density (individuals m <sup>-2</sup> )							Distance to nearest neighbour (cm)
	Whole plot	Distance from target adult (cm)					25–30	
		0–5	5–10	10–15	15–20	20–25		
All plants	78 ± 6	64 ± 14	93 ± 15	71 ± 11	52 ± 7	49 ± 6	37 ± 4	7 ± 1
Seedlings	28 ± 5	17 ± 8	40 ± 12	31 ± 8	24 ± 6	19 ± 4	8 ± 2	10 ± 1
Juveniles	23 ± 4	13 ± 7	27 ± 9	20 ± 4	14 ± 3	15 ± 3	12 ± 2	12 ± 1
Adults	26 ± 2	34 ± 10	27 ± 5	20 ± 5	13 ± 2	15 ± 2	17 ± 2	11 ± 1

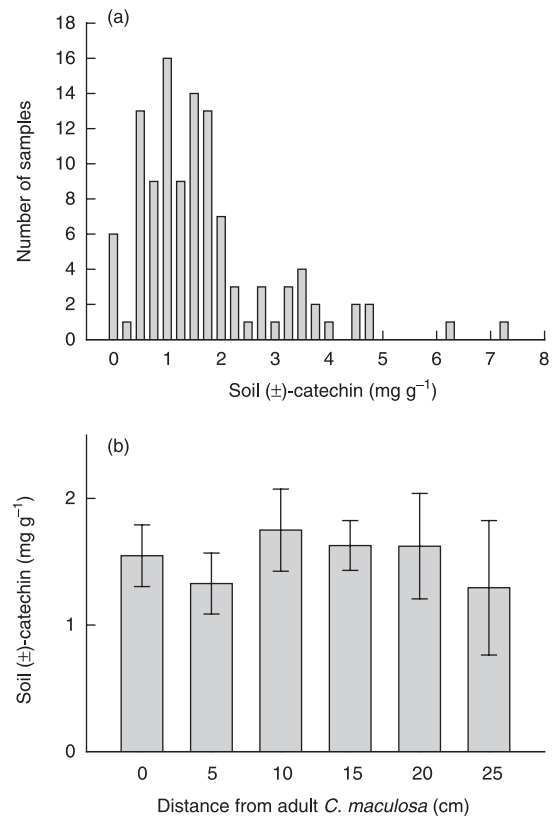


**Fig. 1** Mean seedling establishment (%) in 10 × 10 cm field plots sown with 100 *C. maculosa* seeds. Plots around adult *C. maculosa* received activated carbon with soil disturbance, soil disturbance alone or no soil disturbance (control). Different letters indicate significantly different means (Tukey HSD,  $P < 0.05$ ). Error bars are 1 SE of the mean.  $n = 15$ .

the seedling density in the soil disturbance controls. Seedling densities continued to be 11–31% greater in soil amended with activated carbon than in the soil disturbance controls 41 days, 54 days and 68 days after sowing (data not shown). There was no significant interaction between treatment and sampling date in a repeated-measures ANOVA that included all six sampling dates. Soil disturbance without activated carbon addition did not significantly increase *C. maculosa* seedling density (Tukey HSD,  $P > 0.05$ ), indicating that soil disturbance did not account for the effects of activated carbon.

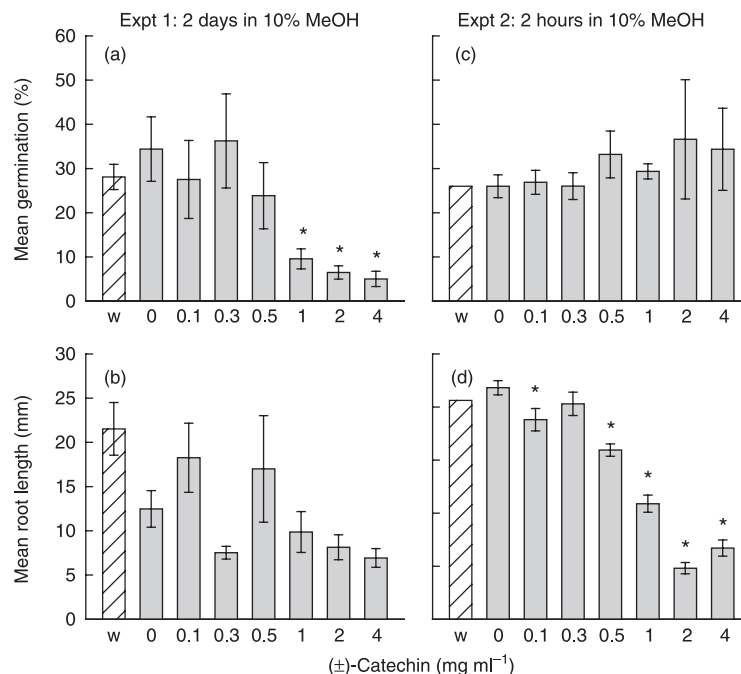
#### FIELD SOIL (±)-CATECHIN CONCENTRATIONS

The mean (±SD) soil (±)-catechin concentration in the 112 samples was 1.55 ± 1.27 mg g<sup>-1</sup> dry soil. Catechin was not detected in six of the samples and concentrations in the remaining 106 samples varied between 0.18 mg g<sup>-1</sup> and 7.10 mg g<sup>-1</sup> (Fig. 2a). Eighty-two per cent of the samples contained ≥ 0.5 mg (±)-catechin g<sup>-1</sup> and



**Fig. 2** (a) Frequency distribution of soil (±)-catechin concentrations in 5-cm-deep soil cores collected at 5-cm intervals along transects between 11 pairs of adult *C. maculosa*. (b) Mean soil (±)-catechin concentrations at different distances from the adult plants. To generate the means in (b), each transect was divided in half, and the data from each half were assigned to the adult at each end. Because some pairs of adults were separated by < 50 cm,  $n = 22$  for the 0-, 5-, 10- and 15-cm distances, but  $n = 17$  and 6 for the 20- and 25-cm distances, respectively. Error bars are 1 SE of the mean.

60% contained ≥ 1.0 mg g<sup>-1</sup>. Soil (±)-catechin concentrations did not change significantly with distance up to 25 cm from *C. maculosa* adults (Fig. 2b). However, mean (±)-catechin concentrations differed among transects (ANOVA,  $F_{10,84} = 2.78$ ,  $P = 0.005$ ), between plants at either end of each transect (ANOVA,  $F_{11,84} = 1.95$ ,  $P = 0.044$ ) and considerably, although not predictably, along each transect.



**Fig. 3** Effects of (±)-catechin on *C. maculosa* (a) germination and (b) root elongation in an experiment in which (±)-catechin was maintained in solution in 10% methanol for 2 days before the methanol was removed. These trends differed from effects of (±)-catechin on (c) germination and (d) root lengths when the methanol was removed immediately after (±)-catechin treatment. Means significantly smaller than the methanol controls (0) (Dunnnett's one-tailed *t*-test) are marked (\*). Data for water-only controls (patterned bars) are also shown. Error bars are 1 SE of the mean. *n* = 4.

Mass spectrometry of the catechin fractions collected by HPLC from two randomly selected samples confirmed that the peaks used to quantify concentrations consisted almost entirely of (±)-catechin. Of the other compounds present, none exceeded 10% of the abundance of (±)-catechin and only five compounds were present at > 5% of the abundance of (±)-catechin. Three of those five compounds were also present at low abundance in a (±)-catechin standard solution, and have molecular weights equal to the mass of frequently observed plasticizer contaminants (121.1, 173.0 and 212.1), which may have been present on laboratory glassware or in the HPLC or gas chromatography/mass spectrometry equipment.

#### BIOACTIVITY OF SOIL (±)-CATECHIN

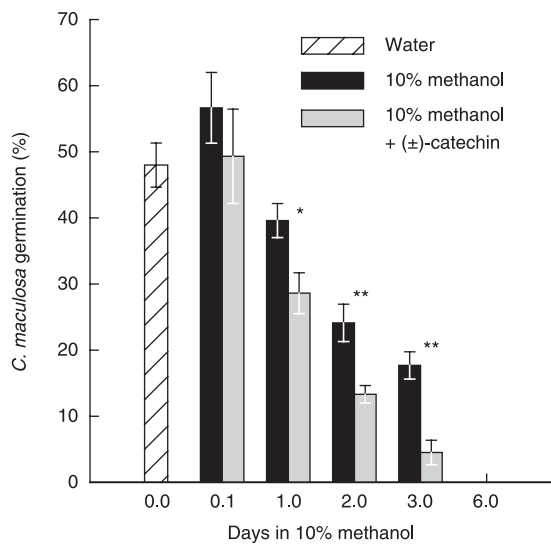
Treatment with both the crude extracts and the (±)-catechin collected by HPLC elution from *C. maculosa* soil resulted in darkened, stunted roots in *Arabidopsis thaliana* and complete mortality after 7 days. These symptoms were similar to those of *A. thaliana* treated with 100 µg mL<sup>-1</sup> (±)-catechin obtained from Sigma Co. *Arabidopsis thaliana* treated with methanol alone did not exhibit these symptoms.

#### EFFECTS OF (±)-CATECHIN ON *C. MACULOSA* GERMINATION AND ROOT ELONGATION

When *C. maculosa* seeds were treated with (±)-catechin suspended in 10% methanol for 2 days before the

methanol was allowed to evaporate, (±)-catechin reduced *C. maculosa* germination by > 70% at concentrations ≥ 1.0 mg mL<sup>-1</sup> (Fig. 3a). In addition, high (±)-catechin concentrations (≥ 1.0 mg mL<sup>-1</sup>) resulted in dark brown *C. maculosa* roots among germinated seedlings. However, we did not detect a significant effect of (±)-catechin on *C. maculosa* seedling root length, perhaps because root elongation was poor in the methanol controls (Fig. 3b). By contrast, when *C. maculosa* seeds were treated with (±)-catechin suspended in 10% methanol for only a few hours before the methanol was allowed to evaporate, (±)-catechin did not reduce *C. maculosa* germination (Fig. 3c) but did reduce *C. maculosa* seedling root length, even at relatively low concentrations (0.125 mg mL<sup>-1</sup>) (Fig. 3d). *C. maculosa* seedling root lengths were reduced by > 50% by ≥ 1.0 mg (±)-catechin mL<sup>-1</sup>.

A direct test of the effects of methanol on *C. maculosa* responses to (±)-catechin confirmed that (±)-catechin inhibits *C. maculosa* germination only when (±)-catechin is maintained in solution in 10% methanol for at least 1 day (Fig. 4), possibly because (±)-catechin can infiltrate the seed coat only when in solution. (±)-Catechin had no effect on *C. maculosa* germination when the methanol used to dissolve (±)-catechin was allowed to evaporate immediately after (±)-catechin treatment. There was a significant effect when the methanol was allowed to evaporate after 1 day, but both the significance and the extent of the response to (±)-catechin treatment increased with additional time in methanol (Fig. 4). Time spent in methanol also directly reduced *C. maculosa* germination ( $F_{3,33} = 46.3$ ,  $P < 0.001$ ).



**Fig. 4** (±)-Catechin inhibition of *C. maculosa* germination depended on the time that (±)-catechin was maintained in solution in 10% methanol. Methanol was removed by evaporation immediately, or 1, 2, 3 or 6 days after treatment. The (±)-catechin concentration was 4 mg mL<sup>-1</sup>. Significant effects of (±)-catechin are marked (\**P* < 0.05, \*\**P* < 0.01). Error bars are 1 SE of the mean. *n* = 5.

#### EFFECTS OF (±)-CATECHIN ON *C. MACULOSA* SEED VIABILITY

Tetrazolium analyses of (±)-catechin-treated *C. maculosa* seeds maintained in 10% methanol for 2 days and then in water for 8 days revealed that some ungerminated seeds remained viable both with and without (±)-catechin treatment (mean = 22 ± 2% of ungerminated seeds). The percentage of total (±)-catechin-treated seeds that were dead was 67 ± 2% compared with 59 ± 4% in the controls, but this difference was not statistically significant.

#### Discussion

Our results demonstrate that (±)-catechin, the phytotoxin produced by *C. maculosa*, has intraspecific effects in addition to its known interspecific effects. Specifically, (±)-catechin is one of the factors that determines *C. maculosa* recruitment in established populations. First, activated carbon added to the soil around adult *C. maculosa* plants in the field increased *C. maculosa* seedling density by 78% 25 days after sowing and by 34% 32 days after sowing (Fig. 1), suggesting that organic compounds in the soil inhibited or delayed seedling establishment. Second, 95% of the soil samples we examined from an established *C. maculosa* population contained (±)-catechin (Fig. 2a), and 60% of the soil samples contained ≥ 1.0 mg g<sup>-1</sup> dry soil. Even low concentrations of (±)-catechin extracted from *C. maculosa* soil caused mortality in *Arabidopsis thaliana* at rates similar to (±)-catechin obtained from commercial sources, confirming that the extract was bioactive. Finally, treatment of *C. maculosa* seeds with ≥ 1.0 mg (±)-

catechin mL<sup>-1</sup> reduced *C. maculosa* seedling root elongation by > 50%, even when the (±)-catechin was not held in solution, and reduced *C. maculosa* germination by > 70% when the (±)-catechin was held in solution in 10% methanol (Fig. 3). These results demonstrate that *C. maculosa* seedling growth and, perhaps, germination are sensitive to the species' phytotoxin at naturally occurring concentrations, although particular responses may depend on whether and how (±)-catechin is maintained in soil solution. (±)-Catechin inhibition of *C. maculosa* recruitment may account in part for the relatively wide spaces between individuals (mean = 7 cm) in *C. maculosa* populations.

Our observations do not suggest that intraspecific chemical regulation is the only factor that limits *C. maculosa* recruitment. *Centaurea maculosa* seed production can exceed 10 000 seeds m<sup>-2</sup> (Schirman 1981), but we observed a mean of only 28 seedlings m<sup>-2</sup>, and 20% of the plots we examined contained no *C. maculosa* seedlings, suggesting that there are strong pressures limiting recruitment. Seedling density in the control plots of the activated carbon experiment, which were sown with *C. maculosa* (Fig. 1), was much greater than in neighbouring areas (1993 ± 475 m<sup>-2</sup> in sown plots compared with 28 ± 5 m<sup>-2</sup> in random plots), indicating that recruitment is limited in large part by low numbers of viable, non-dormant seed. Furthermore, no *C. maculosa* seedlings were present where the soil samples were collected (L. Perry, personal observation), even though variation in soil (±)-catechin concentrations within the site (Fig. 2a) indicated that some locations did not contain sufficient (±)-catechin to inhibit seedling establishment. Seed predation, particularly by biological control organisms (Muller-Schärer & Schroeder 1993), may limit *C. maculosa* seed densities in the site, and water stress may limit *C. maculosa* seedling survival (Jacobs & Sheley 1998). Nevertheless, (±)-catechin production by *C. maculosa* appears to control establishment of conspecifics where other factors are not limiting.

*Centaurea maculosa* plants might benefit from (±)-catechin inhibition of seedling establishment in several ways. First, adults, by inhibiting seedling root growth or germination, may reduce establishment of intraspecific competitors. Intraspecific competition can be intense, because conspecifics share the same resource requirements and acquisition strategies. Second, *C. maculosa* seedlings begin to exude (±)-catechin within 2 weeks of germination under *in vitro* conditions (Weir *et al.* 2003, 2004a) and may further reduce intraspecific competition by interfering with establishment of siblings or other neighbours. *Centaurea maculosa* seeds most often disperse less than 1 m from their parents (Watson & Renney 1974), perhaps making sibling rivalries particularly important. Third, *C. maculosa* seeds, by postponing germination in response to a chemical signal from adults, may avoid establishing in areas where intraspecific competition would prevent their survival. The abundance of dead *C. maculosa* seeds was slightly, but not significantly, greater among (±)-catechin-treated seeds than

in the controls, suggesting that ( $\pm$ )-catechin may have killed some *C. maculosa* seeds, but that delayed germination must also account for some of the effect. Interference from conspecific adults has been shown to have large effects on the seedling survival of *Centaurea diffusa* Lam., a closely related species (Powell 1990). Dormant *C. maculosa* seeds can survive in the seed bank for at least 8 years (Davis *et al.* 1993), indicating that seeds prevented from germinating by high soil ( $\pm$ )-catechin concentrations would be likely to outlive and, perhaps eventually, replace established *C. maculosa* adults (Boggs & Story 1987).

Assessing the specific role of autoinhibition in regulating *C. maculosa* recruitment will require further research to determine the nature of the effect of ( $\pm$ )-catechin on *C. maculosa* in the field. In the present field experiment, *C. maculosa* seedling density was 78% greater in plots with activated carbon than in the control plots 25 days after sowing, but only 34% greater after 32 days (Fig. 1), suggesting that ( $\pm$ )-catechin inhibition of recruitment may be temporary for at least some seedlings in the field. *Centaurea maculosa* seedlings may develop resistance to ( $\pm$ )-catechin exposure, or soil ( $\pm$ )-catechin concentrations may decline in the spring, allowing some *C. maculosa* seedlings to establish. Although a seasonal delay in establishment could increase seedling survival, an earlier study suggested that survival is greatly reduced for *C. maculosa* seedlings that establish later in the spring (i.e. May) compared with seedlings that establish earlier (Schirman 1981). Thus, short-term delays in *C. maculosa* seedling establishment due to ( $\pm$ )-catechin could result in increased seedling mortality.

Furthermore, the nature of the effect of ( $\pm$ )-catechin on *C. maculosa* may depend in part on whether ( $\pm$ )-catechin is held in soil solution. In our experiments, ( $\pm$ )-catechin inhibited *C. maculosa* germination only when held in solution for at least 1 day in 10% methanol. ( $\pm$ )-Catechin may be unable to infiltrate the seed coat and influence germination when not in solution. The proportion of ( $\pm$ )-catechin that is held in soil solution compared with in crystalline and bound forms in *C. maculosa* soils is not known. In addition, the plant substances in root exudates and other soil compounds that solubilize ( $\pm$ )-catechin in soil may not allow soil ( $\pm$ )-catechin to influence germination in the same way that a 10% methanol solution does. Prolonged exposure to 10% methanol directly reduced *C. maculosa* root elongation and germination, apparently obscuring the effect of ( $\pm$ )-catechin on root growth (Fig. 3b) and perhaps compounding effects of ( $\pm$ )-catechin on germination (Fig. 4). Research on the location and form of ( $\pm$ )-catechin in soil and in *C. maculosa* root exudates is needed to gain further insights into the frequency with which ( $\pm$ )-catechin affects germination compared with seedling growth in *C. maculosa* populations.

The very high soil concentrations that we observed throughout the field site support the more general hypothesis that ( $\pm$ )-catechin production by *C. maculosa* may have important effects on plant community

structure (Bais *et al.* 2003). Soil ( $\pm$ )-catechin concentrations did not decline with distances up to 25 cm from isolated *C. maculosa* adult shoots (Fig. 2b). Because adult *C. maculosa* shoots were rarely separated by more than 50 cm (L. Perry, personal observation), these results suggest that areas of high ( $\pm$ )-catechin concentrations around adults often overlap, creating unfavourable conditions for catechin-sensitive species throughout *C. maculosa* populations. Soil ( $\pm$ )-catechin concentrations did not vary predictably with distance from *C. maculosa* adult shoots, suggesting either (i) that widespread *C. maculosa* root systems allow for wide zones of rhizosecretion around adult shoots or (ii) that ( $\pm$ )-catechin diffuses easily from the rhizosphere and through the bulk soil. Further research is needed to determine the extent to which the presence and concentration of ( $\pm$ )-catechin in particular microsites reflects local root exudation compared with diffusion from nearby or distant roots.

Chemical regulation of *C. maculosa* recruitment, as demonstrated here, suggests a dual role of ( $\pm$ )-catechin as an allelochemical and an autoinhibitor. In the native range of *C. maculosa* in Europe, the importance of ( $\pm$ )-catechin as an allelochemical may be limited by ( $\pm$ )-catechin resistance in European species (Bais *et al.* 2003). Thus ( $\pm$ )-catechin may be more important for self-regulation than for interspecific interference in European *C. maculosa* populations. Examination of intraspecific chemical inhibition in European *C. maculosa* populations may yield further insights into the role of ( $\pm$ )-catechin in the native range of *C. maculosa* (Hierro *et al.* 2005).

### Acknowledgements

This work was supported by grants from Colorado State University Agricultural Experiment Station (to J.M.V.), USDA-WRIPM 2003-05060 to J.M.V. and M.W.P., National Science Foundation (NSF-IBN 0335203 to J.M.V.), US Department of Defense SERDP (CS1388 to J.M.V., M.W.P. and R.M.C.; and CS1145 to M.W.P.), and Invasive Weeds Initiative of the State of Colorado (to J.M.V.). R.M.C. also thanks the NSF, Peter Landres, the Rocky Mountain Research Station, and the Aldo Leopold Wilderness centre for support (RJVA 04-JV-11222044-235). Dr Martin Heil and two anonymous referees provided useful comments on earlier versions of the manuscript. Carbochem, Inc. generously supplied activated carbon. The Colorado Seed Laboratory generously provided supplies for tetrazolium analyses. H. Bais, D. Cooper, C. Hall, C. Johnson and V. Stull provided invaluable field and laboratory assistance.

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*Received 31 December 2004  
revision accepted 11 April 2005  
Handling Editor: Martin Heil*