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Catechin–metal interactions as a mechanism for conditional allelopathy by the invasive plant *Centaurea maculosa*

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Summary

1. Evaluating variation, or 'conditionality', in plant interactions is crucial to understanding their ecological importance and predicting where they might be at play. Much is known about conditionality for competition, facilitation and herbivory, but not for allelopathy, which likely contributes to the equivocal nature of reports on this topic. *Centaurea maculosa* (spotted knapweed) is an invasive species in North America, whose success has been attributed, at least in part, to the allelochemical root exudate (±)-catechin.

2. Understanding the ecological relevance of (±)-catechin necessitates determining how it interacts with various soil components. We found that some metals caused rapid declines in measurable (±)-catechin, while calcium impeded its auto-oxidation, maintaining concentrations higher than for (±)-catechin alone. Certain (±)-catechin–metal complexes were more phytotoxic than (±)-catechin alone, while others showed lower toxicity.

3. The variable phytotoxicity of these complexes suggests that (±)-catechin effects are enhanced, mitigated or otherwise affected by complexation with different metals and perhaps other soil components.

4. *Synthesis*. These findings serve to illustrate that the precise chemical forms, interactions and effects of catechin in the environment are highly variable and that further examination is warranted to increase our understanding of its role in invasion and allelopathy. The conditional effects observed for catechin detection and phytotoxicity likely extend to related allelopathic compounds, other root exudates and potentially other systems involving chemically complex and spatially heterogeneous environments.

Key-words: catechin, *Centaurea maculosa*, conditionality, invasive weed, leaf senescence, metal chelation, phytotoxicity, plant mortality, root exudate

Introduction

Considering variation, or conditionality, in the ways that plants compete for resources, facilitate or indirectly interact with each other has been crucial for understanding the relative importance of these interactions in the organization of plant communities (Tilman 1985; Wilson & Keddy 1986; Kitzberger, Steinaker & Veblen 2000; Levine 2000; Brooker *et al.* 2005; Baumeister & Callaway 2006). Such conditionality may also be important for allelopathic interactions, but to our knowledge there have been no explicit, quantitative studies of conditional allelopathic effects or of the mechanisms that might cause them.

Allelopathic effects of the North American invasive plant *Centaurea maculosa* Lam. (spotted knapweed) have been reported from leaves (Fletcher & Renney 1963; Bohlmann, Burkhart & Zdero 1973; Stevens 1986; Kelsey & Locken 1987) and roots (Ridenour & Callaway 2001). Also, phytotoxic effects of (±)-catechin, a racemic phenolic compound exuded from the roots of *C. maculosa* (hereafter referred to simply as catechin), have been demonstrated *in vitro* (Buta & Lusby 1986; Bais *et al.* 2002; Iqbal *et al.* 2003; Weir, Bais & Vivanco 2003; Perry *et al.* 2005b; D'Abrosca *et al.* 2006; Weir *et al.* 2006; Furubayashi, Hiradate & Fujii 2007; Rudrappa *et al.* 2007; Simões *et al.* 2008), in sand (He *et al.* 2009), in controlled experiments with field soils (Bais *et al.* 2003; Inderjit *et al.*

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2008a,b) and in the field (Thelen *et al*. 2005; Thorpe 2006; Inderjit *et al*. 2008a,b). However, experiments have not always shown catechin to have inhibitory effects (Blair *et al*. 2005; Perry *et al*. 2005a), and effects on other species can vary substantially (Weir, Bais & Vivanco 2003; Perry *et al*. 2005a; Thorpe 2006). Furthermore, field applications of catechin at the same site and to the same plant species show substantial variability between years (Thelen *et al*. 2005; Thorpe 2006). Even in the same growing season, application of catechin dramatically reduced the growth of *Geum triflorum* Pursh in open grassland (Thorpe 2006), but the same concentration had no effect on *G. triflorum* in soils under *Pseudotsuga menzeisii* tree canopies at the same site (G.C. Thelen & R.M. Callaway, unpubl. data). Complicating this variation even more, exudate production rates from seedlings and mature plants *in vitro* range from 0–2.4 μg mL^{-1} (Blair *et al*. 2005), 5–35 μg mL^{-1} (Weir, Bais & Vivanco 2003; Weir *et al*. 2006), 0–113 μg mL^{-1} (Ridenour *et al*. 2008), 83–185 μg mL^{-1} (Bais *et al*. 2003), and 0–33 μg mL^{-1} (R.M. Callaway & J.L. Pollock, unpubl. data). In a related system, Tharayil and co-workers found that 8-hydroxyquinoline exudation by the roots of *Centaurea diffusa* varied on a diurnal basis (Tharayil *et al*. 2009). Natural soil concentrations of chemically pure catechin also vary spatially and temporally, with recent measurements being far lower than those initially reported (Blair *et al*. 2005, 2006; Perry *et al*. 2007). Collectively, these observations and reports suggest that substantial variability in the effects of both spotted knapweed and its exudate catechin may exist.

Variation in the effects of a putative allelopathic chemical and its detectable concentrations in the environment or in experiments could be due to many reasons, including: application of different chemical concentrations in experiments; structural differences in the chemical applied; age or size of target plants; seasonal timing of applications or soil collection; local temperature or moisture conditions; different analytical or methodological techniques; or local differences in the effects of soil chemistry or biota on the chemical. Polyphenolic root exudates (catechin belongs in this class of compounds) have been shown to auto-oxidize as well as interact with various specific metals (Gomah & Davies 1974; McDonald, Mila & Scalbert 1996; Lim, Ginny Lim & Liew 2005). Furthermore, Furubayashi, Hiradate & Fujii (2007) suggested that several allelopathic root exudates containing catechol moieties, as does catechin, are particularly prone to as yet undefined chemical transformations and binding to soil.

Here, we focus on how metals that are commonly found in soils affect the concentration of pure catechin in laboratory and greenhouse conditions, and assess whether catechin–metal complexes (hereafter CMCs) affect target plants differently than catechin itself. It is important to note that current analytical methods for determining catechin concentrations (e.g. Paveto *et al*. 2004; Blair *et al*. 2005) depend on HPLC-based detection of chemically pure catechin as a discrete chromatographic peak at a given elution time whose area represents the concentration of the chemical. As a result, essentially any chemical modification through addition, oxidation, complexation with metals, or full or partial degradation could alter the absorption wavelength of the modified catechin or its retention time on the HPLC column. This would render altered catechin undetectable by current detection methods, even though it could still be present in the system in this modified form and may be more or less phytotoxic than pure catechin itself.

We hypothesized that catechin, through its previously known ability to chelate metals and auto-oxidize, would be difficult to quantify reliably and, further, that such interactions and modifications of pure catechin could alter its phytotoxicity. Our results demonstrate that catechin interacts readily and differentially with various metals commonly found in soil. The resulting CMCs exhibit different levels of toxicity to plants and seeds, thereby demonstrating conditionality in the allelopathic effects of catechin and therefore *C. maculosa*, which produces it. These findings suggest that catechin, and perhaps other root exudates from other plants, may exist in multiple forms in soil after exudation, inducing variable and conditional effects on the surrounding plant community and perhaps other macro- and micro-biota. This conditionality may explain the seemingly equivocal nature of various reports regarding allelopathic and phytotoxic activities of *C. maculosa* and catechin, as well as other potentially allelopathic chemicals.

Materials and methods

EFFECT OF METALS ON CATECHIN IN SOLUTION

To examine the effects of metals commonly found in soils on the concentrations of catechin in simple solutions, we created mixtures of catechin and the following minerals: ferric chloride ($FeCl_3 \cdot 6H_2O$); cupric sulphate ($CuSO_4 \cdot 5H_2O$); calcium chloride ($CaCl_2 \cdot 2H_2O$); magnesium chloride ($MgCl_2 \cdot 6H_2O$) and lead nitrate ($Pb(NO_3)_2$(anh)). (±)-Catechin hydrate ($C_{15}H_{14}O_6 \cdot H_2O$) (racemic mixture; >99% purity) was obtained from Shivambu International (New Delhi, India) and all other chemicals from Sigma (St. Louis, MO, USA). For all solutions, catechin was dissolved into sodium phosphate-buffered solution (PB) [100 mM $NaPO_4$ (pH 7.0)] by stirring and gradually warming the solution to create a final concentration of 1000 μg mL^{-1} (3.25 mM). This stock solution was used to make all other solutions of CMCs and also served as the 'no metal' control. Although catechin is stable in ultra-pure water longer than in PB, using a buffered system allowed us to control for the effects of pH, which would vary substantially among these solutions if water were used rather than PB (J.L. Pollock & W.E. Holben, unpubl. data).

After cooling, catechin solution was dispensed into three replicate sterilized tubes for each treatment, and individual metals were then added to establish the following final concentrations: 4 mg mL^{-1} $FeCl_3 \cdot 6H_2O$; 3 mg mL^{-1} $CuSO_4 \cdot 5H_2O$; 4 mg mL^{-1} $Pb(NO_3)_2$(anh); 2.3 mg mL^{-1} $MgCl_2 \cdot 6H_2O$; and 1.7 mg mL^{-1} $CaCl_2 \cdot 2H_2O$. This produced a suite of treatments of 3.25 mM catechin plus 12–15 mM for each individual metal, which was expected to saturate all potential binding sites of the added catechin. The metals are hereafter referred to simply as Fe, Cu, Pb, Mg or Ca respectively. All tubes were kept stationary in the dark at room temperature for the course of the experiment and sampled periodically to measure by HPLC the amount of pure catechin remaining. All tubes were vortexed briefly before use or sampling because precipitates formed in most of the treatments (Mg being the exception). For catechin analysis, 1 mL of each suspension was transferred into a sterile Eppendorf tube, centrifuged at 15.7 g for 10 min, and the resulting supernatants placed into

HPLC vials to determine catechin concentrations as previously described (Paveto *et al.* 2004). In brief, catechin concentrations were determined by analysis of aqueous samples (15 μL injection volume) with UV detection at 280 nm on a Hewlett-Packard (HP) series 1100 HPLC (Hewlett-Packard, Palo Alto, CA, USA) using a HP ODS Hypersil C18 column (5 μm, 125 × 4 mm) and a 100% methanol : 0.2% phosphoric acid (1 : 3 v/v) mobile phase at a flow rate of 1 mL min^{-1}.

EFFECT OF METALS ON CATECHIN EXTRACTION FROM SAND

A number of studies have reported that catechin concentrations typically decrease rapidly following addition to soils (Blair *et al.* 2005, 2006; Furubayashi, Hiradate & Fujii 2007; Inderjit *et al.* 2008a), presumably because of auto-oxidation, sorption and/or chelation. Therefore, it is inappropriate to consider the concentration of pure catechin added (or exuded from knapweed) to soils or other substrates as the 'effective' *in situ* concentration, which likely is much lower. For the growth and survival experiments described below, in which pre-sterilized silica sand was used as a simplified growth matrix for plants, we wanted to assess the extractable concentration of catechin and the various CMCs immediately after addition to the sand matrix. To accomplish this, unplanted pots were separately amended with PB, catechin, each metal alone, or suspended CMC, then 1 g was immediately sampled and extracted with 100% methanol for catechin analysis by HPLC as described above.

EFFECT OF CMCS ON PLANT GROWTH AND STRESS SURVIVAL

To test the effects of catechin and CMCs on the growth and survival of native plant species, we applied each solution independently (except Mg–CMC, since Mg showed little effect on pure catechin concentrations in the initial abiotic experiment) to *Festuca idahoensis* ($n = 10$) and *Koeleria macrantha* ($n = 9$) plants grown in 300 g of pre-sterilized silica sand (20/30 grit, Lane Mountain Sand, Valley, WA, USA) in 525-cm^3 'rocket' pots. For negative controls, 10 individuals of *Festuca* and 9 individuals of *Koeleria* were transplanted, watered and fertilized as for the other treatments, but only PB was added. Both species were purchased as seed from Wind River Seed Co. (Manderson, WY, USA). Initially, each plant species was grown from seeds for 33 days before administering the first of four doses of the corresponding treatment (one dose every week). However, early and high mortality of these seedlings within 30 days of the onset of treatments (data not shown) indicated that multiple pulses of catechin or CMCs were highly toxic to seedlings. The experiment was therefore repeated, this time using more mature and established plants, which were initially grown in clean sand, then transplanted to the rocket pots containing the abovementioned CMC treatments (from which any surviving seedlings had been removed). Prior to transplanting, *Festuca* and *Koeleria* plants were grown for 64 days in clean potted sand in a greenhouse with natural and supplemental light (*c.* 1600 μmol m^{-2} s^{-1} on a sunny day) at temperatures maintained at a mean daily low of 20 °C and mean daily high of 24 °C. Plants were watered with tap water every other day, and fertilized every 21 days with 50 mL of Peters Excel 15-2-20 Plus Fertilizer solution (The Scotts Co., Marysville, OH, USA), mixed at 0.34 mg mL^{-1}. We maintained these same greenhouse conditions after transplanting, but ambient temperatures increased periodically to > 30 °C as the greenhouse warmed during the summer.

The transplants were allowed to equilibrate for *c.* 2 weeks following transplantation and were seemingly unaffected by the prior treatments with catechin or CMCs. Following this equilibration period, the pots were subjected to four more doses of the treatments (once a week for 4 weeks). For each time point, all treatment doses (PB, pure catechin, each metal alone, each metal–CMC) were pre-aged for 21 days to allow auto-oxidation or CMC formation as in the initial abiotic experiment, and then used to treat the sand in the second experiment. As noted above, precipitates formed in all solutions except the PB and pure catechin treatments, so they were thoroughly resuspended before 42-mL aliquots were added to the respective pots.

To directly assess catechin and CMC effects on plant growth, all green leaves on each plant were counted 7 days after transplanting (prior to treatment) to provide an initial leaf number and then again at day 43 following all catechin or CMC treatments, with the difference in green leaf number used as a measure of growth or inhibition. Leaf number data were analysed using one-way ANOVA with each of the 10 treatments used as an independent factor. For comparisons between all combinations of treatments, the ANOVA was followed by a Tukey HSD comparison of means and Bonferroni adjustment for the number of treatments.

To test the effect of catechin and CMCs on general plant mortality under controlled conditions, all of the plant treatments were exposed to harsh, but environmentally relevant, conditions after the 43-day time point. Briefly, the pots were not fertilized or watered for 3 weeks under ambient greenhouse conditions ranging between 25 °C and 35 °C in daily cycles to simulate summer-like temperatures, drought and nutrient-poor conditions. Plant mortality was recorded after 20 days under these conditions (on day 63). Plants were considered dead when no green leaves were left, and no plants that were recorded as dead later put on green leaves. The mortality of both species was summed for each treatment for analysis in order to provide adequate statistical power for logistic regression analysis. We recorded mortality and analysed differences among treatments using logistic regression analysis (see Efron 1988; SPSS Inc. 2006).

EFFECT OF CMCS ON SEEDLING ESTABLISHMENT

Because the earlier experiment exhibited high levels of mortality in emergent seedlings with multiple catechin or CMC treatments, a second experiment was conducted using single doses at lower concentrations to assess the effect of catechin, CMCs or uncomplexed metals on seedling establishment. In this experiment, Ca, Fe and catechin were tested alone or in CMC forms, since these showed enhanced, decreased or control-level phytotoxicity, respectively, in the prior experiment. In this case, the solutions were made in sterile, ultra-pure water rather than PB to alleviate concerns that PB components or buffering may have somehow contributed to the observed toxicity in the seedling experiment.

To assess effects on seedling establishment, rocket pots were set-up with clean, sterile sand as before, except in this case five *F. idahoensis* or *K. macrantha* seeds were placed on the sand surface in each pot and the pots were treated just once with either 40 mL of ultra-pure water (negative control), 40 mL of catechin solution, 40 mL of a given metal chloride solution without catechin or 40 mL of a given CMC; $n = 10$ pots, each containing five seeds, for each plant-treatment combination. Catechin and CMC preparations were made as before except that the catechin stock solution was 300 μg mL^{-1}, sterile ultra-pure water was used rather than PB and they were used immediately following preparation. The pH of each resulting solution was measured using a Corning 340 pH Meter (Corning Inc., Corning, NY, USA). The concentration of pure catechin remaining in each

solution at the time of application to the pots was measured by HPLC as described above. As before, precipitates formed in both CMC solutions and these were thoroughly suspended before application to the pots. Mortality was low during this experiment, and we scored establishment only as plants that germinated and survived to the end of the experiment. Differences in % emergence among treatments were analysed using repeated-measures ANOVA (SPSS 15.0, 2006), where establishment was measured over time (in days) and time was the repeat variable in the analysis.

Results

EFFECTS OF METALS ON CATECHIN IN SOLUTION

The metals tested varied substantially in their effects on pure catechin concentration in solution as determined by HPLC. All metals except Mg formed precipitates in the presence of catechin in PB. Colour and textural differences clearly distinguishable from those observed in the 'no-catechin' metal treatments used for the seedling growth and seed establishment experiments suggest that catechin reacted with metal phosphates to form catechin–metal complexes, catechin–metal–phosphate complexes, or both (for convenience and simplicity, we refer to these collectively as CMCs). Despite thoroughly suspending these precipitates prior to sampling for HPLC analysis, only the supernatant remaining after centrifugation of samples was analysed by HPLC, meaning that insoluble forms of catechin were removed and only pure catechin remaining in solution was detected. Similarly, auto-oxidized catechin, although still in solution and not removed as precipitate, was not detected as pure catechin in these analyses due to its altered chemical form. It is important to note, however, that bound or oxidized forms of catechin may be more, less or equally biologically active (i.e. phytotoxic) as pure catechin, and thus the entire suspension was added to pots in all experiments.

Pure catechin in solution alone or with individual metals decreased in concentration with time, presumably due to auto-oxidation when alone, complexation with metals forming the corresponding CMCs, or possibly an additive effect of both types of reactions (Fig. 1). In PB, the concentration of pure catechin decreased from 978 ± 3 μg mL^{-1} after *c.* 1 h (the shortest possible time to analysis) to 606 ± 17 μg mL^{-1} after 21 days of incubation, a 38% decrease. The presence of metals with catechin resulted in more dramatic and metal-specific effects on the stability of pure catechin in solution. The greatest impact was observed with Cu, which reduced the concentration of pure catechin from 738 ± 13 μg mL^{-1} at *c.* 1 h to undetectable levels (< 5 μg mL^{-1}; hereafter referred to as ND for 'Not Detected') after 21 days. Fe decreased pure catechin in solution from 851 ± 10 μg mL^{-1} at *c.* 1 h to 78 ± 13 μg mL^{-1} at day 21. Pb and Mg produced more moderate decreases in pure catechin after 21 days, from 953 ± 5 at *c.* 1 h to 293 ± 7 μg mL^{-1} at day 21 and from 958 ± 4 to 514 ± 16 μg mL^{-1} respectively. In contrast to the other metals, Ca caused the concentration of catechin in solution to remain significantly higher in comparison to catechin alone,

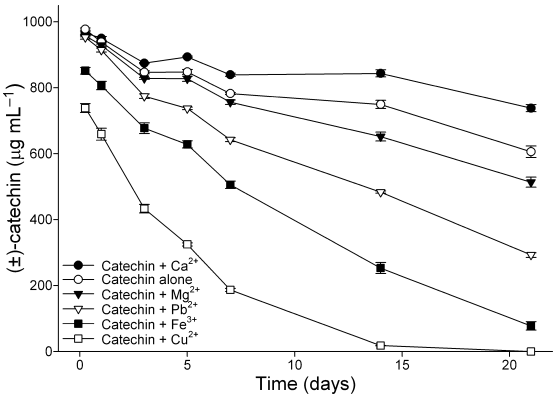

Fig. 1. Mean concentrations of pure catechin in solution with time. Data shown are for catechin alone or in combination with various individual metals (CMCs). Error bars show ± 1 SE of the mean (n = 3).

decreasing only from 966 ± 7 after *c.* 1 h to 738 ± 19 μg mL^{-1} at day 21 (22% higher than the catechin-only control). The effect of the nutrient solution (used to fertilize the plants) on pure catechin stability in PB was also tested and no significant effect was detected compared to the catechin-only control (data not shown).

EFFECT OF METALS ON CATECHIN EXTRACTION FROM SAND

To assess the extractability of catechin from the sand matrix and the effects of metals on extraction, catechin solution or CMCs aged 21 days (as for the initial experiment) were applied to pots and then immediately sampled and analysed for determination of extractable pure catechin concentrations. While the initial input concentration of catechin in each treatment was identical, based on the concentrations of pure catechin remaining in the catechin-only solution, the various CMCs after the 21 day pre-incubation and the 42 mL that were added to each 300 g of sand, the calculated concentrations of pure catechin added to each pot at the onset of this experiment were: no-catechin control and metal-only controls = 0 μg g^{-1} sand; catechin only = 85 μg g^{-1} sand; Ca–CMC = 103 μg g^{-1} sand; Pb–CMC = 41 μg g^{-1} sand; Fe–CMC = 11 μg g^{-1} sand; and Cu–CMC = ND.

The concentrations of pure catechin extracted from sand immediately after application of the above treatments were as follows: no-catechin control and metal-only controls = ND; catechin-alone = 8.0 ± 1.0 μg g^{-1} sand; Ca–CMC = 19 ± 2.0 μg g^{-1} sand; Pb–CMC = 14 ± 0.45 μg g^{-1} sand; Fe–CMC = 5.8 ± 0.72 μg g^{-1} sand and Cu–CMC = ND (Fig. 2). Interestingly, the Pb–CMC treatment yielded more pure catechin than the catechin-alone treatment. Thus, the catechin-alone treatment and all of the CMCs exhibited at least some loss of recoverable pure catechin to the sand matrix in this short time interval, with the catechin-only treatment showing the greatest magnitude of reduction.

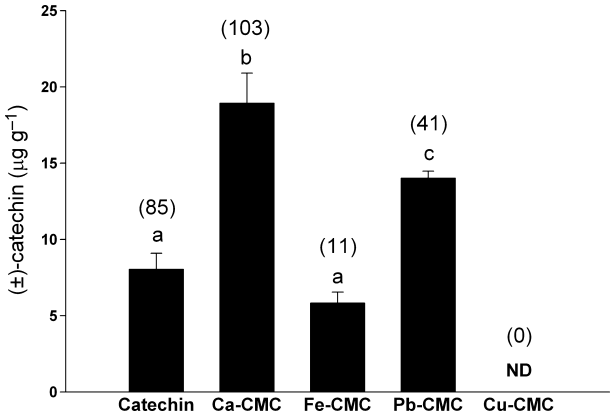

Fig. 2. Pure catechin extracted from sand immediately after treatment with aged catechin or CMCs. Error bars show ± 1 SE of the mean (n = 6). Shared letters above indicate no significant difference in post-ANOVA HSD Tukey tests (P < 0.05). Numbers in parentheses above bars represent the calculated concentrations (μg g^{-1}) of pure catechin added to the sand for one dose.

EFFECT OF CMCS ON PLANT GROWTH AND STRESS SURVIVAL

The effects of catechin and CMCs on plant survival and growth were measured in experiments where, following transplantation and treatment, increased leaf number compared to the PB-only control indicated increased growth, decreased leaf number indicated decreased growth, and visual indications of plant death were scored as mortality. Plants of *Festuca idahoensis* treated with PB-only control solution added 7 ± 1 new green leaves over the course of the experiment (43 days). In contrast, *Festuca* exposed to the catechin-only treatment showed a decrease of 9 ± 2 leaves per plant (Fig. 3). When metals alone (in PB) were added to the pots, only *Festuca* exposed to Cu showed a significant decrease in growth. However, Ca–CMC, Pb–CMC and Cu–CMC inhibited growth of *Festuca* significantly more than the PB-only control solution. Fe-alone and Fe–CMC showed no significant effect on leaf growth. Importantly, no CMC decreased growth significantly more than catechin alone (unlike the case for the mortality measurement), suggesting that catechin complexation into CMCs can create substantial conditionality in the phytotoxicity of catechin to *Festuca*.

For *Koeleria macrantha*, the effect of catechin-alone on leaf growth was significant when compared to the PB-only control, but not as strong as for *F. idahoensis* (Fig. 3). Applied without catechin, Ca and Pb did not affect leaf growth, but Cu, Fe and Fe–CMC significantly reduced growth, while Ca–CMC and Pb–CMC caused strong decreases in growth. Notably, Ca–CMC, Pb–CMC and Cu–CMC had stronger effects on leaf growth than metal alone, suggesting that complexation with metals *increases* catechin phytotoxicity for *Koeleria*, illustrating a species-specific element to catechin and CMC conditionality.

Metals alone had no significant effects on plant mortality relative to the PB-only control (Fig. 4). However, catechin-alone increased mortality relative to the PB-only control, 45% vs. 15% respectively. Ca–CMC and Pb–CMC induced even higher mortality rates (85%, and 75% respectively) than catechin-alone. The effects of Cu–CMC and Fe–CMC on

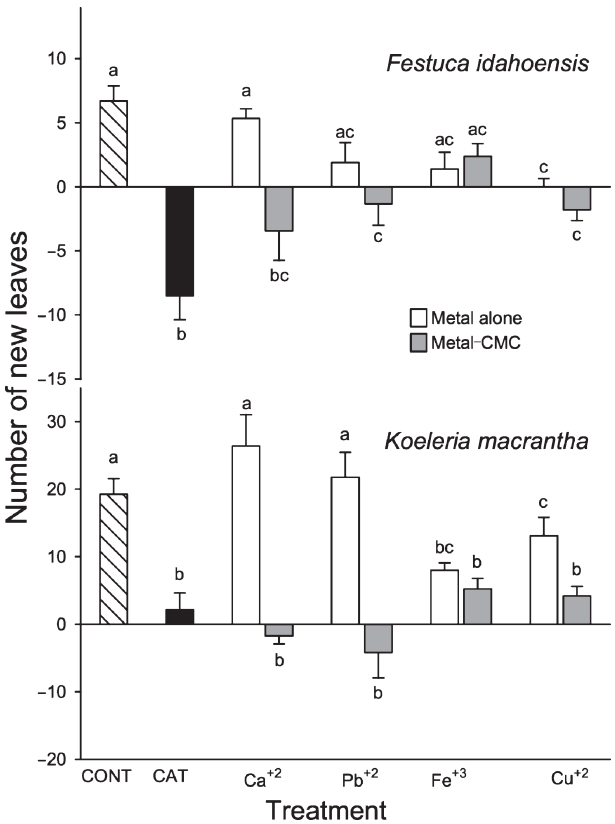

Fig. 3. Number of new leaves produced by *Festuca idahoensis* and *Koeleria macrantha* in sand culture with the following solutions added: controls with no catechin added, CONT; catechin added, CAT; metals-alone, Metal-alone; and CMCs, Metal-CMC. Error bars show ± 1 SE of the mean. With each treatment considered independently in a single ANOVA, $F_{treatment}$ = 16.45; d.f. = 9,101; P < 0.001. Shared letters above indicate no significant difference in post-ANOVA HSD Tukey tests (P < 0.05).

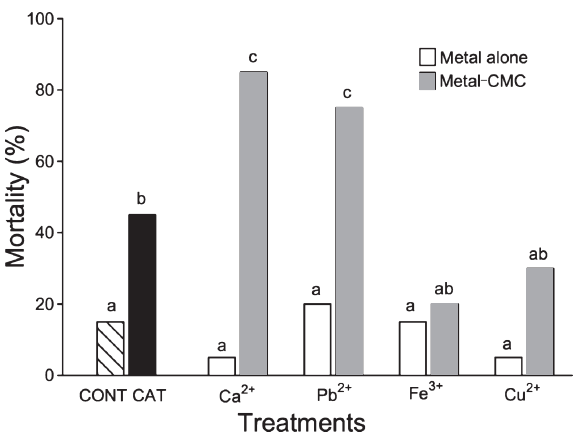

Fig. 4. Percent mortality of *Festuca idahoensis* and *Koeleria macrantha* (combined) under drought-like conditions for controls with no catechin added, CONT; catechin added, CAT; metals-alone, Metal-alone; and CMCs, Metal-CMC. Shared letters above designate no significant differences among means (P < 0.05) as determined from paired logistic regression comparisons (SPSS 15.0, 2006).

mortality did not differ significantly from either the catechin-only treatment or the control (Fig. 4).

EFFECT OF CMCS ON SEEDLING ESTABLISHMENT

Ca (alone) and Fe (alone or in CMC form) had no effect on seedling establishment relative to the water-only control for either plant species (Fig. 5). However, catechin-alone and Ca–CMC inhibited the establishment of both *Festuca* and *Koeleria*. As these treatments were prepared in ultra-pure water and not buffered, the pH of each solution was measured just prior to use. The results were as follows: water-only control, pH 7.1; catechin-alone, pH 5.5; Ca-alone, pH 5.7; Fe-alone, pH 2.1; Ca–CMC, pH 5.3; Fe–CMC, pH 2.0.

Pure catechin concentrations in each treatment were determined by HPLC just prior to addition to the pots with the following results: water-only control, ND; catechin-alone, 291 μg mL^{-1}; Ca-only, ND; Fe-only, ND; Ca–CMC, 290 μg mL^{-1}; Fe–CMC, ND. Note that, unlike in the buffered treatments, Fe rapidly reduced pure catechin concentrations to undetectable levels in pure water. It is very important to the interpretation of these findings to note that these measures reflect the amount of pure catechin remaining in solution in the treatments at the time of application and also that each treatment was prepared with the same initial amount of catechin. As such, each treatment ultimately contains the same molar amount of catechin *per se*, but it exists in one or more different derivative forms in each catechin and CMC treatment applied.

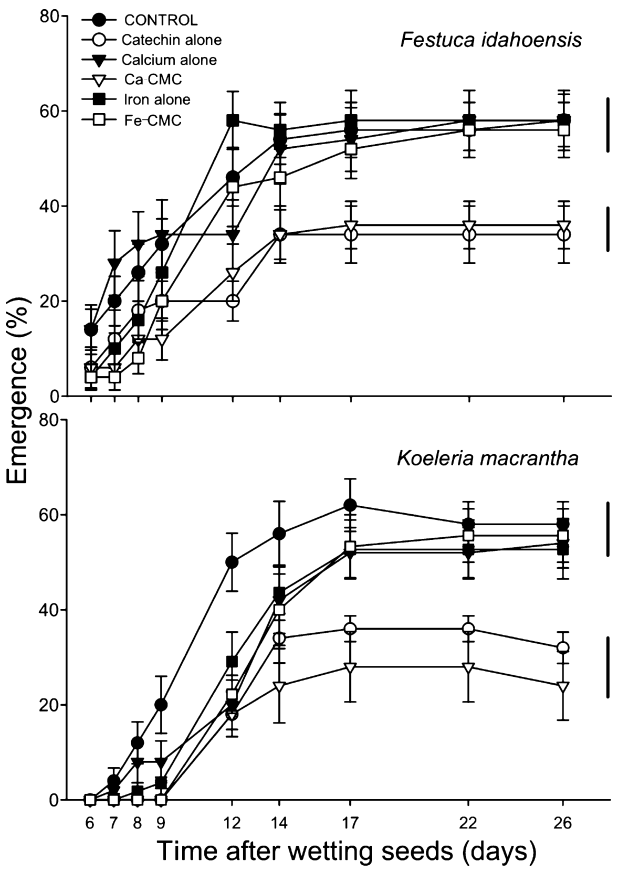

Fig. 5. Impact of catechin, selected metals and CMCs on percentage emergence of *Festuca idahoensis* and *Koeleria macrantha* in sand culture. Error bars show ± 1 SE of the mean. Final means sharing a vertical bar are not significantly different as determined by post-repeated measures ANOVA Tukey HSD tests. For *Festuca*, repeated measures ANOVA, $F_{treatment} = 6.464$; d.f. = 5,55; $P < 0.001$). For *Koeleria*, repeated measures ANOVA, $F_{treatment} = 9.676$; d.f. = 5,55; $P < 0.001$).

Discussion

Numerous prior studies have reported catechin phytotoxicity (Bais *et al*. 2002, 2003; Weir, Bais & Vivanco 2003; Perry *et al*. 2005a; Thelen *et al*. 2005; Thorpe 2006; Rudrappa *et al*. 2007; Inderjit *et al*. 2008a,b; Simões *et al*. 2008) and data from the current study corroborate those findings. Conversely, Blair *et al*. (2006) have suggested that *C. maculosa* does not exhibit phytotoxic activity on failing to extract and measure significant amounts of catechin from *C. maculosa*-inhabited soil in their own study. Based on this study, we suggest that rapid transformation of catechin to oxidized or CMC forms, and perhaps pulsed releases, contribute to the observed variation in soil catechin concentrations where *C. maculosa* is present and may help explain the conditionality in the effects of catechin and in the general allelopathic effects of *C. maculosa*.

We showed that common soil metals dramatically and differentially altered the concentration of pure catechin in solution, suggesting a specific mechanism for the loss of pure catechin after it is added to sand or soil, and for the conditionality in the observed effects of catechin on plants. The results also demonstrate that very low or even undetectable concentrations of pure catechin in sand cultures can inhibit native grass species where catechin is complexed with certain metals (e.g. refer to the Cu–CMC data in Figs 2 and 3). These pure catechin concentrations are comparable to or lower than recent extensive measurements in the field where *C. maculosa* is present (Blair *et al*. 2006) and far lower than some periodic measurements previously observed (Perry *et al*. 2007).

In addition, our results show that high amounts of pure catechin must be applied, even to clean, sterile sand in order to recover and measure pure catechin concentrations comparable to those observed in the field using published protocols (1–100 μg g^{-1} soil) as recently suggested by Inderjit *et al*. (2008a) for soils. However, even these concentrations can be ephemeral, as single large applications may not be detectable immediately after applying catechin. Indeed, our results show that even when 42 mL containing 738 μg mL^{-1} of pure catechin are added to sterile sand and immediately extracted, the concentrations detected can be very low. This suggests that very high levels of catechin may need to be released from *C. maculosa* roots in order to reach field concentrations that have been recently reported (Blair *et al*. 2006; Perry *et al*. 2007). We note, however, that high soil concentrations of catechin have not been consistently observed, with the exception of what appear to be periodic pulses (Perry *et al*. 2007).

It is striking that the most redox-active metals (Fe and Cu) caused the most rapid loss of pure catechin from solution

under abiotic conditions (refer to Fig. 1). Less redox-active metals (Mg and Pb) also decreased the concentration of pure catechin in solution with time, but to a lesser extent. Catechin alone was subject to a modest rate of auto-oxidation, reducing the concentration of pure catechin during the course of the experiment, and that form was also least extractable from sand (Fig. 2). Interestingly, Ca seemed to protect catechin from auto-oxidation (Fig. 1).

Given the chelating properties of catechin and related compounds (Gomah & Davies 1974; McDonald, Mila & Scalbert 1996; Lim, Ginny Lim & Liew 2005), it seems plausible that Pb, Cu or other metals in CMC form might have increased solubility and bioavailability relative to pure metals. This could result either in enhanced availability of micronutrients, as suggested for Fe by Tharayil *et al.* (2009), or in increased metal toxicity to plant species. The experiments and data presented in Figs 2–4 address this last possibility, but warrant careful interpretation because metals in CMC form also are complexed with catechin.

For example, using leaf number as a measure of growth (increased number) or senescence (decreased number) indicated that all CMC forms, with the possible exception of Ca–CMC, were less inhibitory than pure catechin for *F. idahoensis*, while pure catechin and all forms of CMCs showed similar inhibition of *K. macrantha*. This suggests species-specific variability in phytotoxicity in that the pure catechin form was most toxic to the former, while pure and complexed catechin were equally toxic to the latter compared to metal-only controls (refer to Fig. 3). However, we also note that the metal-only controls in this experiment exhibited differential inhibition within and between plant species as well.

It is particularly interesting that Cu–CMC was more toxic than Cu-only, at least for *K. macrantha*, since the Cu–CMC solution added to the pots showed no detectable catechin (Fig. 2) and no pure catechin was recovered from the Cu–CMC treatment in the extraction experiment. This suggests that Cu–CMC exhibits a phytotoxic effect that is not dependent on residual pure catechin concentrations. Extrapolation of this finding to soil suggests that *C. maculosa* may exert catechin-based phytotoxicity in the absence of measurable catechin, perhaps explaining the seemingly contradictory findings of Blair *et al.* (2006), who suggested that there was no evidence for phytotoxicity by *C. maculosa* in their study since there was no catechin detected.

The effects of iron on pure catechin retention in solution and its phytotoxicity were different than for Cu. Fe ultimately decreased the final concentration of pure catechin in solution by *c.* 90% (Fig. 1). However, Fe–CMC did not cause greater stress-related mortality than the negative, metal-only, or catechin-only controls (Fig. 4). Fe–CMC also did not significantly decrease leaf growth of *Festuca* relative to these controls and apparently mitigated toxicity compared to the catechin-only control (Fig. 4). By contrast, Fe-alone and Fe–CMC both decreased leaf growth of *Koeleria* to the same extent as pure catechin. Collectively, these results suggest that, in contrast to the case with Cu, a substantial component of the effects of Fe–CMC may have been due to the metal itself, at least at the higher concentrations utilized in the first growth and mortality experiments. Interestingly, the lower concentrations of Fe-alone and Fe–CMC solution employed later had no effect on seedling establishment in sand (Fig. 5).

In contrast to Fe and Cu, Pb and Mg are far less redox-active, as reflected in their apparent reaction kinetics with pure catechin, showing slower rates of reduction and higher concentrations of pure catechin in solution after 21 days. Similarly, Pb- and Ca-only controls showed lower toxicity and mortality to plants, while their corresponding CMC forms showed greater mortality than even the pure catechin control. Ca–CMC also exhibited enhanced inhibition of seedling establishment (Pb–CMC was not tested for this), and even showed a trend toward greater inhibition than the pure catechin control.

The mechanisms by which the various metals alter catechin concentration are not clear, but binding or chelation of catechin to a variety of di- and trivalent cations is the most likely explanation. Catechin is highly reactive, switching between its reduced form (the one commonly referred to as (±)-catechin), to a more oxidized form (possessing a (semi)-quinone moiety on the B-ring) and conversion to various catechin dimers when exposed to copper (Es-Safi, Cheynier & Moutounet 2003). It is probable that other metal species found in soils are capable of similar abiotic reactions with catechin and thereby decrease its phytotoxic effects by altering its available concentration. Thus, the most redox-active metals produced the greatest reduction in pure catechin concentrations, thereby limiting catechin's toxicity and mortality to plants, while less redox-active metals (e.g. Ca, Mg and Pb) have less or even a positive effect on the persistence of pure catechin.

Chelation processes could affect plants in several different and context-dependent ways including, but not limited to, making toxic metals more bioavailable, mitigating toxic biochemical effects through competitive or transformational effects, increasing the stability and/or availability of phytotoxic biochemicals and thereby creating synergistic effects (as suggested here for Pb and Ca), or otherwise altering the toxic effects of metals in the environment. If the stability of catechin is affected by different di- and trivalent cations in the environment, soils varying in the concentration of different minerals may also vary in how they retain catechin and alter the phytotoxicity of the compound through purely abiotic reactions. Further, spatial heterogeneity in the distribution, speciation, chelation, etc. of metals and other soil components may have a profound effect on the local distribution and behaviour, or even production, of particular root exudates.

Rapid oxidation (1–3 days) of catechin is commonly observed as a red coloration of media in Petri dishes (our unpublished data) and seedling establishment experiments (e.g. Perry *et al.* 2005a; Inderjit *et al.* 2008a), or as a chemical darkening of roots (Bais *et al.* 2003; Weir, Bais & Vivanco 2003); therefore, available pure catechin concentrations in such tests are likely to be much lower than the added amount. Where the chemical and physical properties of an environment are more complex (e.g. in soil), phenomena such as those reported here may enhance or inhibit the concentration and

availability of pure catechin and thus either enhance or mitigate apparent phytotoxicity.

The high levels of plant mortality reported here for catechin and CMCs have not been reported before, either *in vitro* or in the field. The drought-like conditions imposed during the last 3 weeks of our experiment produced 15% mortality in the negative control plants, which is unusually high for such experiments (our unpublished observations), suggesting that there was substantial abiotic stress not associated with catechin treatments. We suggest that the plant mortality induced by catechin and CMCs in this study was more prominent because of the abiotic stress imposed, and such stress may have exacerbated the effects of catechin and CMCs. For example, drying of the sand substrate may have increased aqueous phase concentrations of catechin and CMCs at the root, leading to greater phytotoxic effects. Alternatively, the effects of catechin on mortality may have been due to reduced root length and biomass as suggested by Perry *et al*. (2005a), and drought stress simply exacerbated a fundamental synergistic mechanism driving the deleterious effects of catechin on plants in the field. Such abiotic stresses might also contribute to the conditional effects of catechin.

To summarize, our experiments suggest strong but conditional effects of low and ecologically relevant concentrations of (±)-catechin on native North American plants. We observed different metal-specific effects on the amount of pure catechin in solution, its extractability from a sand-based matrix and its inhibition of growth and exacerbation of mortality under stressful conditions. Such phenomena may help to explain some of the observed spatial variation in soil catechin concentrations and toxicity, and the seemingly equivocal reports of the prevalence and importance of this root exudate to the invasive success of *C. maculosa* and related systems.

These observations have significant impact on our thoughts regarding catechin phytotoxicity, bioavailability and behaviour in soil. Measurable pure catechin present in soil in the presence of *Centaurea* plants may have little to do with the observed degree of phytotoxicity at a given site. While this complicates the interpretation of data related to the role of catechin in *Centaurea* invasion, it serves to illustrate that the precise chemical forms, interactions and effects of catechin in the environment are highly variable and unpredictable and that further examination is warranted to increase our understanding of its effects and role in invasion. Finally, we suggest that the conditional effects observed for catechin likely extend to related compounds, other root exudates and other systems involving chemically complex and spatially heterogeneous environments and that care should be taken in the design and interpretation of studies of such phenomena.

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