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Structure-Dependent Phytotoxicity of Catechins and Other Flavonoids: Flavonoid Conversions by Cell-free Protein Extracts of *Centaurea maculosa* (Spotted Knapweed) Roots

HARSH PAL BAIS,[†] TRAVIS S. WALKER,[†] ALAN J. KENNAN,[‡] FRANK R. STERMITZ,[‡] AND JORGE M. VIVANCO*,[†]

Department of Horticulture and Landscape Architecture and Department of Chemistry, Colorado State University, Fort Collins, Colorado 80523-1173

Invasive plants are believed to succeed in part by secretion of allelochemicals, thus displacing competing plant species. *Centaurea maculosa* (spotted knapweed) provides a classic example of this process. We have previously reported that spotted knapweed roots secrete (±)-catechin and that (−)-catechin, but not (+)-catechin, is phytotoxic and hence may be a major contributor to *C. maculosa's* invasive behavior in the rhizosphere. In this communication, we explore both structure/activity relationships for flavonoid phytotoxicity and possible biosynthetic pathways for root production of (±)-catechin. Kaempferol and dihydroquercetin were shown to be phytotoxic, while quercetin was not. Kaempferol was converted to dihydroquercetin and (±)-catechin when treated with total root protein extracts from *C. maculosa*, but quercetin was not. This finding suggests an alteration in the standard flavonoid biosynthetic pathway in *C. maculosa* roots, whereby kaempferol is not a dead-end product but serves as a precursor to dihydroquercetin, which in turn leads to (±)-catechin production.

KEYWORDS: ***Centaurea maculosa*; allelopathy; (−)-catechin; (±)-catechin; kaempferol; phytotoxicity; flavonoid biosynthesis; in vitro**

INTRODUCTION

Plants produce a plethora of relatively low molecular weight compounds termed secondary metabolites, which can be selectively produced in different parts of the plant. Plant roots, for example, show a remarkable diversity in the production and secretion of secondary metabolites (*1*, *2*). Through the exudation of a wide variety of compounds, roots play an important role in regulating the soil microbial community in their immediate vicinity, in the control of herbivores, and in the growth inhibition of competing plant species (*3*, *4*). A classic example of root-secreted phytotoxicity is provided by the Asian native *Centaurea maculosa* L. (spotted knapweed). This noxious weed, one of the most economically destructive exotic invaders of western North America, displaces other weeds and crops in part by synthesizing phytotoxic root exudates (*5*). We have identified the flavan-3-ol (−)-catechin as the compound responsible for *C. maculosa*'s phytotoxicity (*6*).

Flavonoid biosynthesis is one of the best-described plant secondary metabolic pathways, and the genes encoding flavonoid biosynthetic enzymes have been cloned and characterized in various plant species (*7*). Products synthesized in the early steps of the flavonoid pathway are found in bryophytes and ferns (*8*), whereas gymnosperms and angiosperms accumulate additional classes of flavonoids, possibly reflecting the recruitment of additional genes to flavonoid biosynthesis, as well as the evolution of novel functions for these compounds (*9*, *10*). In angiosperms, flavonoids function to protect plants from predators and infectious agents, shield plants from UV-B radiation, act as signaling molecules in plant−bacterium symbioses, and are the primary pigments that attract pollinators and seed dispersers (*11*). Despite the wide distribution of this large group of compounds among the flowering plants, particular classes of flavonoids have distinct functions in different plant groups. For example, flavonols are essential for male fertility in petunia (*12*), but in Arabidopsis, flavonols do not have such a role (*13*). Isoflavonoids are the major phytoalexins in legumes (*14*), whereas 3-deoxyanthocyanidins fulfill similar functions in *Sorghum bicolor* and other grasses (*15*). The flexibility of this and other secondary metabolic pathways suggests that the selective pressures upon genes encoding enzymes involved in secondary metabolism are quite variable.

We have previously shown that (±)-catechin can be isolated from *C. maculosa* root exudates, that it is phytotoxic, and that the phytotoxicity was entirely due to the (−)-catechin (**1**) enantiomer, while (+)-catechin (**2**) was inactive (*6*). The potent phytotoxicity we observed for (−)-catechin seems fairly rare for a natural flavonoid. Hence, we tested a number of flavonoids

* Correspondence and requests for materials should be addressed to J.M.V. (E-mail jvivanco@lamar.colostate.edu; fax 970-491-7745).
[†] Department of Horticulture.
[‡] Department of Chemistry.

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in order to better determine the presence and extent of such toxicity in this group of secondary metabolites. In addition, we fed a number of possible catechin precursors to a cell-free protein extract from *C. maculosa* roots in order to obtain preliminary evidence which might point to the genesis of the exuded (±)-catechin.

MATERIALS AND METHODS

Plant System. Seeds of *Centaurea maculosa* and *Centaurea diffusa* were obtained from natural populations in Larimer County, CO. Seeds of *Kochia scoparia* (kochia) and *Linaria dalmatica* (dalmatian toad flax) were obtained from natural populations in Larimer and Routt Counties, CO. Seeds of *Lycopersicon esculentum* and *Triticum aestivum* (wheat) were obtained from Quality Seeds (The Rocky Mountain Seed Co., Denver, CO). Seeds of *Arabidopsis thaliana* were purchased from Lehle Seed Co. (TX).

Chemicals. Catechin, naringenin, quercetin, kaempferol, epicatechin, and enantiomers were obtained from Sigma Chemical Co. (St. Louis, MO). (±)-Dihydroquercetin (taxifolin) was obtained from Indofine Chemical Co. (Hillsborough, NJ) and (+)-dihydroquercetin (+35°, MeOH) from the Stermitz Laboratory collection. All other chemicals and solvents were of HPLC grade.

Culture Conditions. Seeds were washed in running tap water and surface sterilized using sodium hypochlorite (0.3% v/v) for 10−15 min, followed by three or four washes in sterile distilled water. Surface-sterilized seeds were inoculated on static MS (*16*) basal media in Petri dishes for germination purposes. Seeds were allowed to germinate for 10 days until roots and shoots emerged and were incubated under 16 h light and 8 h dark at 25 ± 2 °C. The light intensity in the growth chamber was 24 μmol m^{-2} s^{-1}. Ten-day-old seedlings were transferred to 50-mL culture tubes with 10 mL of liquid MS basal media. Plant cultures were maintained on an orbital platform shaker set at 90 rpm (Lab-Line Instruments, Inc., Melrose Park, IL).

Inhibitory Bioassay. All the flavonoids were dissolved in absolute methanol and were administered at 50−250 μg mL^{-1} levels in MS basal medium containing 10-day-old seedlings of the tested plants. Ten-day-old seedlings and surface-sterilized seeds of *C. maculosa*, *C. diffusa*, *K. scoparia*, *A. thaliana*, *L. dalmatica*, *T. aestivum*, and *L. esculetum* were placed on MS 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 Co.). Commercially obtained flavonoids were dissolved in methanol in different concentrations (50−250 μg mL^{-1}), filter sterilized using a 0.45-μm filter, and added over the surface-sterilized seeds and seedlings of tested plants to analyze their phytotoxic effects. The phytotoxic minimum inhibitory concentration (MIC) of the flavonoids was compared to the MIC of 2,4-D. Growth parameters such as length of shoots, number of shoots, and length of the primary root of the treated and untreated plants along with germination efficiency were measured and were denoted as units for shoot and root differentiation.

Total Protein Isolation and Incubation Experiments. Roots of 1-month old *in vitro* grown *C. maculosa* plants were harvested, and the total soluble proteins from the roots were extracted (*17*). Different concentrations of total proteins (0.5−30 μg mL^{-1}) and flavonoids (0.5−30 μg mL^{-1}) were incubated with a buffer comprised of 100 mM Tris HCl, 167 mM KCl, and 100 mM $MgCl_2$, pH 7.2, for 24 h at 4 °C under dark conditions. The incubation mixture was extracted with hexane overnight, and the hexane solutions were concentrated to dryness under N_2 and subjected to HPLC analysis.

Isolation and Analysis. The powdered fractions were dissolved in 1 mL of methanol (Fisher Co., Pittsburgh, PA). The samples were prepared for HPLC analysis after filtration through a 0.2-μm filter (Gelman Sciences, Ann Arbor, MI). 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 (Dionex Corp., Sunnyvale, CA) consisted of P580 pumps connected to an ASI-100 automated sample injector (Dionex Corp.) with absorbance at 280 nm measured by a PDA-100 photodiode array variable UV/vis detector. Mobile-phase solution A consisted of double-distilled water, and solution B was absolute methanol. A multistep gradient of

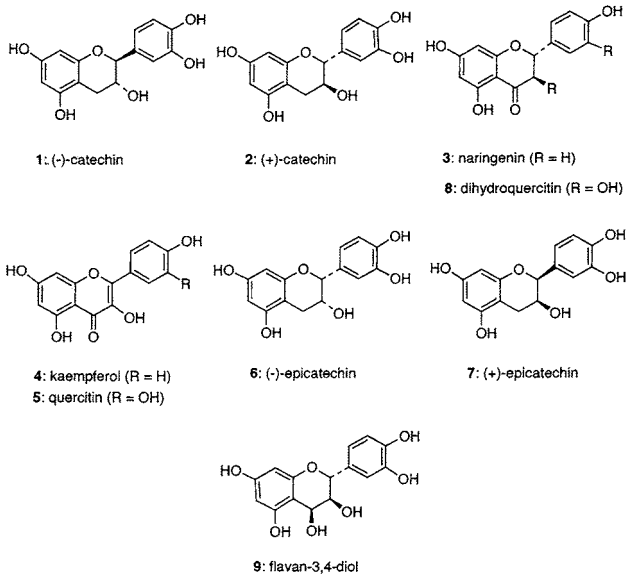

Figure 1. Chemical structures of all the flavonoids tested for phytotoxicity.

double-distilled water and methanol was used for flavonoid estimation (*6*), with an injection volume of 15 μL and a flow rate of 1 mL min^{-1}. Flavonoids from the incubation experiments were compared with the standards on the basis of retention time and peak area. Chromelon software (Dionex Corp.) was used for peak integration analyses. The eluted peaks were collected, evaporated, and analyzed by ^{1}H NMR and CD spectroscopy.

^{1}H NMR and Circular Dichroism (CD) Spectroscopy. ^{1}H NMR spectra of flavonoids isolated from the incubation experiments were compared to those of standards using a Varian INOVA 400-MHz Fourier transform spectrometer. Spectral data also corresponded to published spectra in the Aldrich library of ^{1}H FT NMR spectra (*18*).

Circular dichroism (CD) spectra were obtained in methanol on an Aviv model 202 spectrometer in methanol. (+)-Catechin showed a Cotton effect at 280 nm ($\Delta\epsilon = -1.511$) (*19*) and (+)-dihydroquercetin at 293 ($\Delta\epsilon = -8.249$) and 326 nm ($\Delta\epsilon = +2.740$) (*20*).

RESULTS AND DISCUSSION

Phytotoxicity of Flavonoids on Various Weeds and Crop Plants. To assess possible phytotoxicity among some compounds and intermediates in the flavonoid pathway, (−)-catechin (**1**), naringenin (**3**), kaempferol (**4**), quercetin (**5**), (−)-epicatechin (**6**), (+)-epicatechin (**7**), and (±)-dihydroquercetin (**8**) (**Figure 1**) were assayed for their effect on phenotypic response in terms of shoot and root differentiation and germination efficiency in various plants. These plants included the weeds *C. maculosa* (spotted knapweed), *C. diffusa* (diffuse knapweed), *L. dalmatica* (dalmatian toad flax), *K. scoparia* (kochia), the model plant *A. thaliana*, and crops such as wheat (*T. aestivum*) and tomato (*Ly. esculentum*). As a preliminary experiment, we tested these flavonoid compounds at different ranges to determine a minimum inhibitory concentration (MIC). We found that a concentration of 250 μg mL^{-1} in all flavonoids tested showed a distinct plant inhibitory profile. Generally, the phytotoxic and seed germination activities were around 250 μg mL^{-1}, except for (−)-catechin, which was active at 50 μg mL^{-1}. All plants except *C. maculosa* showed mortality on the 14th day after the addition of **1**, **4**, and **8** to in vitro cultured plants (**Figure 2**). (+)-Epicatechin (**7**) was active against all the tested plants, including *C. maculosa* (**Figure 2**). Wilting symptoms prior to senescence with reduced shoot and root differentiation also occurred (data not shown). Naringenin (**3**), **5**, and **6** failed to show any phytotoxic effects on the tested plants. Addition of **1**, **4**, and **8** inhibited seed germination of all the tested plants

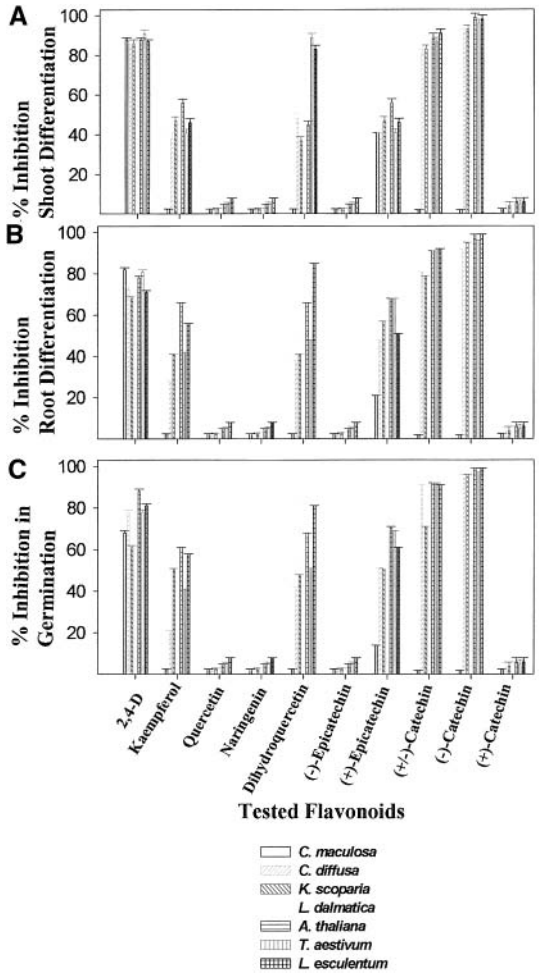

Figure 2. Effect of flavonoids on shoot (A) and root (B) differentiation and inhibition of seed germination **(C)** of different weeds and crop plants on the 14th day after treatment. Flavonoids were dissolved in methanol and administered (250 μg mL^{-1}) to the media containing 10-day-old seedlings. Tubes and Petri plates were subsequently incubated under a 16 h light and 8 h dark photoperiod in an incubator. The data represent the percent inhibition relative to the untreated control in shooting, rooting, and germination efficiency response in various tested seeds and seedlings. The seeds and seedlings tested against *C. maculosa* exudates were *C. maculosa*, *C. diffusa*, *K. scoparia*, *L. dalmatica*, *A. thaliana*, *T. aestivum*, and *Ly. esculentum* (values are mean ± SD, $n = 10$).

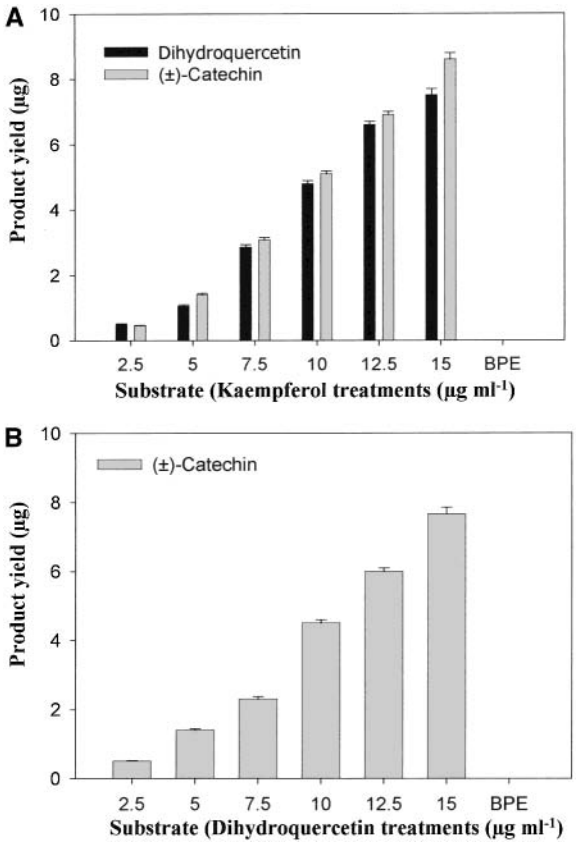

Figure 3. Production of racemic catechin upon incubating with kaempferol **(**A**)** and dihydroquercetin **(**B**)** with total protein extracts isolated from roots of *C. maculosa* grown in vitro. Experiments were performed by incubating total proteins extracted from root cultures of *C. maculosa* (0.5–30 μg mL^{-1}) with the flavonoids (0.5–30 μg mL^{-1}). The incubation mixture was extracted with hexane overnight, and the hexane layer was dried, concentrated under N_2, and subjected to HPLC. (BPE in the inset depicts boiled protein extracts as a control to confirm the enzymatic involvement for conversions.)

except *C. maculosa* (**Figure 2**). (+)-Epicatechin (**7**) inhibited seed germination of all the tested plants, including *C. maculosa*, while **3**, **6**, and **5** did not inhibit germination.

Clearly, none of the flavonoids are as potent as (−)-catechin, but there nevertheless appears to be some structural dependence on activity. (+)-Epicatechin represents an interesting case since it is the only flavonoid found to have some activity against *C. maculosa*. As might be expected, it was not found in *C. maculosa* roots and root exudates. Both enantiomers of epicatechin occur naturally, with (−)-epicatechin being widespread in plants and (+)-epicatechin a minor component of a few species, such as *Camellia sinensis* (the tea plant) (*21*). This uneven occurrence of enantiomers is also true of catechin, where (−)-catechin, the phytotoxic enantiomer, occurs very rarely (*22*) compared to the widespread enantiomer (+)-catechin. Roots of some plants have yielded glucosides of both (+)- and (−)-catechin (*23*, *24*). Isolation of (±)-catechin has been reported from plant bark and cotton cell cultures (*25*). Phytotoxic (−)-catechin and (+)-epicatechin have the 2*S* configuration, so this feature may be important for phytotoxic activity; the 2,3-trans relationships in (−)-catechin appears to provide additional potency.

Incubation of Flavonoids with Cell-free Total *C. maculosa* Root Proteins. Although studies have been reported on aspects of the biosynthesis of the common enantiomer (+)-catechin, nothing is known regarding the genesis of (−)- or (±)-catechin as natural products. One possibility might include (+)-catechin production followed by racemization in the root or during the exudation process. Alternatively, there could be a deviation from the normally observed highly stereo- and enantiospecific biosynthesis steps. In exploratory experiments which could lead later to a detailed study of enzymatic processes, we incubated commercially available possible catechin precursors with total protein extracts from *C. maculosa* roots. Both (−)- and (+)-catechins were also incubated and were recovered unchanged, suggesting that no racemase enzyme was present. This experiment also provided a control for the other incubations.

Possible catechin precursors **3**, **4**, **5**, and **8** were incubated with *C. maculosa* total root proteins. Of these, only **4** (kaempferol) and **8** (dihydroquercetin) were converted to catechin (**Figure 3**). Kaempferol (15 μg mL^{-1}), incubated overnight with 15 μg mL^{-1} of total protein extracts, and dihydroquercetin (20 μg mL^{-1}), incubated with 10 μg mL^{-1} of total protein extracts, resulted in the maximum bioconversion to catechin (**Figure 3**). The catechin recovered from kaempferol incubation experiments

was racemic, as evidenced by its CD spectrum. Dihydroquercetin was also isolated after incubation of kaempferol with the root protein extract and also proved to be racemic. Although these results are preliminary, they suggest that the root processes for catechin production are not enantiospecific, either in the requirement for a chiral precursor or in the conversions. The direct application to the intact plant system of such in vitro studies, of course, has not been established. For example, *in vitro* experiments demonstrated broad synthetic capabilities for anthocyanin synthase, unrelated to its presumed natural function (*26*, *27*). Thus, incubation of 3,4-dihydroxyflavan with anthocyanin synthase indeed produced small amounts of the expected anthocyanin, but the major products were dihydroquercetin (both cis and trans) and quercetin; incubation of the unnatural substrate naringenin led to dihydrokaempferol.

In recent years, much effort has been directed at identifying genes involved in flavonoid biosynthesis (*7*). In the first step, a chalcone is converted to a flavanone, such as naringenin, by the action of chalcone flavanone isomerase (CHI). Subsequent conversions lead to what has been termed (*28*) a metabolic grid, with (+)-catechin being formed from the flavan-3,4-diol, **9**. In this pathway, cyclization to form naringenin, subsequent 3-hydroxylation, and reduction of dihydroquercetin to the diol are all stereo- and enantiospecific processes. Kaempferol and quercetin are generally regarded as end products rather than intermediates for further transformations. In contrast, the products from *C. maculosa*'s root protein incubations appear to be formed from kaempferol nonenantiospecifically through dihydroquercetin to (±)-catechin. The results do not rule out dihydrokaempferol as an intermediate. The correlation of our *in vitro* experiments to the root exudation process is yet to be determined, but our data should provide a starting point for further study. Physical transport pathways from synthesis to exudation sites need to be elucidated, along with whether the usual flavonoid glycosylation steps are still operative or are bypassed in *C. maculosa*.

Evidence that Arabidopsis flavonoid biosynthetic enzymes physically interact *in vivo* (*9*) suggests that the formation of different multiprotein complexes may contribute to the biosynthesis of each group of flavonoids (*29*). Such complexes could form a different configuration in roots compared to other plant tissues and might not exist in protein extracts such as those employed here. Differences between soluble and endoplasmic reticulum-bound flavonoid pathway enzymes and compartmentalization in flavonoid biosynthesis are certainly factors to consider in the final evaluation of our experiments (*30*). Our results are not what might have been expected on the basis of knowledge of the usual pathways in other plant parts; in the case of *C. maculosa* root exudates, the final result seems to have been the production of a potent aid for survival and expansion of invasive *C. maculosa.*

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