Scientific Correspondence

Enantiomeric-Dependent Phytotoxic and Antimicrobial Activity of (±)-Catechin. A Rhizosecreted Racemic Mixture from Spotted Knapweed[1]

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In this communication, we unravel part of the mystery surrounding the allelopathic capability of the noxious weed spotted knapweed (*Centaurea maculosa*). We have found that (−)-catechin is a root-secreted phytotoxin that undoubtedly contributes to spotted knapweed's invasive behavior in the rhizosphere. Although spotted knapweed roots exude (±)-catechin, only the (−)-catechin enantiomer was phytotoxic. (+)-Catechin had antibacterial activity against root-infesting pathogens, which (−)-catechin did not show. This suggests the biological significance for the exudation of racemic catechin, with each enantiomer contributing separate properties for plant aggression and defense.

The soil immediately surrounding a plant root constitutes a unique physical, biochemical, and ecological environment. The rhizosphere is to a large extent controlled by the root system itself through chemicals exuded/secreted into the surrounding soil. Root exudates include low-M_r compounds such as amino acids, organic acids, sugars, phenolics, and various secondary metabolites, and high-M_r compounds like mucilage and proteins (Roshina and Roshina, 1993; Schultze et al., 1994). Through the exudation of a wide variety of compounds, roots may regulate the soil microbial community in their immediate vicinity, cope with herbivores, encourage beneficial symbioses, change the chemical and physical properties of the soil, and inhibit the growth of competing plant species (Nardi et al., 2000). Countering a challenge, roots may respond by secreting certain chemicals such as secondary metabolites, proteins, and even volatiles (Shulaev et al., 1997; De Moraes et al., 2001). Root secretions may play symbiotic or defensive roles as a plant ultimately develops a positive or negative communication, depending on the other elements of its rhizosphere. An example of a negative communication is provided by the Asian native spotted knapweed. This noxious weed, one of the most economically destructive exotic invaders of western North America, displaces other weeds and crops by mounting a chemical warfare mediated by root exudates (Callaway et al., 1999; Callaway and Aschehoug, 2000). Although allelopathy was suggested as the displacing mechanism as early as 1832 (DeCondolle, 1832), there has been minimal success in characterizing any responsible allelochemical(s) from knapweed. Considering the complexity of isolating and characterizing metabolites exuded from roots into the soil, our laboratory addressed this issue by developing a system where knapweed roots, grown in vitro, can secrete or be induced to secrete the allelochemical from its roots into sterile media in a way comparable with secretion into the rhizosphere (Bais et al., 2001).

Root exudates of in vitro-grown spotted knapweed plants were assayed for effect on the phenotypic response and germination efficiency of various weeds, including Dalmatian toadflax (*Linaria dalmatica*), common mullein (*Verbascum thapsus*), downy brome (*Bromus tectorum*), kochia (*Kochia scoparia*), diffuse knapweed (*Centaurea diffusa*), the model plant Arabidopsis, and crops such as wheat (*Triticum aestivum*) and tomato (*Lycopersicon esculentum*). All the plants showed mortality on the 14th d after addition of root exudates from spotted knapweed (Fig. 1, A and B). Plants showed wilting symptoms prior to senescence with reduced shoot and root differentiation after administration of the root exudates (Fig. 1A). Upon elicitation of in vitro-grown spotted knapweed plants with fungal cell wall preparations from *Phytophthora cinnamoni*, a fungal pathogen that infects the roots of several plant species (Agrios, 1997), the allelochemical activity of root exudates increased dramatically over the nonelicited exudates (Fig. 1B). The fungal cell wall-elicited allelochemical(s) did not inhibit the growth of *P. cinnamoni* (data not shown). Spotted knapweed root exudates also behaved as inhibitors of seed germination for all the weeds and crop plants tested (Fig. 1, A–C). The degree of involvement of the microbial communities during plant-plant allelopathic interactions remains un-

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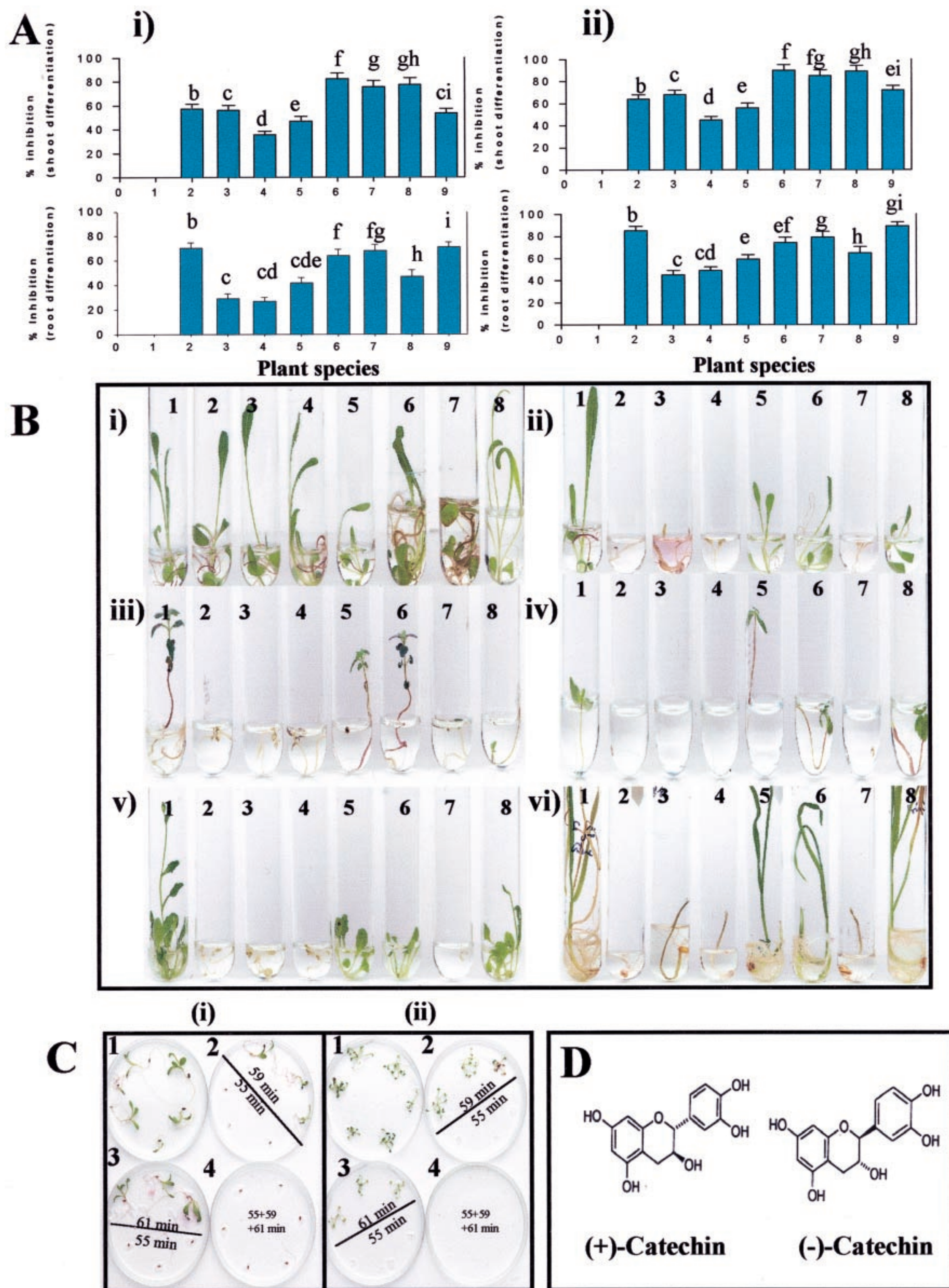

Figure 1. Purification and characterization of the allelochemical activity from spotted knapweed root exudates. A, i, Effect of nonelicited root exudates of spotted knapweed on shoots and root differentiation of different weeds and crop plants on the 14th d. Root exudates collected from nonelicited cultures of spotted knapweed were administered in different concentrations (1–3 mL [v/v]) in the Murashige and Skoog basal liquid media containing different seedlings to analyze phenotypic explant response. ii, Effect of fungal cell wall extract (CWE)-elicited root exudates of spotted knapweed on shoots and root differentiation of different weeds and crop plants on the 14th d. Fungal CWEs from *P. cinnamoni* and *Rhizoctonia solani* were used. The fungal cell wall elicitors were prepared and used according to McKinley et al. (1993). Fungal elicitors

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known. Our results and previous ecological results of other groups (Callaway et al., 2001) suggest that microbes may play a role in triggering root exudation of allelochemicals (Fig. 1, B and C), implying a cross talk between root-root and root-microbe interactions in the rhizosphere. In this way, fungi may induce spotted knapweed's secretion of allelochemicals and thus favor infection of other plant species weakened by the allelochemical (Callaway et al., 2001).

Extracts of freeze-dried medium in which spotted knapweed had been grown were subjected to HPLC analysis and collected fractions were used for bioassay. Essentially all the activity was confined to a single HPLC peak, which was shown to be due to the flavanol (±)-catechin (Fig. 1D). The exudate racemic catechin showed no optical activity, neither at the sodium D line nor in the CD spectrum from 225 to 300 nm, which confirms that (+)- and (−)-catechin are secreted by the roots in an equal ratio.

(+)-Catechin, a widespread plant bioflavanoid, is a well-known antioxidant-free radical scavenger, reported as a component of green tea (*Camellia sinensis*; Kim et al., 1997), as an antitumour agent (Du et al., 2001), and as an insect repellent (Kiderlen et al., 2001). (−)-Catechin has been found (Nahrstedt et al., 1987) much more rarely than the (+) isomer and the racemic form only occasionally as well (Karimdzhanov et al., 1997; Ono et al., 1997). We confirmed that pure (±)-catechin isolated from spotted knapweed root exudates had more potent herbicidal activity than that of the crude root exudates against all the weeds and crop plants tested (Fig. 1, A and B). (±)-Catechin did not show inhibitory activity when tested against an array of soil-borne fungi including *P. cinnamoni* (data not shown). The commercially available racemic catechin had the same effect as root-exudated (±)-catechin (Fig. 2, A and B). Spotted knapweed was resistant to its own exudates and to the purified (±)-catechin, suggesting a possible detoxifying activity within the roots against its own toxin (Fig. 1, B and C).

(±)-Catechin herbicidal activity was compared with that of 2,4-D by feeding Arabidopsis roots with these chemicals. The minimum MIC of (±)-catechin was approximately 100 μg mL^{-1} as tested on Arabidopsis shoot cultures in vitro, compared with 10 μg mL^{-1} for 2,4-D (Fig. 2, A and B). (±)-Catechin was exuded from spotted knapweed roots at doses as high as approximately 83.2 μg mL^{-1}, and approximately 185.04 μg mL^{-1} upon treatment with *P. cinnamoni* cell wall elicitors. Upon close examination, (−)-catechin was found to account for the allelochemical activity at doses as low as approximately 50 to 60 μg mL^{-1} (Fig. 2, A and B), whereas (+)-catechin did not show such activity (Fig. 2, A and B). To our knowledge, this is the first report on bioactivity of (−)-catechin. Our results show that although racemic catechin is exuded by spotted knapweed roots, only (−)-catechin accounts for the allelochemical activity. On the other hand, (+)-catechin was inhibitory to soil borne bacteria. Of the six bacterial strains tested, most showed inhibition of growth in response to (+)-catechin treatment (Fig. 2C). Thus, *Xanthomonas campestris*, *Pseudomonas fluorescens*, and *Erwinia carotovora* showed a distinct inhibition under (+)-catechin treatment. In contrast, *Agrobacterium rhizogenes* (15834) was not affected even at higher concentrations of (+)-catechin (Fig. 2C). (−)-Catechin failed to show any antibacterial activity against all the tested soil-borne pathogens.

It has been previously shown that introduced diffuse knapweed has stronger allelochemical effects on North American weed species than on grasses from its original native communities in Asia (Callaway et al., 1999; Callaway and Aschehoug, 2000). Although we did not study the effect of spotted knapweed

Figure 1. (*Legend continued from facing page.*)
were administered at various concentrations as described in the text (http://lamar.colostate.edu/~jvivanco.) Media exudates from these elicited plants were collected after 14 d and were added in different concentrations (1–3 mL [v/v]) to the various plants tested. Tubes were subsequently incubated under 16-h-light and 8-h-dark photoperiod in an incubator. The data represent the percent inhibition relative to the untreated control in shooting and rooting response in various tested seedlings against nonelicited and elicited root exudates of spotted knapweed. The seedlings tested against spotted knapweed exudates were as follows: 1, spotted knapweed; 2, Dalmatian toadflax; 3, common mullein; 4, downy brome, 5, kochia; 6, diffuse knapweed; 7, Arabidopsis 8, wheat; and 9, tomato. (Means of different letters at the top of each bar indicate significant difference [$P < 0.05$, SE ± 52 df]; http://lamar.colostate.edu/~jvivanco; Steel and Torrie, 1980). B, Effect of purified HPLC-fraction from spotted knapweed root exudates on morphological differentiation of various weeds and food crops. HPLC peaks were collected and administered into 5 mL of Murashige and Skoog basal medium at 100 μg mL^{-1} concentration; fractions were added in different permutations. i, Spotted knapweed; ii, diffuse knapweed; iii, *K. scoparia*; iv, Dalmatian toadflax; v, Arabidopsis; vi, wheat. Treatments were as follows: 1, untreated control; 2, plain root exudates (3 mL); 3, fungal elicited root exudates (3 mL); 4, 55-min fraction (100 μg mL^{-1}); 5, 59-min fraction (100 μg mL^{-1}); 6, 61-min fraction (100 μg mL^{-1}); 7, admixture of 55- + 59- + 61-min fraction (100 μg mL^{-1} each); and 8, mere fungal elicitors from CWE from *P. cinnamoni* (600 μL/5 mL of Murashige and Skoog media). C, Effect of bioactive HPLC eluant from elicited cultures of spotted knapweed on germination efficiency of various seeds. i, Diffuse knapweed seeds; ii, Arabidopsis seeds. Treatments were as follows: 1, untreated control; 2, 55- + 59-min fraction (100 μg mL^{-1} each); 3, 55- + 61-min fraction (100 μg mL^{-1} each); 4, admixture of 55- + 59- + 61-min fraction (100 μg mL^{-1} each). D, Structure of both enantiomers of catechin, with (−)-catechin identified as a potent allelochemical and (+)-catechin showing antibacterial activity. Isolated racemic catechin from spotted knapweed root exudates showed no optical activity and had the same ^{1}H and C^{13}-NMR spectra as those of the commercial racemic catechin.

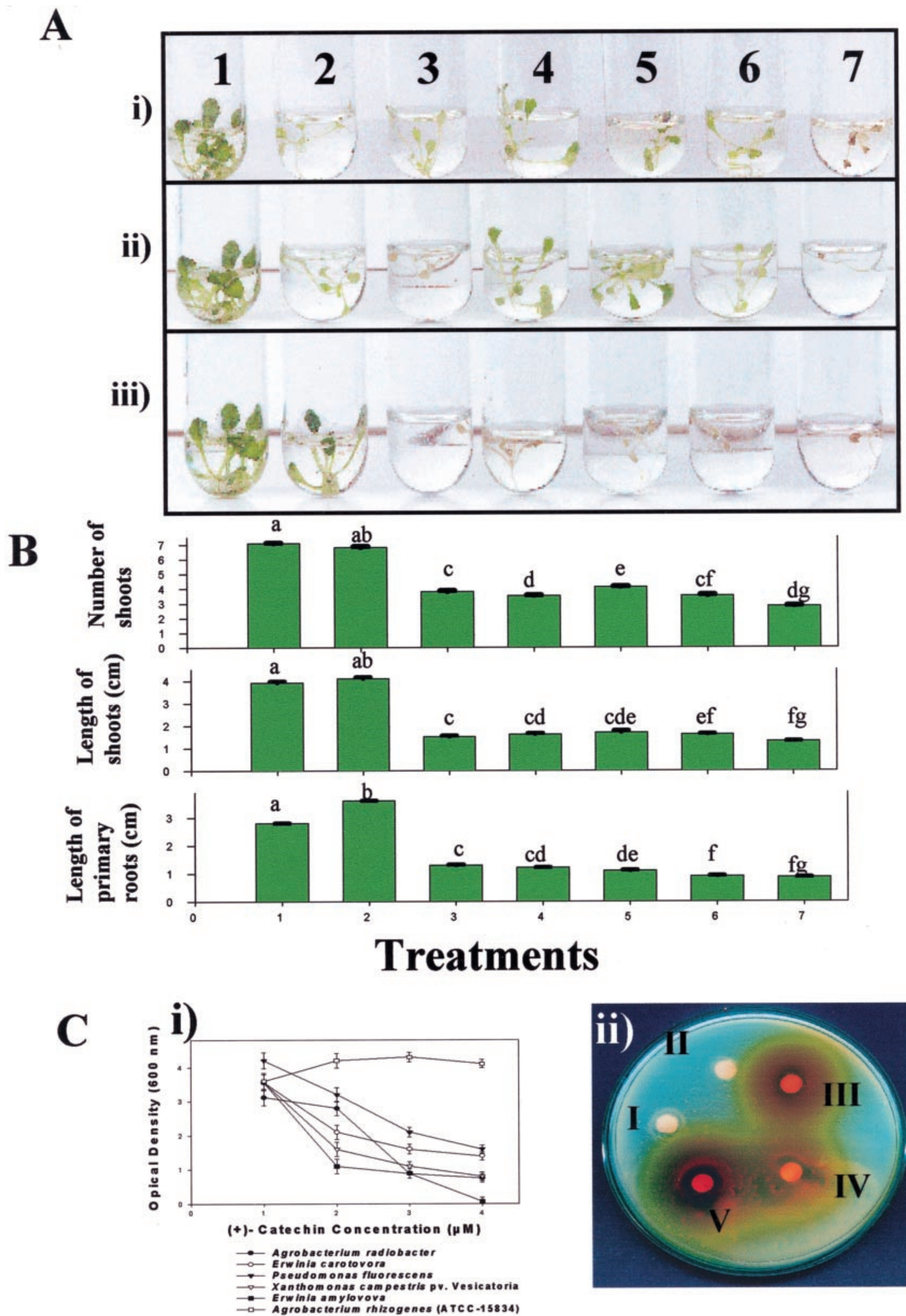

Figure 2. Comparative influence of racemic catechin isolated from spotted knapweed root exudates with commercially obtained enantiomers, racemate and 2,4-dichlorophenoxyacetic acid (2,4-D) on morphological differentiation and phenotypic response in Arabidopsis seedlings. A, Racemic catechin isolated from spotted knapweed root exudates and commercially available racemic catechin was tested against its two commercially available isomers (+),(−), and 2,4-D (http://lamar.colostate.edu/~jvivanco). Catechin and 2,4-D were administered in Murashige and Skoog basal medium at different

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exudates or (−)-catechin on Asian grasses, we have otherwise observed broad-spectrum allelochemical activity of (−)-catechin against a diverse range of plant species including the closely related diffuse knapweed (Fig. 1, A–C).

We recently have found racemic catechin in soil extracts of spotted knapweed-invaded fields, which validates our in vitro approach and proves spotted knapweed's invasive behavior through exudation of (±)-catechin in the soil. It was observed that (±)-catechin concentration in the soil varied with its proximity to the taproot (approximately 389.8 ± 28.6 $\mu g\ g^{-1}\ cm^{-2}$–291.6 ± 17.8 $\mu g\ g^{-1}\ cm^{-2}$), differences in soil sampling zones (approximately 361.8 ± 21.4 $\mu g\ g^{-1}\ cm^{-2}$–315.2 ± 15.3 $\mu g\ g^{-1}\ cm^{-2}$), and age of spotted knapweed's invasion (data not shown). These findings allow us to conclude that (±)-catechin is a naturally exudated and stable flavanol in the soil that is responsible for spotted knapweed's phytotoxicity and antimicrobial activity.

In summary, our results revealed that (−)-catechin, a root exudate component, has broad-spectrum herbicidal activity, suggesting an important role in spotted knapweed's allelochemistry and invasive nature. (−)-Catechin status as a natural plant product suggests fewer biosafety concerns should it prove useful as a herbicide. Our report clearly shows the untapped potential of root exudates as a general system for isolating biologically active secondary metabolites.

ANNEXURE

This section will be incorporated in this web address: http://lamar.colostate.edu/~jvivanco.

CULTURE CONDITIONS AND ELICITATION

Seeds of spotted knapweed, diffuse knapweed, and common mullein were obtained from natural populations in Larimer County (CO). Seeds of Dalmatian toadflax, downy brome, and kochia were obtained from natural populations in Larimer and Routt Counties (CO).

Seeds of tomato and wheat were obtained from Quality Seeds (The Rocky Mountain Seed Co., Denver). Above seeds were washed in running tap water and were surface sterilized using sodium hypochlorite (0.3% [v/v]) for 10 to 15 min, followed by three to four washes in sterile distilled water. Surface-sterilized seeds were inoculated on static Murashige and Skoog (Murashige and Skoog, 1962) basal media in petri dishes for germination. Seeds were allowed to germinate for 10 d until roots and shoots emerged. The light intensity within the growth chamber was 4.4117 $J\ m^{-2}\ s^{-1}$. Ten-day-old seedlings were transferred to 50-mL culture tubes with 10 mL of liquid Murashige and Skoog basal media. Plant cultures were maintained on an orbital platform shaker set at 90 rpm (Lab-Line Instruments, Inc., Melrose Park, PA). Ten-day-old spotted knapweed plants grown in 10 mL of nutrient-enriched Murashige and Skoog basal medium were elicited with fungal cell wall preparations, jasmonic acid, salicylic acid, and chitosan. Fungal CWEs from different fungi such as *P. cinnamoni* and *Rhizoctonia solani* were used. The fungal cell wall elicitors were prepared and used according to McKinley et al. (1993). Fungal elicitors were dispensed at various concentrations (1–3 mL [v/v]) into 50-mL culture tubes containing 10 mL of Murashige and Skoog basal media. Solutions of salicylic acid and jasmonic acid were prepared in ethanol and were added individually to the spotted knapweed seedlings at final concentrations of 50 to 200 and 100 to 500 μM, respectively. Media exudates from elicited, spotted knapweed plants were collected after 30 d and were added in different concentrations (1–3 mL [v/v]) to the various test plants. Media exudates from a nonelicited control were also harvested during the same period for secondary metabolite analy-

Figure 2. (*Legend continued from facing page.*) concentration range (1–200 $\mu g\ mL^{-1}$) to check for the minimum inhibitory concentration (MIC). i, Ten micrograms microliters^{-1}; ii, 60 $\mu g\ mL^{-1}$; iii, 100 $\mu g\ mL^{-1}$. Treatments were as follows: 1, untreated control; 2, (+)-catechin; 3, (−)-catechin; 4, (±)-catechin; 5, (±) co-administered catechin; 6, (±) catechin isolated from root exudates of spotted knapweed; and 7, 2,4-D. B, Effect of catechin on shoot and root differentiation in Arabidopsis plants on the 14th d. Catechin was compared for phytoxicity against 2,4-D and was administered at 100 $\mu g\ mL^{-1}$ concentration in Murashige and Skoog basal medium (5 mL). Treatments were as follows: 1, untreated control; 2, (+)-catechin; 3, (−)-catechin; 4, (±)-catechin; 5, (±) co-administered catechin; 6, (±) catechin isolated from root exudates of spotted knapweed; 7, 2,4-D. (Means of different letters at the top of each bar indicate significant difference [$P < 0.05$; SE ± 52 df); http://lamar.colostate.edu/~jvivanco; Steel and Torrie, 1980.) C, Antibacterial activity of (+)-catechin on different bacterial strains. i, Tube antibacterial assay. 1, Control [without (+)-catechin]; 2, (+)-catechin, 100 $\mu g\ mL^{-1}$; 3, (+)-catechin, 150 $\mu g\ mL^{-1}$; 4, (+)-catechin, 200 $\mu g\ mL^{-1}$. Bacterial cultures were grown overnight at 24°C in liquid Luria-Bertani broth media to an optical density of 0.2 at 600 nm. Different concentrations (100–200 $\mu g\ mL^{-1}$) of (+)-catechin were administered to the bacteria-containing Luria-Bertani broth media. Optical density at 600 nm was checked after 24 h of incubation to measure bacterial inhibition in tubes. Antibacterial activity was assayed on both tubes and 35-mm plates. ii, Antibacterial activity of (+)-catechin on *Pseudomonas fluorescens* analyzed in a petri dish assay. Treatments on petri dish refers to the following concentrations: I, methanol [(+)-catechin solvent] was used as a control; II, (−)-catechin (100 $\mu g\ mL^{-1}$); III, (+)-catechin (100 $\mu g\ mL^{-1}$); IV, (±)-catechin (200 $\mu g\ mL^{-1}$); and V, (±)-catechin (200 $\mu g\ mL^{-1}$) isolated from root exudates of spotted knapweed. Antibacterial plate assay was performed on an overnight-grown bacterial culture; both the enantiomers and the racemic catechin were added to the paper discs and allowed to dry under laminar hood conditions. Antibacterial activity is depicted by the inhibitory halo surrounding the filter paper.

ses. A time course experiment was established, wherein media samples from all the elicited treatments were taken weekly and analyzed for the presence of novel secondary metabolites in the root exudates. Soil samples were collected from a spotted knapweed's vegetation near North Riverdale Estates (Larimer County, CO).

EXTRACTION

The root exudates (1 mL) from all treatments were extracted using 5 mL of hexane (Fisher Co., Pittsburgh). The extracts were vortexed and stored for 24 h at 4°C. The supernatant was transferred with a Pasteur pipette to a separate test tube, and 1 mL of hexane (Fisher Co.) was added. The supernatant was further concentrated by freeze drying (VirTis, Genesis, Gardiner, NY), and the weighed powder was redissolved in 500 μL of absolute methanol (Fisher Co.) for HPLC analyses. Similarly, roots of spotted knapweed were extracted for metabolic profiling of the roots. Roots were harvested and 200 mg of fresh wet tissues were extracted in 2 mL of absolute methanol for 24 h at 4°C (Fisher Co.). The extracts were centrifuged at 10,000 rpm for 10 mins; supernatants were concentrated under vacuum and were resuspended in 500 μL of methanol for HPLC analyses. Soil obtained from spotted knapweed's habitat was extracted with absolute methanol for 24 h at ambient temperature, and extract was further concentrated with nitrogen and was subjected to HPLC analysis.

HPLC-MASS SPECTROMETER (MS) ANALYSIS

Compounds in the elicited root exudates, roots, and soil samples were chromatographed by gradient elution on a reverse phase 5-μm, C_{18} column (25 cm × 4.6 mm; Supelco Co., Bellefonte, PA). The chromatographic system (Summit Dionex, Sunnyvale, CA) consisted of P_{580} pumps (Dionex) connected to an ASI-100 Automated Sample Injector (Dionex Co). The visible A_{210} was measured by a PDA-100 Photodiode array variable UV/VIS detector (Dionex). Mobile phase solution A consisted of double distilled water and solution B (acetonitrile; Fisher Co). A multistep gradient was used for all separations with an initial injection volume of 15 μL and a flow rate of 1 mL min^{-1}. The multistep gradient was as follows: 0 to 5 min, 5.0% (v/v) B; 5 to 10 min, 20.0% (v/v) B; 15 to 20 min, 20.0% (v/v) B; 20 to 40 min, 80.0% (v/v) B; 40 to 60 min, 100% (v/v) B; 60 to 70 min, 100% (v/v) B; and 70 to 80 min, 5.0% (v/v) B. Different peaks resulting from various elicitation treatments were collected for the bioassay against various other invasive weeds and crop plants. Various peak eluants were concentrated under vacuum at 30°C and further purified by injecting them back into HPLC under similar conditions and were collected at similar retentions. The eluant showing biological activity was dried under vacuum at 30°C resulting in 4 mg of an amorphous powder. We checked whether its occurrence could be ascribed to contamination by microorganisms, but this was not found to be the case. The biological activity was detected in the whole fraction, but was missing in fractions collected before and after 55 min. The HPLC eluant passed through a UV detector with a flow rate of 0.25 mL min^{-1} was delivered into the electron spin-MS (Finnigan LQ Qizmo, 1100 series, Hewlett-Packard, Palo Alto, CA). The MS parameters were optimized to maintain a high gas temperature (200°C) and gas flow (50 psi). Ions were referred to both positive and negative splits. Scan ranges of 100 to 750 milli-absorbance units were used for negative ions. A step size of 1 milli-absorbance unit and dwell time of 1 ms was used during the analysis. The active eluant had m/z 289 (M^+-1) for $C_{15}H_{14}O_6$.

COMPOUND IDENTIFICATION BY NMR

The ^{1}H and ^{13}C NMR spectra of the HPLC-purified active exudate component were essentially identical to those of commercial (Sigma-Aldrich, St. Louis) (±)-catechin, (+)-catechin, (−)-catechin, and literature values for the latter two compounds previously reported in Nahrstedt et al., 1987.

INHIBITORY BIOASSAY

Ten-day-old seedlings and surface sterilized seeds of spotted knapweed, Dalmatian toadflax, common mullein, downy brome, kochia, diffuse knapweed, Arabidopsis, wheat, and tomato were placed on Murashige and Skoog basal medium in petri dishes after initial surface sterilization. Petri dishes were kept under a 16-h-light and 8-h-dark photoperiod in an incubator (Lab-Line Instruments). An additional objective was to check the effect of allelochemicals from spotted knapweed on growth and differentiation of food crops such as wheat and tomato. This was of interest because wheat is known to produce an allelopathic effect with its root exudates (Wu et al., 2000). Root exudates collected from nonelicited and elicited cultures of spotted knapweed were administered in different concentrations (1–3 mL [v/v]) over the surface sterilized seeds and seedlings to analyze their phytotoxic effects. Root exudates were subjected to autoclaving at 120°C for 30 min at 1.25 kg cm^{-2} pressure, and were added at the concentrations mentioned above to the germinating seeds and seedlings. This procedure was performed to narrow down the effect to a secondary metabolite. Similarly collected fractions (approximately 100 μg mL^{-1}) were administered in different permutation and combination to assess their phytotoxic activity. Arabidopsis was used to assess the phytotoxicity MIC of racemic catechin and each enantiomer in comparison with the MIC for 2,4-D. After incubation growth

parameters such as length of shoots, number of shoots and length of primary root of the treated and untreated plants were measured.

STATISTICAL ANALYSIS

Results were subjected to statistical analyses of variance appropriate to completely randomized design and the means were separated using Duncan's new multiple range test (Steel and Torrie, 1980).

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