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Bioavailability of Allelochemicals As Affected by Companion Compounds in Soil Matrices

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Multicompound allelochemical interactions were studied using *Centaurea maculosa* as a model source to understand how the bioavailability of complex allelochemical mixtures is modified in soil−microbial systems. Litter decomposition of *C. maculosa* in sandy loam soil yielded five phenolic acids, namely, hydroxybenzoic, vanillic, protocatechuic, *p*-coumaric, and ferulic acids. The degradation studies were conducted by exogenous application of catechin, the primary allelochemical exuded by *C. maculosa*, and the phenolic acid cosolutes in a sandy loam and silt loam soil. Compared to a single-solute system, in a multisolute system the persistence of individual allelochemicals was significantly increased in both soils. Oxidation and sorption were primarily involved in the disappearance of allelochemicals. Mass spectrometric data showed that catechin rapidly underwent polymerization to form procyanidin dimer both in soil and in bioassay medium, resulting in reduced persistence and phytotoxicity. Hence, catechin phytotoxicity could occur only under conditions that would inhibit these condensation reactions. This study clearly demonstrates that various soil mechanisms including competitive sorption and preferential degradation would increase the persistence of allelochemical mixtures in a soil matrix.

KEYWORDS: Allelopathy; catechin; *Centaurea maculosa*; procyanidin; phenolic acids; interaction index; competitive sorption

INTRODUCTION

Allelopathy, plant-to-plant interactions mediated by plant secondary metabolites, has gained importance in present-day agricultural and ecological sciences. In agriculture, due to their less persistent and more environmentally friendly nature, allelochemicals are investigated as a supplemental, if not, alternative method of weed control (*1*). From an ecological perspective, allelopathy is occasionally implicated as one of the major factors enhancing the invasiveness of a non-native plant species (*2*).

Despite competent research efforts, the occurrence and significance of allelopathy at ecosystem levels are still highly debated (*3*). The interaction of allelochemicals in the soil matrix remains as one of the least understood areas in the research on allelopathy (*4*). Although volatile compounds exuded by plant shoots are implicated in allelopathy (*5*), most of the allelopathic interactions take place in the soil, where allelochemicals are concentrated and exuded through roots (*2, 6*) or are released during decomposition of plant litter (*7, 8*). Thus, soil forms the primary medium for the transport of allelochemicals from a donor to a receiver plant. During this transportation, the soil matrix is capable of altering the bioavailability of allelochemicals by various processes including sorption and chemical and microbial degradation (*4, 9, 10*). Because allelochemicals are secreted in quantities far less than needed to overwhelm the soil processes, at the field level, the soil matrix becomes the governing factor in the allelochemical phytotoxicity. Thus, in many cases allelochemicals are not found in phytotoxic quantities under field conditions (*11–15*).

Another aspect of allelopathy which has received less attention is the fact that the allelochemicals are always released as mixtures with other compounds (*16*). The degradation pattern of individual allelochemicals in soil matrices has been studied before (*9, 10*); however, the fate of allelochemicals when present in multicompound mixtures is still less understood. Along this line, we investigated sorption of phenolic allelochemical mixtures onto a soil matrix and reported the phenomenon of preferential sorption, where cinnamic acid derivatives increased the effective concentration of benzoic acid derivatives in a microbe-free soil environment (*4*). Various compounds in plant root exudates and those produced during litter decomposition vary widely in their phytotoxicity (*19*). Hence, preferential sorption and degradation of compounds in a mixture could protect and prolong the bioavailability of its cosolutes, thus modifying the overall toxicity of a mixture. Thus, allelochemicals, even at subtoxic concentrations, can exhibit an enhanced phytotoxicity when present in a multisolute mixture.

Centaurea maculosa, spotted knapweed, is a noxious and economically destructive invasive weed inhabiting >4 Mha in

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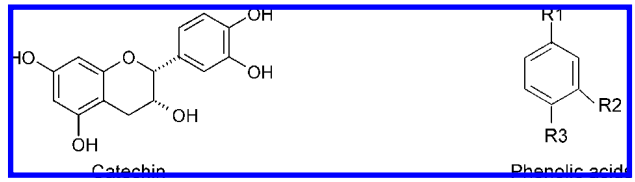
Table 1. Characteristics of Allelochemicals Used in This Study (*4*)



Catechin Phenolic acid

phenolic acid	R1	R2	R3	pK_a[a]	ionized fraction (pH 5.15)	water solubility[b] (g L^{-1})
p-hydroxybenzoic acid (HYB)	COOH	H	OH	4.5	0.82	23.6 ± 0.1
protocatechuic acid (PRC)	COOH	OH	OH	4.5	0.82	15.2 ± 0.1
vanillic acid (VAN)	COOH	OCH_3	OH	4.5	0.82	9.2 ± 0.1
p-coumaric acid (COU)	CH=CHCOOH	H	OH	4.7	0.73	3.4 ± 0.1
ferulic acid[c](FER)	CH=CHCOOH	OCH_3	OH	4.7	0.73	3.1 ± 0.1

[a] pK_a of COOH. [b] At pH 5.1 and 24 ± 1 °C. [c] 99% trans form.

North America (*20*). Recently the invasiveness of this species was attributed to the root exudation of a racemic (±)-catechin [*2*; 3,3′,4,5,7- pentahydroxyflavan; referred to as catechin hereafter]. Although catechin was initially reported to be present in phytotoxic concentration under *C. maculosa* stands (*2, 21*), further studies by Blair et al. (*11, 14*) contradicted this, reporting concentrations 3 orders of magnitude less than the required phytotoxic concentration. Furthermore, catechin was found to be very weakly phytotoxic to the test species (*11*).

Catechin is a natural flavanoid having a very high antioxidant property and is found in many plant species including tea, grapes, and apples. Because of the high reactivity attributed to the five hydroxyl groups (**Table 1**), catechin is unstable in aqueous solutions and is oxidized by dissolved oxygen (*22*). At alkaline pH catechin undergoes nucleophilic addition reaction to form catechenic acid derivatives and quinones (*25*), whereas acidic pH conditions result in the formation of dimers from radical coupling (*26, 27*). Due to this highly reactive nature of catechin its persistence in a highly heterogeneous environment such as a soil matrix is always questionable. Therefore, the objectives of this study were to understand the soil dynamics of catechin and its cosolutes when present in a multicompound mixture, to identify the important soil processes that contribute to the persistence of allelochemical mixtures, and to evaluate the physiological joint action of catechin and cosolutes for enhanced phytotoxicity.

MATERIALS AND METHODS

Soil and Chemicals. Degradation studies were carried out in a silt loam soil and a sandy loam soil. The soils were air-dried, sieved (0.5 mm), and stored at 15 °C. The soil characteristics were as follows: sandy loam, Typic Udorthent (42° 16′ 37″ N, 72° 24′ 5″ W), 60% sand, 35% silt, 5% clay, organic matter 2.5%, pH 6.1, Fe 5 μg/g, Mn 0.8 μg/g; silt loam soil, Typic Udifluvent (42° 28′ 37″ N, 72° 36′ 2″ W), 28% sand, 60% silt, 12% clay, organic matter 5.1%, pH 5.8, Fe 30 μg/g, Mn 12 μg/g. The water-holding capacity of the soils was determined by a pressure-plate membrane apparatus.

Five phenolic acids [protocatechuic acid (PRC), *p*-hydroxybenzoic acid (HYB), vanillic acid (VAN), *p*-coumaric acid (COU), and ferulic acid (FER)], and (±)-catechin (50:50 racemic mixture; CAT) of analytical grade (>99% purity) were purchased from Sigma-Aldrich (St. Louis, MO). The chemical properties of the compounds used are given in **Table 1**.

Litter Decomposition Study. The organic compounds co-occurring with catechin in a soil matrix were identified on the basis of a litter decomposition study of *Centaurea maculosa*. The upper two-thirds of the senescing *C. maculosa* plants was harvested from its invasive stands in Montana (46.87° N, 113.97° W) during September 2006. This litter mainly consisted of woody stems, leaves, flowers, and seed-heads. Plant litter was air-dried, chopped to 10 mm mesh size, and mixed thoroughly. The homogenized sample had a C/N ratio of 50:1. Litter decomposition studies were carried out both in sandy loam and in silt loam soils at a litter/soil ratio of 1:4 (100 g of litter to 400 g of soil). A high litter/soil ratio was chosen on the basis of preliminary experiments which showed that further decreasing this ratio results in poor phenolic acid recoveries. The same litter/soil ratio could exist in top soils in knapweed monoculture stands. The litter−soil was moistened to 80% water-holding capacity and incubated in complete darkness at 25 °C and 95% relative humidity.

The litter was extracted at 4, 7, 14, and 21 days after incubation. Because we were interested in the allelochemicals that will be released to soil solution during the decomposition process, the litter was extracted with double-distilled water. The extraction method consisted of eluting the litter with 300 mL of water after the litter−soil had been transferred to a perforated container lined with Whatman no. 1 filter paper. This ensured an initial soaking of the litter by water followed by slow elution under gravity. The eluted samples were further purified and concentrated using solid phase extraction (SPE) procedures as follows: buffered samples (50 mM KH_2PO_4, pH 6.8) were loaded onto Strata X-AW (Phenomenex, Torrance, CA), a reverse phase, polymeric, weak anion exchange resin cartridges (500 mg, 6 mL), followed by washing with 6 mL of 30% methanol/acetonitrile (2:3, v/v). The retained phenolic compounds, excluding the higher molecular weight compounds, were eluted with 60% methanol/acetonitrile (2:3, v/v) in 5% H_3PO_4. Preliminary SPE studies with spiked standards in sample matrix yielded 80 ± 7% recovery.

Degradation of Allelochemicals. Decomposition studies of the allelochemicals in a single-solute system and in a multisolute system were conducted in sandy and silt loam soils. The initial CAT concentration in a single-solute as well as the multisolute studies was 100 μg/g of soil. This CAT concentration is well below the reported field level (*21*). The phenolic acids selected for the multicompound degradation studies were based on the litter decomposition studies, and the individual concentrations of phenolic acids were 72 μg/g of soil. The degradation studies involve using 5 g of soil in 20 mL glass vials with glass wool stoppers to facilitate air circulation. The soils were maintained at 80% field capacity. The vials were incubated in complete darkness at 24 °C and 95% relative humidity. The control consisted of double-distilled water with no allelochemicals. Triplicate samples were extracted with double-distilled water at 0, 5, 10, and 24 h and 2, 3, 4, 5, 6, 8, 10, 12, 16, and 20 days after incubation. Water extracted levels can indicate availability of phenolic compounds to plants and microbes (*13, 31*). The extracts were centrifuged at 3000*g* for 30 min, filtered through a 0.2 μm nylon membrane filter, and analyzed using high-performance liquid chromatography (HPLC). Background allelochemical concentrations of the tested soils were below our detection limits. Subsamples of extracts were analyzed with inductively coupled plasma atomic emission spectrometry (Perkin-Elmer) for iron and manganese concentrations.

Soil Sorption Study. Sorption of HYB, VAN, COU, and FER onto silt loam soil was studied using a batch equilibration technique in a microbe-free environment. Preliminary studies with the sandy loam soil did not yield significant sorption of phenolic acids to have enough data

points for the sorption isotherm. The experiments were conducted at 24 °C with sodium azide (200 μg/mL) as bioinhibitor (*4*). The soil/solution ratio for HYB, VAN, and COU was kept at 1:4, and for FER the ratio was 1:8. Competitive sorption experiments for a FER−VAN binary system were carried out under the same experimental conditions as described above at a fixed initial concentration of FER (3 mM). Sorption and competitive sorption data were fitted using a logarithmic form of the Freundlich equation

$$\log S = \log K_F + N \log C \quad (1)$$

where S is the sorbed concentration of phenolic acid to soil (μg/g), C is the solution-phase equilibrium concentration (μg/mL), and K_F [(μg/g)(μg/mL)$^{-N}$] and N (dimensionless) are constants. The Freundlich exponent (N) and affinity constant (K_F) were determined by linear regression of log-transformed data.

Chromatographic Analysis. All of the samples were analyzed on a series 200 quaternary pump HPLC system equipped with an autosampler, inline degasser, UV−vis diode array detector (DAD), and fluorescent detector (Perkin-Elmer, Waltham, MA) in tandem. Separations were performed on a 250 mm × 4.6 mm, i.d., 5 μm particle size Discovery C-18 column (Supelco, Bellefonte, PA). The absorbance of the samples were monitored at 254 and 280 nm with the DAD and at excitation−emission wavelengths of 205−315 and 280−315 nm with the fluorescent detector. Fluorescent spectroscopy (Perkin-Elmer fluorescence spectrometer LS45) studies of CAT showed that with excitation−emission (Ex−Em) of 205−315 nm, the fluorescent intensity was 9 times higher compared to Ex−Em of 280−316 nm. All of the data were acquired and processed with Totalchrom 6 (Perkin-Elmer). The mobile organic phase consisted of MeOH/MeCN (60:40) in 0.5% (v/v) glacial acetic acid, and the aqueous phase consisted of 0.5% (v/v) glacial acetic acid. The separation was achieved by increasing the organic phase by 5%, from the initial 15% to a final 30% at 10 min intervals followed by re-equilibration for 10 min. This gave a minimum peak resolution (R_s) of 4. The limit of detection (LOD) was defined as a signal-to-noise (S/N) ratio of 10, and all values reported are based on peak area. The detection limit of different phenolic acids with DAD was 5 ng, whereas the CAT detection limit with fluorescence detector was 0.1 ng. The standards were prepared in elution solvent matrix to avoid the interference of sample matrix on the quantification of analytes. Identification of compounds in the samples was accomplished by comparing the retention time and the spectra of the unknowns with the standards.

Data were analyzed using one-way ANOVA. Duncan's new multiple-range test (DNMRT) was used to compare K_d and the Fe and Mn release across the systems. Significance is reported at the 95% confidence interval. All statistical tests were performed with SAS 9.1 (SAS Institute, Cary, NC).

Plant Bioassay. The effect of phenolic acids (HYB, VAN, COU, FER) in modifying the toxicity of CAT was investigated using radicle elongation bioassays. The test species included two monocot species, perennial rye grass (*Lolium perenne*) and barnyard grass (*Echinochloa* spp.), and two dicot species, lettuce (*Letuca sativa* var. Buttercrunch) and cucumber (*Cucumis sativus*). The bioassays were carried out in glass Petri plates (100 × 20 mm, Pyrex brand) using Whatman no. 1 filter paper as medium. Each Petri plate received 15 surface-sterilized (6% NaOCl for 6 min) seeds and 6 mL of the test solution (pH adjusted to 6); thereafter Petri plates were sealed with parafilm and incubated in complete darkness at 23 °C. All treatments had four replicates and were repeated twice. Radicle length was measured to the nearest millimeter 5 days after incubation. CAT stability in the bioassay medium was tested in two of the four replicates at 5 days after incubation. The toxicity of individual compounds (effective concentration 50, EC_{50}) was estimated using a dose−response curve with at least 12 concentrations spanning from no response to total inhibition of root growth. The highest concentration of all the phenolic acids was below 70% of the maximum water solubility of the acids [**Table 1** (*4*)]. On the basis of preliminary studies looking at autoxidation and solubility, the maximum CAT concentration was kept at 250 μg/mL. Joint action of CAT with PRC and FER was tested at 1:1 ratio on the basis of the relative potency of individual compounds. Combined phytotoxic effect of the binary mixture was computed on the basis of the interaction index (*28*).

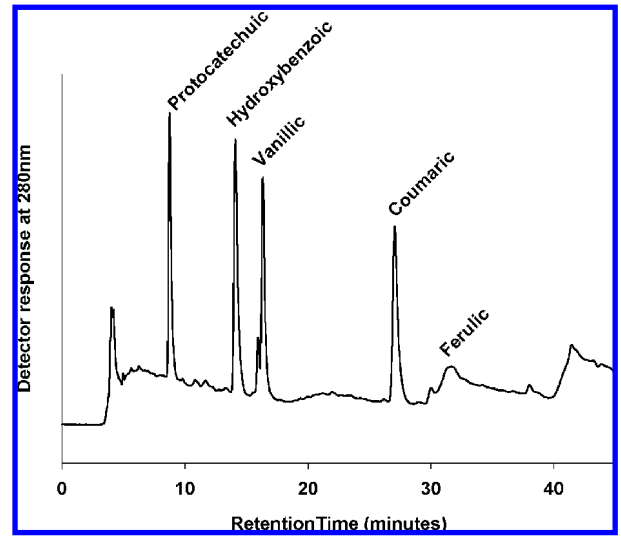


Figure 1. LC-DAD chromatograms of the compounds in water extracts during the decomposition of *Centaurea maculosa* litter in a sandy loam soil after 7 days of incubation.

The interaction index (γ) is defined as Z_{mix}/Z_{add}. The overall combination effect is taken as synergistic if $\gamma < 1$, additive if $\gamma = 1$, and antagonistic if $\gamma > 1$. Z represents the desired effect level or EC_{50}. Z_{mix} is the observed EC_{50} of the mixture; Z_{add} is the theoretical EC_{50} of the mixture, which is based on the observed individual EC_{50} of its components and is calculated as follows: $Z_{add} = fA + (1 - f)B$, where A and B represent the individual EC_{50} values of the two compounds A and B in the binary mixture and f is the fraction of EC_{50} of the reference compound (either A or B) in the mixture. The binary combination (1:1) is made such that their fractions add to unity [f and $(1 - f)$].

Because the maximum dose of CAT tested did not elicit any significant reduction in radicle elongation, CAT was considered to be an inactive compound in the mixture. Hence, Z_{add} was computed as $Z_{add} = A/\rho_A$, where ρ_A is the proportion of potent compound (A, phenolic acid) in the binary mixture. Proportion (ρ) can be related to fraction of EC_{50} (f) as follows: $f = \rho B/(A + \rho B - \rho A)$. The standard error (SE) was calculated as $SE(Z_{add}) = SE(A)/\rho_A$.

RESULTS AND DISCUSSION

Litter Decomposition Study. Decomposition of plant litter produces a wide array of compounds (*29*). To avoid matrix interferences that compromise the detection, and because phenolic acids are the major products of plant-litter decomposition (*8*), our sample purification and detection methods were tailored for the qualitative detection of phenolic acids alone. The litter exudates were analyzed for 12 phenolic acids belonging to benzoic and cinnamic acid groups. The recovery of phenolic acids varied primarily with soil type and days of incubation. Highest water extractable phenolic acids were obtained from sandy loam soil (**Figure 1**), whereas no detectable amount of phenolic acid was obtained from silt loam during the incubation. The lower extractability of phenolic acids from silt loam soil could be explained by the high sorption affinity of this soil (**Figure 2**). Seven days into litter decomposition, the water extract from sandy loam soils yielded three benzoic acid derivatives (PRC, HYB, VAN) and two cinnamic acid derivative (COU and FER) (**Figure 1**). These phenolic acids have been found associated with other plant litter decompositions (*30*). We did not detect CAT during any stages of litter decomposition. This is in agreement with Bais et al. (*2*), who reported CAT to be present only in root tissues.

Although litter decomposition progressed with incubation duration, the phenolic acid recovery showed an inverse trend with the number of phenolic compounds decreasing from 7 to

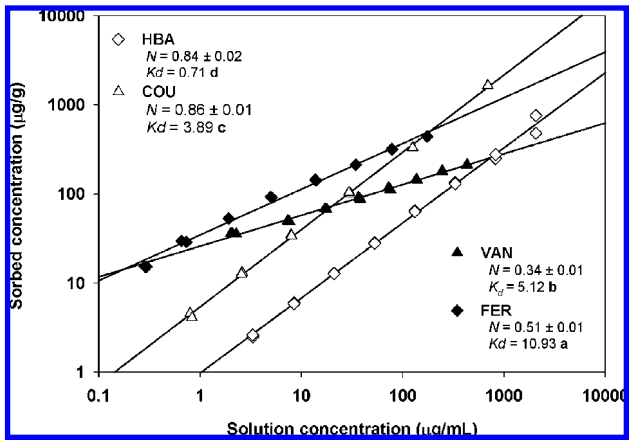


Figure 2. Sorption isotherms of various phenolic acids in silt loam soils. The concentration-dependent sorption coefficient (K_d) is at solution concentration of 10 μg/mL. Abbreviations: HYB, *p*-hydroxybenzoic acid; VAN, vanillic acid; COU, *p*-coumaric acid; FER, ferulic acid (FER). Means of K_d with the same letters are not significantly different at $P < 0.05$ (DNMRT).

14 days. Fourteen days after incubation, the amount of phenolic acids present significantly decreased and only PRC and HYB were detectable. Apart from matrix interference due to excess amount of organic compounds formed, this could be due to the further polymerization (*32*) or microbial degradation of simple phenolic compounds (*18*). Also, decomposing litter could itself act as a sink for the phenolic acids, further reducing their availability (*33*). Being less reactive and highly hydrophilic among the phenolic acids (*4*), HYB was present in soil aqueous extracts even 24 days after incubation (data not shown).

Decomposition Study of Allelochemicals. *Sandy Loam Soil.* In a single-solute system the concentration of all the allelochemicals decreased to <1% of that initially applied within 6 days. CAT and PRC showed a significant decrease, with >50% of the initial quantity disappearing within the first 12 h (**Figure 3A**). When present in a multisolute system, the residence time of allelochemicals was significantly increased (**Figure 3B**). Six days after incubation, which corresponds to complete degradation of all allelochemicals in a single-solute system, >65% of the total added phenolic acids remained in a multisolute system, and recoveries of COU, HYB, and VAN were >80%.

Their presence in a multisolute system did not delay the initial rapid disappearance (>40%, 12 h) of both CAT and PRC (**Figure 3B**). Following this, although CAT degradation continued, PRC concentration decreased much more slowly, with 32% remaining 12 days after incubation. Degradation of phenolic acids in a single-solute systems resulted in the accumulation of intermediary compounds in both soils. Degradation of FER coincided with the appearance of COU, VAN, and PRC, whereas degradation of both VAN and COU resulted in HYB and PRC accumulation. Thus, the buildup of PRC, following its initial rapid disappearance in the sandy loam soil, can be related to the breakdown of FER, COU, and VAN (schematic degradation pathway of the phenolic acids identified on the basis of HPLC separation and spectral studies given in the Supporting Information). Accumulation of PRC as a result of degradation of phenolic compounds has also been reported by Haider and Martin (*33*) and Venturi et al. (*34*). HYB and VAN were slow to decline from soil solution, with >90% of both present up to 5 days after incubation in a multisolute system. This initial steady concentration ($P < 0.05$, $n = 3$) of HYB, VAN, and COU in sandy loam soil can also be related to the rapid breakdown of FER (**Figure 3B**). At the end of the experiment (20 days after incubation) >10% of the initial PRC, VAN, and HYB remained in soil solution (**Figure 3B**).

Silt Loam Soil. In a single-solute system the reaction of allelochemical was rapid, with >80% of the initial PRC and CAT disappearing within 5 h after incubation (**Figure 3C**). In soil matrix phenolic acids can sorb onto organic matter and to clay minerals through cation bridging, undergo polymerization on mineral surfaces (*4, 32*), form inner and outer sphere complexation with iron and aluminum hydroxides, or be oxidized by Fe and Mn (*10, 35*). Compared to sandy loam soil, silt loam soils have more reactive clay fractions, organic matter, and Fe and Mn (see Materials and Methods), which reduce their aqueous extractability (**Figure 2**).

Sorption isotherms of allelochemicals were fitted to a straight line using least-squares regression of log−log transformed data. The data fitted well to the Freundlich model, and the sorption isotherms did not plateau at higher concentrations, which indicates sorption site abundance. The Freundlich exponent (N) denotes the curvature of the isotherm. Soils that are homogeneous at the macroscopic level are heterogeneous at a finer scale as different matrices and interfaces may exhibit varied affinities for organic solutes. As this reactivity is unevenly distributed in different soil fractions, N can be used to infer the energy distribution of the sorption sites (*36*). CAT and PRC underwent continuous degradation in silt loam and did not reach equilibrium even after 30 days, and hence their sorption isotherms could not be computed. Sorption isotherms of phenolic acids onto silt loam soil were nonlinear, and N value follows the order VAN > FER > COU > HBA (**Figure 2**).

Because the sorption isotherms were nonlinear, precise comparison of the sorption affinities of different phenolic acids could not be made using the Freundlich affinity coefficient (*4, 36*). Therefore, the concentration-dependent sorption coefficient (K_d) was computed at a solution concentration value of 10 μg/mL for comparison across the different systems. The numerical value of K_d is equal to the slope (S/C) of the sorption isotherm at a given value of C and has units of milliliters per gram. The K_d value follows the order FER > VAN > COU > HBA ($P < 0.05$, DNMRT, **Figure 2**). Higher values of K_d indicate that the solute is more strongly held onto the sorption sites, and this order of affinity could partly be explained by their hydrophobicity [PRC > HYB < VAN < COU < FER; (*4*)]. The sorption affinity of VAN was decreased 10-fold ($K_d = 0.49$), and the isotherm became more linear ($N = 1.0$) in a FER−VAN binary system (data not shown), which suggests competition for sorption sites. Thus, the slower disappearance of VAN in a multisolute system in this soil could partly be due to its competitive displacement by FER and due to the accumulation of breakdown compounds formed during degradation. Rapid degradation of FER resulted in HYB concentration being higher ($P = 0.04$,; **Figure 3D**) than the initially applied up to 15 h after incubation.

The Fe and Mn concentrations in the silt loam soil solution showed an inverse pattern with the phenolic acid disappearance (**Table 2**), with a rapid increase in Mn and Fe concentration within 1 h of incubation with PRC or CAT. A plot of solution Mn concentration with the percent allelochemical disappearing in silt loam soil gives a correlation coefficient of 0.98, indicating oxidation as the primary mechanism for the disappearance of PRC or CAT from this soil (**Table 2**). The oxidation was very abrupt in the first hour of incubation. When present in a multisolute system, the rapid disappearance of CAT was significantly reduced to 26% (compared to 84% in a single-

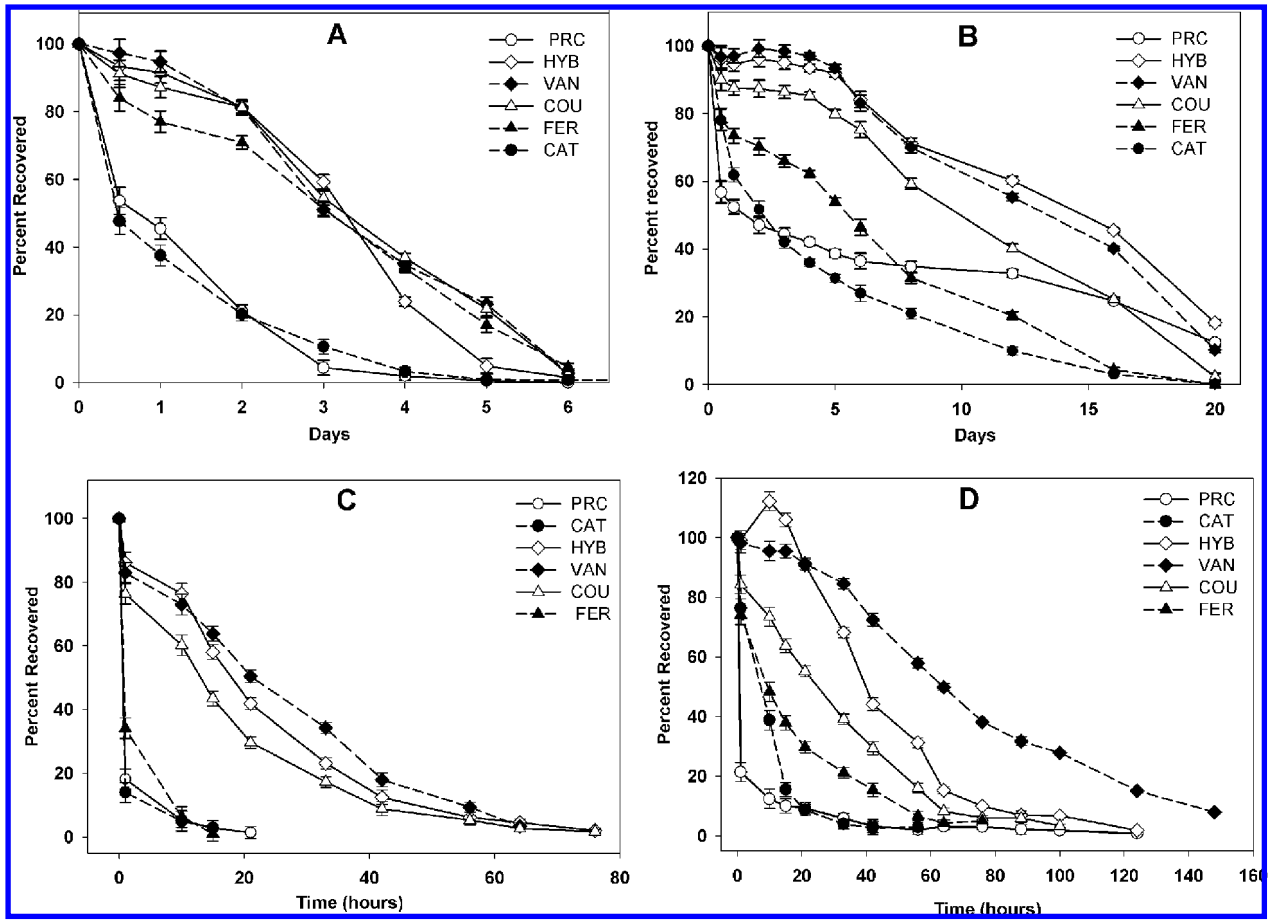


Figure 3. Degradation of allelochemicals in a sandy loam soil: (**A**) single-solute system; (**B**) multisolute system and silt loam soil; (**C**) single-solute system; (**D**) multisolute system. Abbreviations: PRC, protocatechuic acid; CAT, catechin; FER, ferulic acid; HYB, hydroxybenzoic acid; VAN, vanillic acid; COU, coumaric acid. LSD bars (0.05).

Table 2. Release of Fe and Mn from Silt Loam Soil at 1 and 10 h after Incubation with Various Allelochemicals[a]

	1 h		1 h	10 h
compound	Mn (μmol/g of soil)	Fe (μmol/g of soil)	Mn (μmol/g of soil)	Fe (μmol/g of soil)
protocatechuic acid	64.0 a	51.8 a	86.3 a	46.0 ab
p-hydroxybenzoic acid	18.6 d	36.1 c	20.8 e	40.6 bc
vanillic acid	20.6 d	32.2 cd	34 d	46.7 ab
p-coumaric acid	21.7 d	36.2 c	25.5 e	36.1 c
ferulic acid	37.2 c	49.2 b	51.9 c	49.5 a
catechin	55.1 b	46.4 ab	71.7 b	46.2 ab
water	23.1 d	25.9 d	23.3 e	29.5 d

[a] Means with the same letter are not significantly different ($P < 0.01$, $n = 3$).

solute system; **Figure 3C**) in the presence of PRC; however, the reduction of PRC concentration was statistically similar to that in a single-solute system ($P < 0.05$; **Figure 3C**,**D**). This further demonstrated that PRC undergoes preferential oxidation due to its high reactivity with Fe and Mn in silt loam soils, thus protecting CAT. The number of phenolic hydroxyl groups of a molecule increases its oxidative reactivity; accordingly, the higher reduction of Mn as a function of phenolic hydroxyl moieties has been shown in other soil systems (*10*). On the basis of our results CAT was the second most reactive compound in terms of its reductive potency (**Table 2**). The *o*-dihydroxy moiety of the CAT forms the site of nulcleophilic attack where the adjacent phenolic oxygen can form metal chelates (*37*).

The disappearance of allelochemicals was delayed when present in a multisolute mixture from both soils (**Figure 3B**,**D**). This slow disappearance of allelochemicals in a mixture could be due to the combined effect of preferential degradation, where compounds with a stable ring structure and without a 3-C (acrylic) side chain are less susceptible to degradation, and competitive sorption, where less hydrophobic molecules are displaced into soil solution. Microbial degradation of substrate in soil matrix is in accordance with the biological activity of the compound, where toxic compounds are degraded slowly (*38*). Addition of a more soluble and energy-efficient carbon source has been shown to reduce the microbial decomposition accompanying complex substrates (*18*). Competition for sorption sites arises if the same sites can be occupied by more than one nonidentical molecule (*4, 36*). This competition for sorption sites in a soil matrix could increase the effective concentration of phenolic acids in soil solution (*4*).

CAT has the maximum UV absorbance at 279 nm (**Figure 4**); however, UV spectrophotometry studies after incubation of CAT in soil for 48 h showed a decrease in absorbance at 279 nm and a corresponding increase in absorbance at 379 nm (**Figure 4**). Oxidation of CAT could result in the production of semiquinone radical (one-electron oxidation) or *o*-quinone (two-electron oxidation), which result in yellow color development with the observed absorbance peak shift (*26*). The semiquinone radicals formed undergo coupling reaction, forming dimers, which are more hydrophobic than CAT (*26*). HPLC separation showed the degradation products to be more hydrophobic and eluted later. The degradation products were further identified using a quadrupole ion-trap tandem mass spectrometer (Bruker Esquire MS/MS; Mass Spectrometry Facility, University of

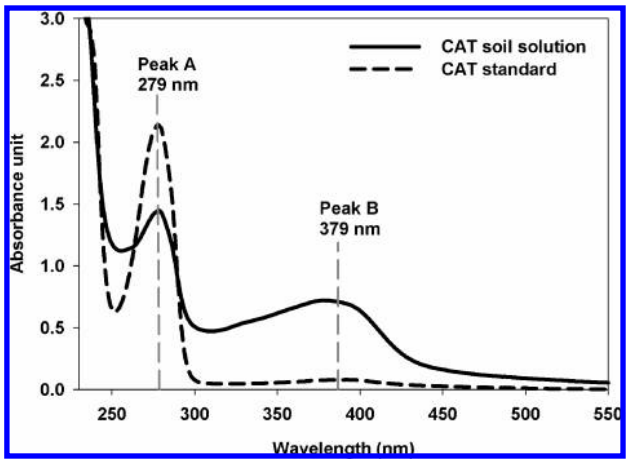


Figure 4. Background-corrected UV−vis spectrum of the degradation product formed after incubating (±)-catechin (CAT) in soil for 48 h. Peaks: A, catechin; B, degradation product.

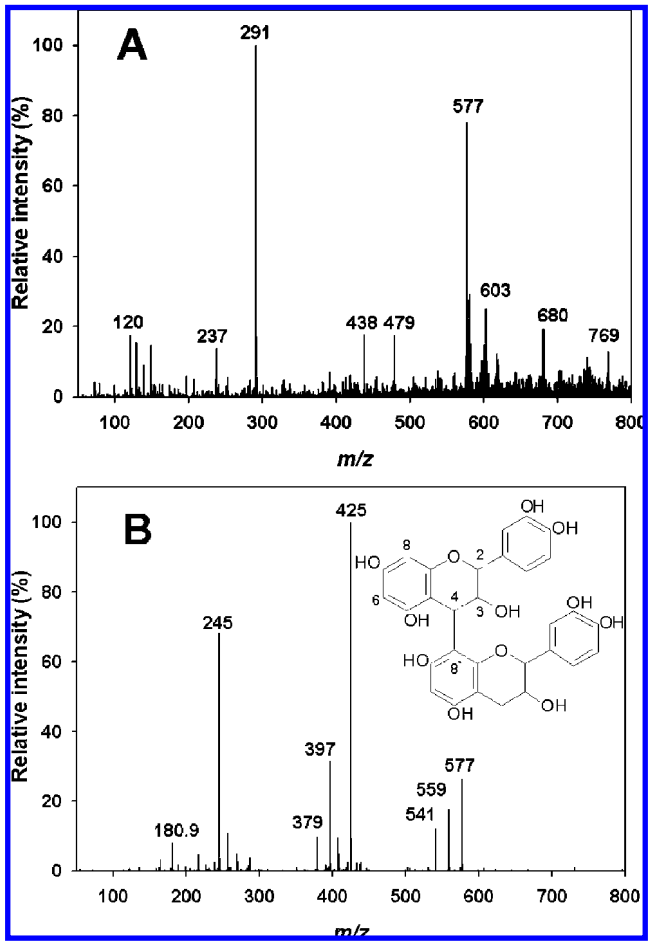


Figure 5. ESI-MS/MS spectra of degradation products of catechin (**A**) and the product ion spectra and molecular structure of procyanidin dimer *m/z* 577 (**B**). *m/z* 291 corresponds to catechin.

Massachusetts−Amherst), with an electronspray ionization interface (ESI-MS/MS). The samples were directly infused into the mass spectrometer at 2 μL/min, and spectra were acquired in both positive (capillary exit = 127 V, skimmer = 47 V) and negative (capillary exit = –127 V, skimmer = –47 V) ion modes in a scan rage from *m/z* 50 to 800. The full-scan positive ion mass spectra clearly show the predominance of polymerization product of CAT (*m/z* 577; $M + H^+$; **Figure 5A**). The product-ion mass spectra of this ion further confirmed it to be a CAT

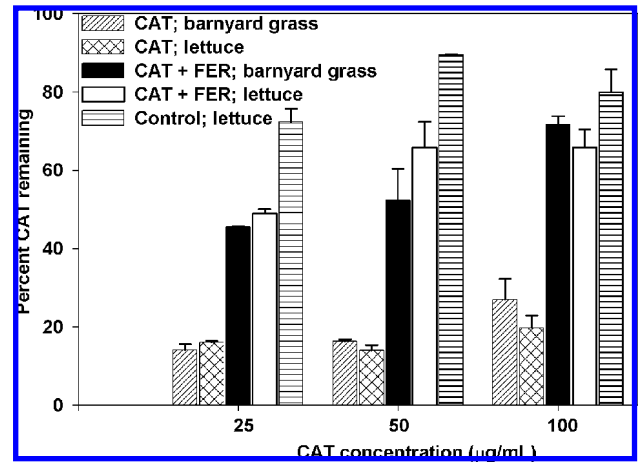


Figure 6. Percent of initially applied (±)-catechin (CAT) remaining in bioassay medium after 5 days of incubation. Control consisted of seeds with embryo removed. Values are means ± range bars ($n = 2$).

Table 3. Toxicity of Phenolic Acids, Measured as EC_{50}, to Dicot and Monocot Species

	EC_{50} (mM)			
phenolic acid	lettuce	ryegrass	baryardgrass	cucumber
p-hydroxybenzoic acid	1.4 ± 0.2	6.4 ± 0.2	9.6 ± 0.5	1.6 ± 0.1
vanillic acid	0.7 ± 0.1	6.4 ± 0.3	5.4 ± 0.4	1.4 ± 0.1
protocatechuic acid	1.4 ± 0.2	2.1 ± 0.1	3.9 ± 0.2	1.2 ± 0.2
p-coumaric acid	2.6 ± 0.1	3.6 ± 0.2	1.0 ± 0.1	3.4 ± 0.1
ferulic acid	2.3 ± 0.1	4.3 ± 0.1	0.8 ± 0.1	3.1 ± 0.1

dimer (procyanidin dimer) with the following fragmentation characteristics (**Figure 5B**): 559 $(M - H_2O + H)^+$, 541 $(M - 2H_2O + H)^+$, 425 [retro-Diels−Alder cleavage of heterocyclic flavanoid ring; (*39*)]. Cross-ring cleavage of the dimer (removing C3 and C4 and their respective functional groups) will result in ion fragments *m/z* 245; cleaving of C4−C8′ and C3−C2 bonds of the dimer molecule will result in a monomer linked to extra quinone (*m/z* 397) and the remaining bicyclic compound (*m/z* 180). Further polymerization of CAT to form trimers (*m/z* > 577) was also evident from the parent-compound spectra (**Figure 5A**), which could result in production of condensed tannins (proanthocyanidins). Mass spectrometry analyses of procyanidins are further discussed in Karchesy et al. (*39*), and flavanoid polymerization is detailed in Dixon et al. (*40*).

Bioassay. In general, the benzoic acid derivatives (HYB, VAN, PRC) were more toxic to the dicot species (lettuce and cucumber), whereas the cinnamic acid derivatives (COU, FER) were more toxic to monocot species (ryegrass and barnyard grass; **Table 3**). Phenolic acids are reported to affect normal plant water uptake, inhibit catalase and peroxidase activities, disrupt nutrient uptake, and inhibit phytohormones (*41*).

CAT, at its highest concentration, limited by its water solubility, did not cause significant reduction in radicle elongation of any of the test species, and hence the EC_{50} could not be computed. This is in contrast to earlier reports of CAT being highly phytotoxic to both monocots and dicots [(*2*); possible reasons for this contrast are further discussed in the Supporting Information]. CAT concentration decreased in the bioassay system with time (data not shown). This decrease was not species specific. The CAT concentration in the control, seeds with embryo removed, remained >75% of the initial at all concentrations. In agreement with the earlier results (*2*), the presence of CAT caused browning of roots of the test species. However, root-browning was not accompanied by a reduction

in radicle elongation due to a significant reduction of CAT in the bioassay medium (**Figure 6**). The root browning could be caused by the oxidation of CAT mediated by polyphenol oxidase and/or peroxidase enzymes, which are very active during seed germination (*42*). Enzymatic oxidation of CAT has shown to yield dimers and browning of the medium (*22, 26*). In the presence of polyphenol oxidase (PPO), semiquinone radicles of CAT could be formed, which undergo further coupling forming dimers (*26*). Peroxidases could also facilitate similar reaction. Although we did not measure the enzyme activity directly, the observation that control treatments with embryo removed did not result in significant reduction of CAT concentration points to enzyme-induced degradation of CAT during the seed germination. The UV absorption spectral shift of CAT from 279 to 379 nm (**Figure 4**) was observed in the bioassay medium except for the control (embryo-less seeds). This further suggests the degradation/condensation of CAT in bioassay medium mediated by enzymes. CAT dimerization in bioassay medium was further confirmed by mass spectral analysis.

We further tested two phenolic acids (PRC, FER) in binary systems for modulating the phytotoxicity of CAT in barnyard grass. The presence of both PRC and FER increased the persistence of CAT in bioassay systems (**Figure 6**); however, no increase in toxicity was observed with the binary mixture. CAT in both PRC and FER binary systems produced antagonistic and additive phytotoxic effect (data provided in the Supporting Information). The better stabilization of CAT in binary bioassay systems (e.g., less loss of CAT in CAT + FER treatment, **Figure 6**) could be an indirect effect of the phytotoxicity of the phenolic cosolute.

It was shown that the bioavailabilities of five phenolic acids added to the soil as measured by aqueous extractible fractions are significantly different in the soil matrix. *C. maculosa* litter decomposition study showed the existence of allelochemicals in multicompound mixtures in a soil matrix. All of the phenolic acids exhibited a prolonged residence time when present in a multicompound system. Our study demonstrates the dynamic nature of phenolic acids in soil where the degradation of phenolic compounds results in an extended persistence and bioavailability of simple compounds. Although our study was conducted using exogenous application of allelochemicals, considering the fact that most allelochemicals are present in soil matrix as multicompound mixtures, this finding is of significance. The presence of allelochemicals in mixture could increase the effective concentration of more phytotoxic compounds in soil the matrix, as observed in the persistence of PRC in sandy loam soils. This partly explains how allelochemical mixtures containing lower, nontoxic concentrations of individual compounds could cause phytotoxicity in a soil matrix. Our study clearly shows that it is the polymerization to procyanidins that reduces the persistence and toxicity of CAT, and hence CAT bioactivity may occur under conditions that delay these condensation reactions. Considering the reactive nature of CAT, and the heterogeneity of the soil matrix, the occurrence of such microsites could be limited especially in alkaline invaded ranges of *C. maculoso*. However, considering the dynamic nature of rhizosphere chemical composition, the occurrence of such a stabilizing mechanism could not completely be ruled out. On the basis of the litter decomposition study and the toxicity exhibited by phenolic acids in the bioassay, the phytotoxicity of *C. maculosa*, if any, could be brought about by a complex interaction of its different allelochemicals. Furthermore, the litter decomposition study highlights the importance of using the allelopathic properties of cover crops for weed suppression in agricultural fields.

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Supporting Information Available: Discussion on the reduced phytotoxicity of catechin; degradation dynamics of phenolic allelochemicals, and joint toxic action of catechin with phenolic acids. This material is available free of charge via the Internet at http://pubs.acs.org.

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