



Phenolic acids in plant-soil interactions: a microcosm experiment

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ABSTRACT

In soil naturally occurring phenolic acids act as allelochemicals and are involved in several plant-soil interactions. In the present study, a microcosm-style experiment was used to investigate the allelopathic responses induced by individually applied *trans*-cinnamic and ferulic acids and coumarin on the root system of maize seedlings grown for 288 h (12 days) under controlled conditions on a sterile 70/30 (v/v) soil/perlite mixture. Preliminary soil incubation assays were carried out to assess the potential bioavailability of these compounds as affected by abiotic sorption/desorption processes. The concentration-dependent sorption coefficients (Freundlich) showed that phenolic acids were rapidly adsorbed onto the soil surfaces following two distinctive sorption mechanisms. Further, recoveries from a 96-h depletion experiment indicated that the bioavailable amount of *trans*-cinnamic and ferulic acids markedly (-97 and -94%, respectively) and continuously decreased over time; whereas available coumarin strongly decreased (-92%) soon after addition remaining unchanged over time. According to these findings, single (coumarin) and pulsed (*trans*-cinnamic and ferulic acids) applications were established during the microcosm experiment to provide resident soil concentration at 0.02-0.15 $\mu\text{mol g}^{-1}$ still able to induce plant responses. Total root length, biomass allocation, root fineness and tissue density of the whole root system and different root types (radicle, seminal, nodal, lateral of the radicle and seminal roots) of maize seedlings were evaluated. Coumarin appeared to be the strongest phytotoxic compound reducing total root length and completely inhibiting the elongation of radicle root and the formation of the other root types. *Trans*-cinnamic acid exerted a marked and rapid action in reducing the total root length showing also a selective action towards the different root types more than ferulic acid. Further, *trans*-cinnamic acid affected both biomass allocation and structural parameters; whereas ferulic acid mainly influenced the root fineness. Results suggested: i) a specific response of maize root system in relation to the allelochemical and time of exposure; ii) maize root types showed a different response to the phenolic acids, and iii) their morphological parameters were selectively affected.

Keywords: Allelopathy, phenolic compounds, root morphology, soil sorption, *Zea mays* L.

Introduction

Allelopathy is known to play an important role in the agro-ecosystems as it has a great potential in improving crop productivity, maintaining genetic diversity and providing biological control of weeds and pests. The increased public concern about environmental health combined with the emergence of herbicide-resistant weed species have made allelopathy an attractive non-chemical strategy for weed management in sustainable agriculture (Weston and Duke, 2003; Macias *et al.*, 2007). Indeed, due to their low environmental persistence, high bioactivity and multiple mode of action, allelochemicals can be used directly or as template molecules for the development of new environmentally-friendly herbicides (Duke *et al.*, 2000). In spite of considerable literature appeared on the phytotoxic effects of allelochemicals and their

mechanisms of action on plant metabolism, a little attention has been given to evaluation of allelopathic interactions at the plant-soil interface. In soil the fate of an allelochemical is primarily governed by several biotic (microbial activity) and abiotic (chemistry of colloidal soil particles) processes, which both influence their activity, mobility and persistence (Rice, 1984; Cheng, 1992; Cheng, 1995; Blum, 1996). In fact, to be effective in influencing the plant growth under field conditions, allelochemicals have to enter the soil, escape sorption, survive chemical and biochemical breakdown and eventually come in contact with target plants (Choesin and Boerner, 1991). Beside microbial activity, organic matter and clay minerals provide soil particles with highly chemically-reactive surface areas, which are able to control the availability and the phytotoxic potential of allelochemicals towards the target plants (Leham and Blum, 1999; Blum, 2006; Tharayil *et al.*,

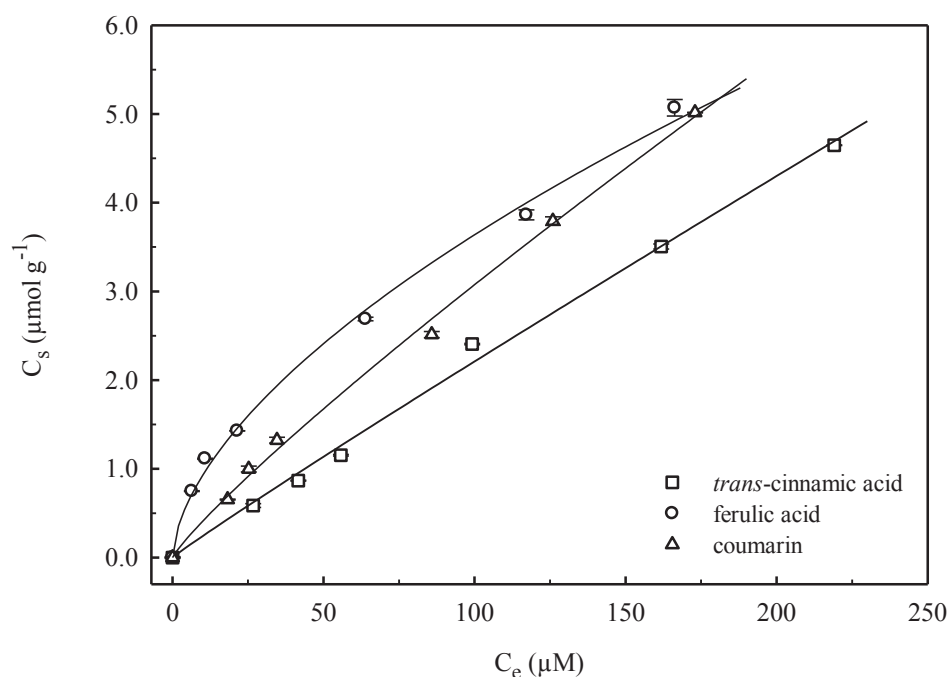


Figure 1. Freundlich adsorption isotherms of *trans*-cinnamic acid, ferulic acid and coumarin on sterile growing substrate. Values are means $\pm$ standard error bars ($n = 3$). Sorption parameters are shown in Table 1.

2006). Allelopathic substances include a range of secondary metabolites such as alkaloids, terpenoids, and phenolics. Among them, phenolic compounds are common and ubiquitous molecules entering the soil through leaching, root exudation and rhizodeposition, litter degradation and microbial synthesis (Bates-Smith, 1956; Harborne, 1998; Blum, 2004 and 2006). Phenols drive complex soil-plant-microbes interactions and they are considered to play a pivotal role in both natural and agricultural ecosystems (Rice, 1984). They inhibit the root growth of many different species (Fujita and Kubo, 2003; Abenavoli *et al.*, 2004), interfere with water and nutrient uptake (Pospíšil *et al.*, 1987; Booker *et al.*, 1992; Yu and Matsui, 1997), and control plant succession in natural ecosystems (Inderjit and Mallik, 2002; Vermerris and Nicholson, 2006; Zeng *et al.*, 2008).

For instance, using different mixtures of plant phenolic acids representative of plant exudates composition, Tharayil *et al.* (2006) confirmed the important role of site-specific soil sorption in influencing the availability and the phytotoxic action of various plant exudates. Within this context it becomes of great interest to investigate how the potential bioavailability of allelopathic compounds is governed by sorption/desorption processes occurring at the soil surface level.

In the present study the soil-plant responses to some phenolic compounds in microcosm-

style experiment were studied. In particular, the present work was addressed to investigate the morphological responses (length, fineness, density) of the root system in maize seedlings grown for 288 h (12 days) under controlled conditions into a sterile soil-based substrate amended with *trans*-cinnamic acid, ferulic acid and coumarin. In order to realistically describe these processes and provide a reliable estimate of the bioavailable amount of these phenolic compounds as derived by abiotic interactions occurring in the soil matrix, equilibrium concentration and persistence of single compounds was previously investigated by using sorption and depletion experiments.

Material and Methods

Chemicals

Three phenolic acids, namely *trans*-cinnamic acid (*trans*-3-phenyl-2-propenoic acid), ferulic acid [3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid] and coumarin (1,2-benzopyrone) of analytical grade (> 99%) were purchased from Sigma Chemical Co. (St. Louis, MO). Aqueous phenolic solutions were purified by passing them through a sterile 0.22 μm-pore cellulose acetate filter (Millex®-GV, Millipore Corporation). All solvents were of HPLC grade.

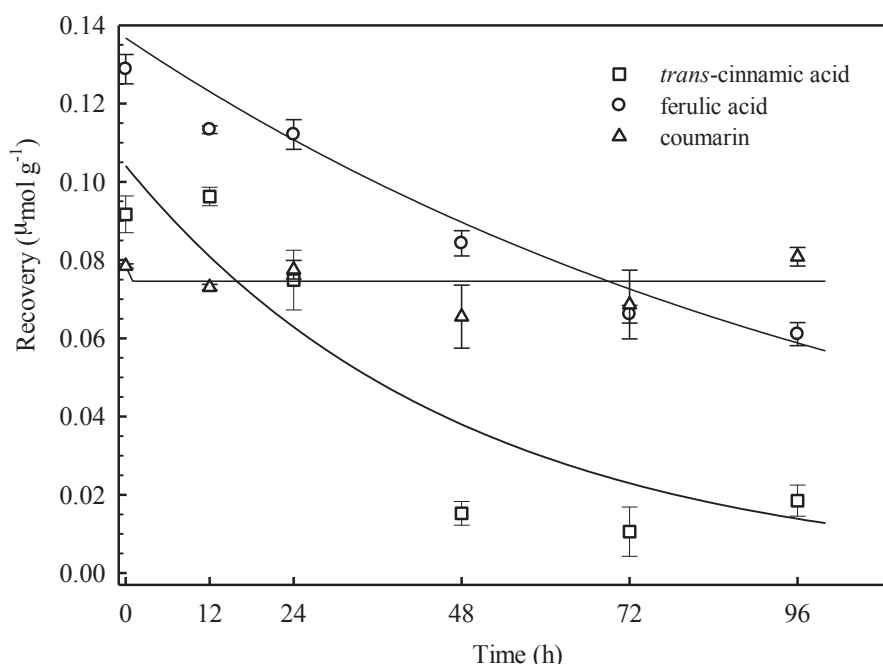


Figure 2. Kinetics of depletion of *trans*-cinnamic acid, ferulic acid and coumarin (added amount was 1 $\mu\text{mol g}^{-1}$ growing substrate) from sterile growing substrate under short-term incubation assays. Recoveries following 0.5 M K_2SO_4 extraction are shown as means $\pm$ standard error bars ($n = 3$) and were fitted to the Eq. (3). Depletion parameters are shown in Table 2.

Soil and growing substrate

The soil used for the microcosm experiment was an arable clay loam soil (sand 34.1%, silt 32.2%, clay 33.7%, $\text{pH}_{\text{H}_2\text{O}}$ 7.2 ± 0.1 , total organic C 19.3 ± 0.4 g kg^{-1} dw soil, total N 1.8 ± 0.2 g kg^{-1} dw soil, C/N ratio 10.7, cation exchange capacity (CEC) 20.9 ± 1.3 $\text{cmol}_{(+)}$ kg^{-1} dw soil, total CaCO_3 8.4 ± 0.5 g kg^{-1} dw soil, electrical conductivity ($\text{EC}_{1:1}$) at 25°C 0.271 ± 0.021 dS m^{-1} , bulk density 1.23 ± 0.04 kg dm^{-3}) freshly collected from the Ap layer (0–15 cm) of an agricultural field located at the Agricultural Experimental Station of the University of Reggio Calabria (South Italy). Soil properties were determined according to the standard methods recommended by the Soil Science Society of America (Sparks, 1996). After collection, the arable soil was air-dried (1 day), coarse sieved at <4 mm particle size, and then thoroughly mixed with commercial perlite (Agrilit[®]3, purchased from Perlite Italiana s.r.l., Milano, I) to prepare a 70/30 (v/v) soil/perlite mixture (growing substrate). Perlite properties were: particle size 2–5 mm, bulk density 100 ± 20 kg m^{-3} , pH 6.5–7.5, CEC 0.79 $\text{cmol}_{(+)}$ kg^{-1} , EC at 25°C 0.02 dS m^{-1} (1 dS $\text{m}^{-1} = 1000$ $\mu\text{S cm}^{-1}$), water content at -1 kPa 26.30%, water availability 12.97%. The growing substrate was autoclaved at 120°C and 1 bar for 20 min, incubated at 25°C for 24 h and autoclaved an additional 20 min, according to Wolf and Skipper (1994). Absence of culturable

microorganisms was confirmed by plating soil dilutions on half-strength nutrient agar.

Sorption experiment

Batch adsorption isotherms on sterile growing substrate were determined at $25 \pm 2^\circ\text{C}$. Samples of 1 g of sterile growing substrate were equilibrated in sterile polyallomer centrifuge tubes with 5 ml of the aqueous phenolic acid solution ranging from 0 to 3.2 mM, consistently with the water solubility of each allelochemical. Tubes were stirred using Teflon-coated magnetic stir bars for 4 h in the dark. Preliminary studies indicated that 95% adsorption occurred within the first 30 min. After the equilibrium was reached, the suspension was centrifuged (5000 g, 5°C , 15 min) and the clear supernatant recovered for HPLC analysis. The pH of aqueous suspensions was also checked. All studies were done in triplicate and included a blank tube (reference) containing the solution without the sterile growing substrate.

Depletion experiment

Depletion of available phenolic acids was determined by incubating the sterile growing substrate with *trans*-cinnamic acid, ferulic acid or coumarin at a fixed concentration (1 $\mu\text{mol g}^{-1}$ growing substrate) under controlled conditions (temperature

25±1°C, relative humidity 75%, day/night 16/8 h, photosynthetically active radiation, "PAR" 350 $\mu\text{mol m}^{-2} \text{s}^{-1}$) for 0, 12, 24, 48, 72 and 96 h. At each sampling time, the growing substrate was extracted with 0.5 M K_2SO_4 (1:4, w/v, soil:solution) under agitation (120 rpm, 60 min) at room temperature and then the soil slurry was filtered through a Whatman® No. 42 filter paper. Clear extracts were stored at -20°C prior to analytical determination. All studies were done in triplicate and included a blank tube (reference) containing the solution without the sterile growing substrate.

Microcosm experiment

The microcosm-scale experiment was carried out in a growth chamber located at the University of Reggio Calabria for 288 h (12 days). The microcosm units consisted of 1.5 L polyvinyl chloride (PVC) pipes (30 cm height x 8 cm i.d.) closed at the bottom by a layer of 35 μm -thick nylon stocking material (Normesh Limited, Lancashire, UK; code NN35/16115R). Before using, the PVC pipes were cleaned carefully and surface sterilized with GNeoSterixidina® (GOLMAR® SpA, I) and then immediately filled with the sterile 70/30 (v/v) soil/perlite mixture (growing substrate). A sterile soil-based substrate was used to give prominence to abiotic sorption processes. The microcosms were randomly set up in a 2 x 2 x 3 experimental block (3-time replicated in the experimental design) with 2 plant treatments (with and without maize plant), 2 soil treatments (with and without phenolic acids) and 3 phenolic acids. At the beginning of the trial (time zero), phenolic acid-amended microcosms were supplied with sterile aqueous solutions of *trans*-cinnamic acid, ferulic acid or coumarin (each at an equivalent concentration of 1 $\mu\text{mol g}^{-1}$ growing substrate). Additional amounts of *trans*-cinnamic acid and ferulic acid (at 1 $\mu\text{mol g}^{-1}$ growing medium) were added at 48, 96 and 192 h. A same amount of sterile distilled water was added to control (no phenolic acid amendment) soil microcosms. Phenolic acids in soil materials were extracted with 0.5 M K_2SO_4 (as seen for the desorption study) at time zero, immediately (30 min) before and after supplementary additions at 48, 96, 192 and then at 288 h to quantify short-term depletion of phenolic acids. Unamended microcosms were monitored accordingly. Pre-germinated maize seeds (*Zea mays* L., var. Cecilia, PR34N84-250, kindly provided by Pioneer HI-Bred Seeds Italy s.r.l., PR, I) (~1 cm root length) were transplanted onto each soil microcosm (1 plant for microcosm) (start of the trial) and let grow for 288 h. Growth chamber conditions were as previously reported in the decay experiment. No nutrients were provided during the growing period.

After 48, 96, 192 and 288 h from transplanting, soil microcosms were destructively sampled and maize plants were gently removed from soil, separated into shoot and root, rinsed with de-ionized water and their fresh weight determined. The root system was stained with 0.1% (w/v) toluidine blue O for 5 min and then scanned (WinRhizo STD 1600, Instruments Régent Inc., Canada) at a resolution of 600 dpi for morphological analyses (root length Lr, root volume Vr). Afterwards, root dry weight (DWr) was determined by oven-drying at 70°C until a constant weight was reached. Root fineness ($\text{Fr} = \text{Lr}/\text{Vr}$) and tissue mass density ($\text{TMDr} = \text{DWr}/\text{Vr}$) were then calculated.

HPLC analysis

Before the chromatographic analysis, a 2-ml aliquot of supernatant was filtered through a sterile 0.22- μm pore cellulose-acetate filter (Millex®-GV, Millipore Corporation, MA) and then a 1-ml aliquot of the filtrate was further purified by passing it through a Waters Sep-Pak® classic C_{18} cartridge (Millipore Corporation). The high-performance liquid chromatography (HPLC) analysis was carried out using a PerkinElmer LC 250 binary pump (Norwalk, CT) equipped with a Rheodyne 7125 injector (20- μl loop), a 4.0 x 3.0 mm SecurityGuard™ Max-RP (C_{12}) cartridge (Phenomenex®, CA) and a 250 x 4.6 mm i.d. Synergi™ Hydro-RP C_{12} (4 μm) analytical column (Phenomenex®). The mobile phase (0.7 ml min^{-1} flow rate) was acetonitrile/acetic acid/water (20:1:79 by vol) for the ferulic acid elution and acetonitrile/acetic acid/water (15:1:84 by vol) for the *trans*-cinnamic acid and coumarin elution. A PerkinElmer LC 135 diode array detector operating at 275 nm (*trans*-cinnamic acid and coumarin) or at 325 nm (ferulic acid) was used for analytical detection. Identification and processing of the chromatographic data was done by a PerkinElmer Omega-2 Analytical Workstation.

Data analysis

Data were firstly tested for deviation from normality (Kolmogorov-Smirnov test) and homogeneity of within-group variances (Levene's test) (Brown and Forsythe, 1974). After running a one-way ANOVA to check any significant effect of the treatment on the variability of the data (the block effect in the experimental design was found to be not significant at $P < 0.05$), multiple pairwise comparison of means was done by Tukey's HSD (Honestly Significant Difference) test at $P < 0.05$ level of significance.

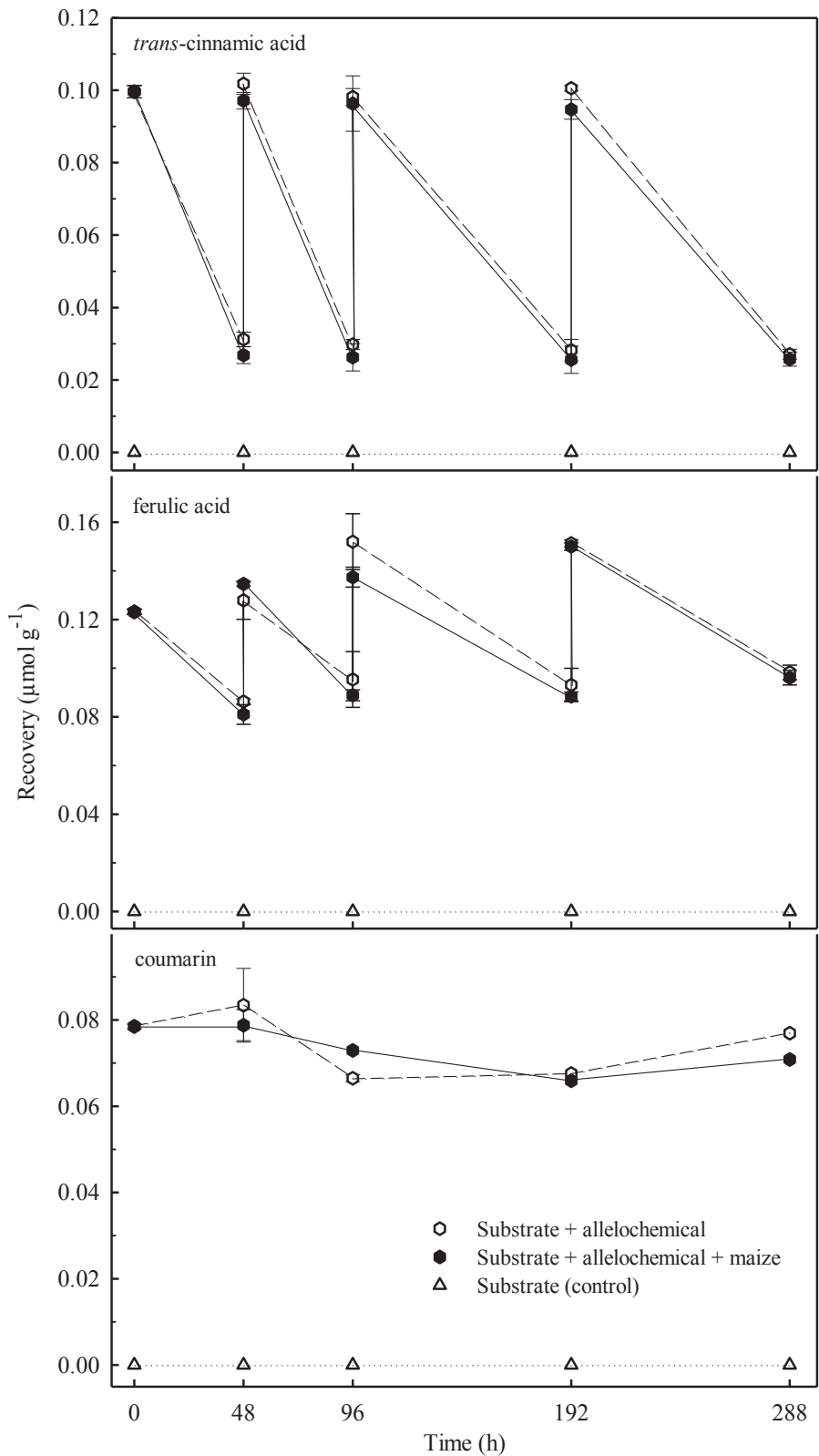


Figure 3. Changes in available amount of *trans*-cinnamic acid, ferulic acid and coumarin in soil microcosms following initial (time zero) addition (1 μmol g⁻¹ growing substrate). Supplementary additions (1 μmol g⁻¹ growing substrate) of *trans*-cinnamic and ferulic acids were done after 48, 96 and 192 h. Recoveries were determined by 0.5 M K₂SO₄ extraction 30 min before and after 48, 96 and 192 h, followed by a final sampling after 288 h. Values are means ± standard error bars (*n* = 3).

The extent of allelochemical sorption on sterile growing substrate was estimated as the difference between initial and final concentration in the solution. Sorption data were fitted to the Freundlich equation $C_s = K_f C_e^{1/n}$ (Eq. 1), where the amount adsorbed per mass of sorbent (C_s , in $\mu\text{mol g}^{-1}$) is plotted *vs* the equilibrium concentration (C_e , in mM). K_f and $1/n$ are empirical constants describing the sorption capacity and sorption intensity, respectively. The distribution coefficient $K_d = C_s/C_e$ was also calculated at $C_e=10 \mu\text{M}$ level.

Recoveries of allelochemical from the sterile growing substrate were used to draw a time-dependent curve showing depletion of phenolic acid from the autoclaved substrate. Depletion kinetics was described according to a mathematical model representing a non-linear, first order equilibrium equation:

$$-\frac{dC}{dt} = k_{ad} \cdot C_t - k_{des} \cdot (A_0 - C_t) \quad (\text{Eq. 2})$$

where A_0 is the initial concentration of each compound; C_t is the recovered amount at time t ; k_{ad} and k_{des} are, respectively, the forward (adsorption process) and reverse (desorption process) rate constants. The Eq. 2 may be written in integrated form as the exponential function:

$$f(t) = \frac{A_0 \cdot (k_{des} + k_{ad} \cdot \exp(-t \cdot (k_{ad} + k_{des})))}{(k_{ad} + k_{des})} \quad (\text{Eq. 3})$$

where $f(t)$ represents the concentration of the allelochemical, in $\mu\text{mol g}^{-1}$ of substrate, in response to the incubation time t , in hours; A_0 , k_{ad} and k_{des} are same as above. Eq. 3 could also be used to describe the phenolic acid depletion as a consequence of adsorption/desorption equilibrium processes occurring at the solid-liquid interface. After a certain period of time, this decline reaches an equilibrium concentration (C_{eq}) that can be estimated according to the following equation $C_{eq}=A_0 \cdot (k_{des}/(k_{ad}+k_{des}))$ (Eq. 4), which is derived from Eq. (3) when longer times are being considered. The best fit of Eq. 1, 2 and 3 to analytical data, and estimates of empirical parameters (K_f , $1/n$, A_0 , k_{ad} , k_{des} and C_{eq}) were carried out through non-linear regression analysis using the algorithm of Levenburg-Marquardt (TableCurve 2D v.5.01 software, SYSTAT Software Inc.). Non-linear regressions were repeated several times in order to minimize the sum of the square of deviation between the predicted and experimental values to less than 0.01% between two consecutive fits. The quality of curve fitting was assessed by F test for non-fit based on analysis of variance at $P < 0.05$. Graphs were drawn by using the SigmaPlot v.10.0 (SYSTAT Software Inc.).

Results and Discussion

Adsorption dynamics of phenolic acids

Sorption isotherms of *trans*-cinnamic and ferulic acids and coumarin showed a different binding mechanism on the sterile growing substrate (Fig. 1). According to the classification proposed by Giles *et al.* (1974), *trans*-cinnamic acid and coumarin isotherms were of C-type, which describes a constant partition of the binding molecule between the aqueous solution and the adsorption sites of the growing substrate. Linearity shows that the number of sites for adsorption remains constant throughout the whole range of solute concentration up to saturation of the substrate. This finding suggests that the more the substrate is being added, the more the sorption can take place. On the other hand, the ferulic acid sorption isotherm was of L-type (Fig. 1). This indicates that sorption becomes increasingly difficult for a bombarding molecule substrate and the affinity between the molecule and the adsorbent surface decreases as more solute is being adsorbed. The occurrence of these two distinctive mechanisms of allelochemical adsorption on the sterile growing substrate was further confirmed by the empirical sorption parameters K_f (capacity) and $1/n$ (intensity), which have been estimated by fitting the empirical Freundlich relationship (Eq. 1) to sorption data. In fact, $1/n$ was close to 1 for *trans*-cinnamic acid and coumarin, and this indicates a sorption curve with steady slope close to linearity (Table 1). Whereas in the experiments with ferulic acid $1/n$ deviated considerably from linearity, thus confirming a concentration-dependent sorption dynamics (Table 1). The sorption affinity coefficient K_f was rather low in all cases and the general order of sorption magnitude was ferulic acid > coumarin > *trans*-cinnamic acid. The distribution coefficient K_d ranked the allelochemicals with the same sorption affinity (Table 1). The occurrence of dynamic sorption/desorption processes represents a key soil factor affecting the environmental fate (i.e. mobility, persistence, etc) and the phytotoxic activity of allelochemicals in soil (Kobayashi, 2004). In fact, soil surfaces are able to adsorb phenolic acids through several different sorption mechanisms involving either the mineral or the organic fractions (Dalton *et al.*, 1989; Blum *et al.*, 1999; Ohno, 2001). In general, such an order of sorption affinity on soil surfaces as observed here (ferulic acid > coumarin > *trans*-cinnamic acid) has previously been reported (Tharayil *et al.*, 2006); and the greater adsorption of ferulic acid was related to the methoxy substitution on the aromatic ring (Dalton *et al.*, 1989). Moreover, the adsorption of *trans*-cinnamic acid and,

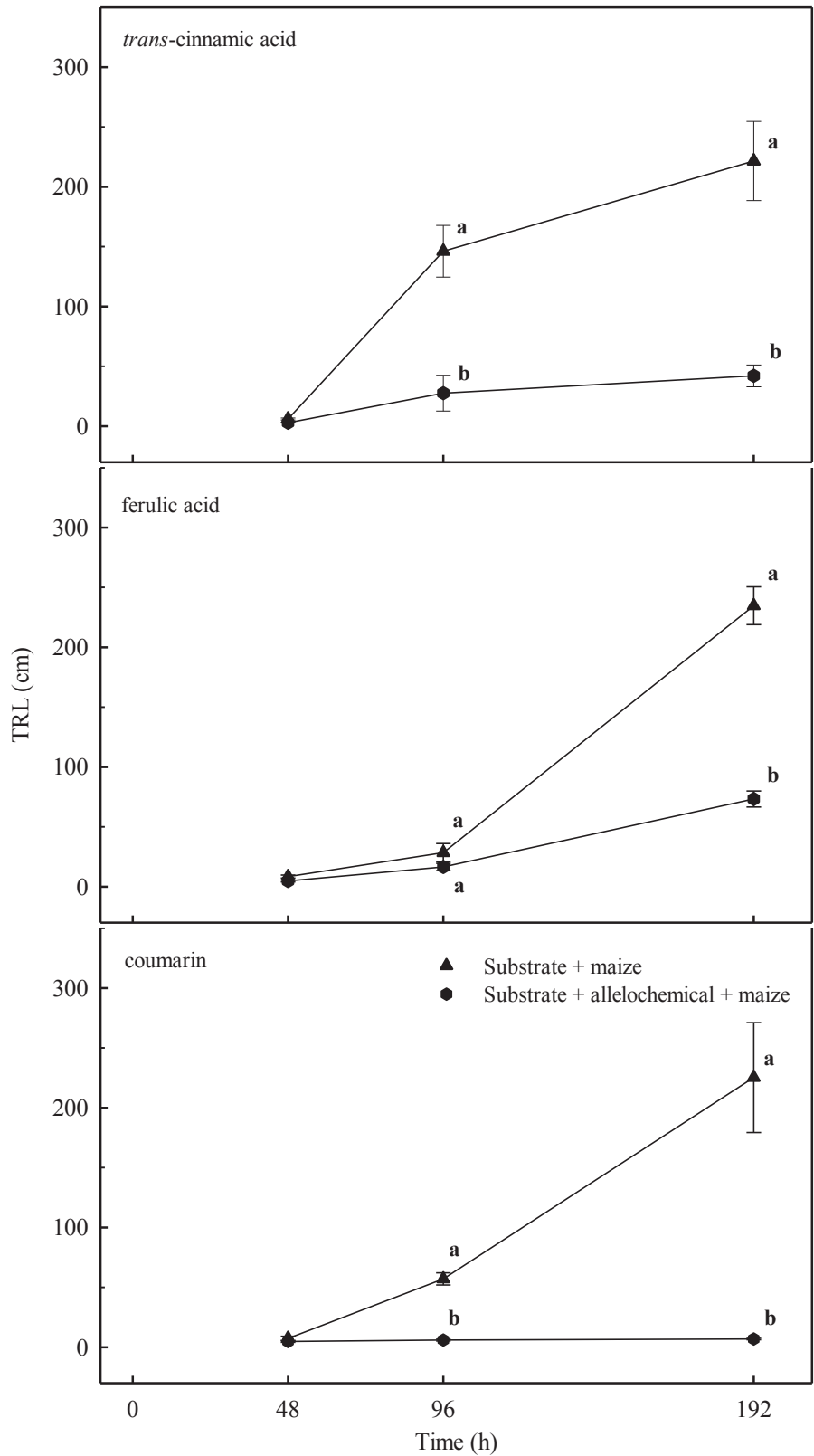


Figure 4. Changes in total root length (TRL) of maize seedling roots as induced by *trans*-cinnamic acid, ferulic acid and coumarin at three sampling times (48, 96 and 192 h) during the microcosm experiment. Values are means $\pm$ standard error bars ($n = 3$). At each sampling time different letters indicate significant differences between treatments (Tukey's test, $P < 0.05$).

Table 1. Freundlich parameters (K_f and $1/n$) and distribution coefficient (K_d) for the adsorption of *trans*-cinnamic acid, ferulic acid and coumarin on sterile 70/30 (v/v) clay arable loam soil/perlite. The coefficient of determination (r^2) and the significance level (as F -stat) for all curves are also shown.

Phenolic acid	K_f	$1/n$	r^2	F -stat [#]	K_d [§]
<i>trans</i> -cinnamic acid	0.03	0.96	0.99	2677.25***	0.02
ferulic acid	0.24	0.59	0.99	1644.32***	0.09
coumarin	0.05	0.88	0.99	2088.71***	0.04

[#] level of significance: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

[§] referred to a $C_e = 10 \mu\text{M}$.

at greater extent, coumarin could be explained by the hydrophobicity of the two molecules, which determines their binding affinity with the organic material. It also true that ionic interactions are also important. It was found that type and concentration of the added phenolic acids affected selectively the pH of the soil slurry. The addition of ferulic and *trans*-cinnamic acids caused a significant dose-response decline of the pH, which constantly decreased from 7.6 (control, 0 mM treatment) to approx 7.0 (3.2 mM treatment). Conversely, coumarin addition did not vary the pH of the soil slurry, which remained stable at 7.6 in all treatments. Because the pK_a value of ferulic and *trans*-cinnamic acids is somewhat lower (4.7) than the pH of the slurry (~7.0), this finding indicates that during the sorption experiment they both occur as phenolic acid anions. On the contrary, coumarin has no hydrolizable functional groups and therefore remains unchanged under the experimental conditions. Then, anionic exchange reactions cannot be invoked as a significant mechanism explaining the observed sorption magnitude between the allelochemicals and soil substrate surfaces. Since microorganisms or soil enzymes were excluded from the growing mixture by repeated sterilization, the decrease in recovery should be primarily attributable to an abiotic process due to physicochemical interactions with colloidal soil surfaces.

Depletion dynamics

Losses of *trans*-cinnamic acid, ferulic acid and coumarin from the growing mixture varied considerably with time and the particular type of allelochemical added (Fig. 2). In fact, the $K_2\text{SO}_4$ -extractable amount of *trans*-cinnamic acid and ferulic acid markedly decreased during the 96-h incubation assay and final recoveries were considerably low: -97 and -94%, respectively. Conversely, the amount of $K_2\text{SO}_4$ -extractable coumarin strongly decreased (-92%) soon after addition but then remained fairly unchanged over time (Fig. 2). The mathematical model of Eq. 3 was applied to the recovery data in order to describe the concentration decline of these

two cinnamic acids as a consequence of adsorption/desorption equilibrium processes occurring at the solid-liquid interfaces. If so, k_f and k_r would indicate the rate kinetic constants for the adsorption (k_{ad}) and desorption (k_{des}) process, respectively. After a certain period of time when the rate of adsorption equals the rate of desorption, the soluble concentration has reached a dynamic equilibrium (Fig. 2) that may be calculated using the kinetic parameters estimated according to the Eq. 3 (Table 2). Estimates of A_0 indicate that a major amount of the initially added phenolic compound is rapidly depleted from soil solution: i.e. 86 and 90% for ferulic and *trans*-cinnamic acids, respectively (Table 2). This finding is in line with what has previously been reported by Blum (1998) and Blum and Shafer (1988), who also used autoclaved soil systems to study phenolic acid interactions in soil. Nevertheless, occurring of complex action mechanisms including adsorption or oxidation processes, particularly acting during early stages (Dalton *et al.*, 1989; Cecchi *et al.*, 2004), or microbial utilization, cannot be ignored. Although a sterile soil-based substrate has been used to give prominence to abiotic processes occurring at the soil interface, the contribution of soil microbes to the allelochemical breakdown during the recolonization process could not been excluded. However, this contribution was equally acting on all microcosms with no difference among treatments. This assumption could help explain the different hierarchy of allelochemical availability observed in the sorption isotherm and in the depletion experiment. It is also worth noting that at longer times the bioavailable amount of ferulic and *trans*-cinnamics acid in soil solution reaches an equilibrium concentration that is consistent with what observed in other soil systems (Martens, 2002). However, the observed values are well below the $0.1 \mu\text{mol g}^{-1}$ soil threshold concentration needed to cause an appreciable allelopathic activity in soil (Blum *et al.*, 2000). Interestingly, even though 92% coumarin added to soil was rapidly retained by absorbing surfaces, then the available amount in soil solution had remained unchanged over time. Nevertheless, coumarin is fairly recalcitrant to microbial breakdown

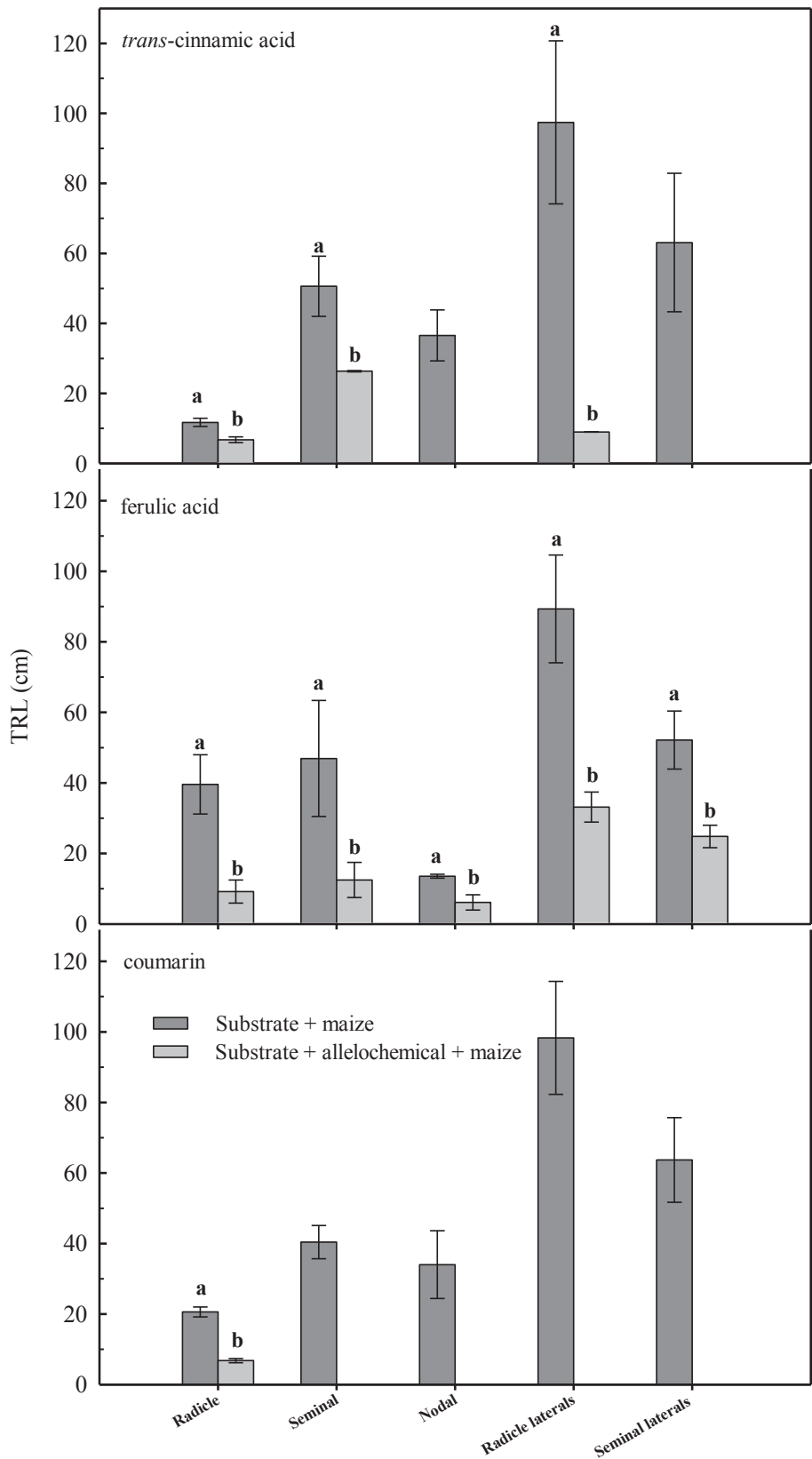


Figure 5. Changes in length of different root types of maize seedlings as induced by *trans*-cinnamic acid, ferulic acid and coumarin at the 192-h sampling time during the microcosm experiment. Values are means $\pm$ standard error bars ($n = 3$). Within each root type, different letters indicate significant differences between treatments (Tukey's test, $P < 0.05$).

Table 2. Parameters related to the depletion dynamics of *trans*-cinnamic acid, ferulic acid and coumarin from the sterile growing substrate as estimated according to the non-linear, first order equilibrium equation of Eq. 3.

<i>trans</i> -cinnamic acid	0.10	0.021	0.001	0.79	24.28***	0.005
ferulic acid	0.14	0.010	0.007	0.87	42.47***	0.061
coumarin	0.08	0.260	5.061	0.12	0.36	0.071

§ estimated concentration at time 0, in $\mu\text{mol g}^{-1}$ growing substrate.
* in h^{-1} .
level of significance: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.
† estimated according to the relationship $C_{\text{eq}} = A_0 (k_{\text{des}} / (k_{\text{ad}} + k_{\text{des}}))$ Eq (4), in $\mu\text{mol g}^{-1}$ growing substrate.

and long persistence in soil environment has been reported (Kunc, 1974). This finding leads to the conclusion that the allelopathic potential of coumarin in soil remains stable over time after an equilibrium has been reached. To sum up, the following hierarchy of allelochemical availability can be inferred from the depletion kinetic parameters: coumarin > ferulic acid > *trans*-cinnamic acid. Differences from what it could be predicted by the sorption isotherm parameters are likely due to the prolonged incubation times used during the assay.

Microcosm experiment: recovery of K₂SO₄-extractable allelochemicals from the sterile growing substrate after repeated additions

The amount of K₂SO₄-extractable phenolic compound occurring in amended soil microcosms was strongly dependent on the allelochemical and was considerably lower than the amount totally added. In particular, the bioavailable amount of *trans*-cinnamic and ferulic acids recovered soon after the initial addition was fairly consistent with what observed in the sorption experiment: the amount of adsorbed *trans*-cinnamic was always greater than that of ferulic acid (Fig. 2; Table 2). Despite the total addition of approx 4.0 $\mu\text{mol trans-cinnamic acid g}^{-1}$, the amount recovered had never exceeded 0.102 $\mu\text{mol g}^{-1}$, the lowest value was 0.026 $\mu\text{mol g}^{-1}$ and the mean value was 0.063 $\mu\text{mol g}^{-1}$ (~1.6% of total amount added) (Fig. 3). Whereas, the amount of ferulic acid recovered slightly increased over time without exceeding 0.152 $\mu\text{mol g}^{-1}$, the lowest value was 0.081 $\mu\text{mol g}^{-1}$ and the mean value was 0.114 $\mu\text{mol g}^{-1}$ (~2.8% of total amount added) (Fig. 3). Coumarin showed a completely different trend. Even though the available amount strongly decreased soon after addition (-92%, as could be predicted according to the depletion experiment, Fig. 2 and Table 2), then the amount recovered remained constant over time at 0.074 $\mu\text{mol g}^{-1}$, which corresponds to 7.4% of the totally added amount (Fig. 3). This finding further confirms that coumarin is a rather stable molecule

in soil and the available amount remains almost unchanged after the sorption/desorption processes have reached a dynamic equilibrium. Because *trans*-cinnamic and ferulic acids were rapidly depleted from soil solution by sorption processes or abiotic surface reactions, additional amounts of these two compounds were added to the soil system to maintain a consistent range of soil solution concentrations over the experimental period. The experimental approach with pulsed applications was already reported in previous studies (Blum and Gerig, 2006; Inderjit *et al.*, 2008). During the microcosm experiment the resident soil concentration of each allelochemical ranged between 0.06 and 0.15 $\mu\text{mol g}^{-1}$ for ferulic acid and coumarin, respectively; while a 0.02-0.10 $\mu\text{mol g}^{-1}$ range was found for *trans*-cinnamic acid. Even though these values appear rather low when compared to similar studies (Djurdjevic *et al.*, 2004; Qu and Wang, 2008), they can be considered still able to determine allelopathic interactions at the soil-plant interface (Blum, 1998; Martens, 2002; Blum and Gerig, 2006). Lastly, no detectable amount of *trans*-cinnamic and ferulic acids or coumarin was found in unamended (control) soil (Fig. 3).

Microcosm experiment: effects of the allelochemicals on maize root system

The most frequently observed effect of allelochemicals, and in particular of phenolic compounds, on plants is the inhibition of root growth (Rice, 1984). *Trans*-cinnamic acid and structurally related compounds such as ferulic, caffeic and *p*-coumaric acids are able to inhibit the root growth in many plant species (Fujita and Kubo, 2003). Also coumarins, which are lactones of *o*-hydroxycinnamic acid, have been shown to strongly affect both root morphology and function (Svensson, 1971; Aliotta *et al.*, 1993; Kupidowska *et al.*, 1994; Abenavoli *et al.*, 2001 and 2004).

The total root length of maize seedlings was strongly affected by exposition to *trans*-cinnamic and ferulic acids and coumarin, and root responses

Table 3. Changes in morphological and structural parameters of maize root seedlings as induced by *trans*-cinnamic acid, ferulic acid and coumarin at 192-h sampling during the microcosm experiment. Within each root type, different letters indicate significant differences between treatments (Tukey's test, $P < 0.05$).

Allelochemical	Treatment	DWr [§]	Fr [‡]	TMDr [#]
<i>trans</i> -Cinnamic acid				
Radicle	Substrate	0.0150 ^a	247.90 ^a	0.2288 ^a
	Substr + allelol	0.0064 ^b	174.30 ^b	0.1610 ^b
Radicle laterals	Substrate	0.0027 ^a	1084.44 ^a	0.0330 ^a
	Substr + allelol	0.0003 ^b	288.71 ^b	0.0089 ^b
Seminal	Substrate	0.0084 ^a	416.51 ^a	0.0694 ^a
	Substr + allelol	0.0028 ^b	369.88 ^a	0.0471 ^a
Seminal laterals	Substrate	- [§]	-	-
	Substr + allelol	-	-	-
Nodal	Substrate	-	-	-
	Substr + allelol	-	-	-
Ferulic acid				
Radicle	Substrate	0.0155 ^a	231.54 ^a	0.0746 ^a
	Substr + allelol	0.0067 ^a	113.08 ^b	0.0969 ^a
Radicle laterals	Substrate	0.0047 ^a	817.69 ^a	0.0430 ^a
	Substr + allelol	0.0029 ^a	600.55 ^a	0.0526 ^a
Seminal	Substrate	0.0092 ^a	224.47 ^a	0.2242 ^a
	Substr + allelol	0.0064 ^b	232.17 ^a	0.2256 ^a
Seminal laterals	Substrate	0.0031 ^a	802.84 ^a	0.0476 ^a
	Substr + allelol	0.0024 ^a	215.03 ^b	0.0486 ^a
Nodal	Substrate	0.0020 ^a	677.75 ^a	0.1570 ^a
	Substr + allelol	0.0009 ^b	425.76 ^a	0.1356 ^a
Coumarin				
Radicle	Substrate	0.0177 ^a	137.35 ^a	0.0530 ^a
	Substr + allelol	0.0132 ^b	139.22 ^a	0.1091 ^a
Radicle laterals	Substrate	-	-	-
	Substr + allelol	-	-	-
Seminal	Substrate	-	-	-
	Substr + allelol	-	-	-
Seminal laterals	Substrate	-	-	-
	Substr + allelol	-	-	-
Nodal	Substrate	-	-	-
	Substr + allelol	-	-	-

[§] root dry weight, in g; [‡] root fineness, in cm cm⁻³; [#] root tissue mass density, in g cm⁻³;

[§] not revealed

varied with time and the allelochemical considered (Fig. 4). In details, *trans*-cinnamic acid and coumarin markedly inhibited the root elongation already after 96 hours of exposure, causing a reduction by 81 and 89%, respectively. The inhibitory activity of *trans*-cinnamic acid remained fairly constant over the following 96 hours, whereas a 97%-inhibition of root growth was reached in coumarin-treated microcosms (Fig. 4). After 288 hours of exposure, either compounds had completely inhibited the root growth (*data not shown*). Conversely, in ferulic acid-treated microcosms a significant reduction

(-69%) of the root growth has been noticed after 192 h of exposure (Fig. 4), and this effect has been maintained over the whole time course (*data not shown*). Coumarin appeared to be the most phytotoxic compound followed by *trans*-cinnamic and ferulic acids. The different phytotoxic activity of tested allelochemicals can be attributable to the chemical structure of the compounds. It is well known that the occurrence of a substituent on the aromatic ring may change the potential phytotoxicity of allelochemicals. For example, hydroxylation decreased the inhibitory activity of *trans*-cinnamic on ion uptake (Yu and

Matsui, 1997), while reduced the negative effect of coumarin on root elongation (Kupidlovska *et al.*, 1994). Furthermore, the position and/or the isomerization of the substitution group defined allelopathic activity. Indeed, *cis*-cinnamic acid was ten times more active than *trans*-cinnamic acid in root growth inhibition of *Arabidopsis thaliana* (Wong *et al.*, 2005). Finally, the lactonization increased the inhibitory effect, *i.e.* coumarin was a stronger inhibitor on the germination compared to *trans*-cinnamic acid (Reynolds, 1989). According to Glass and Dunlop (1974), the results indicated that the presence of a hydroxyl group reduced the inhibition activity of the compounds, as observed with ferulic acid, while in disagreement with Maffei (1999), the methoxyl substituent seemed to play a minor role in defining the allelochemical phytotoxicity. Indeed, ferulic acid was less inhibitory of *trans*-cinnamic acid. Furthermore, in agreement with Reynolds (1989), coumarin, derived through the process of lactonization of *trans*-cinnamic acid, showed a greater inhibitory activity compared to its linear chain precursor. The strong inhibitory activity of coumarin respect to other simple phenolic compounds has already been reported (Kupidlovska *et al.*, 1994).

Several hypotheses have been drawn to explain the inhibition of the root elongation process induced by these allelochemicals. *trans*-Cinnamic acid may block the pumping of protons mediated by the H⁺-ATPase of the plasma membrane, resulting in a reduction of the internal pH (Fujita and Kubo, 2003). Coumarin is supposed to act through several action mechanisms: interference with the auxin metabolism through the activation of peroxidase and IAA oxidase activities (Goren and Tomer, 1971), changes in the distribution of micro-fibrils in the radial direction (Svensson, 1971), alteration of the mitotic process and/or the cell cycle (Kupidlovska *et al.*, 1994).

However, the data on the whole root system provided no information on the specific responses of different root types to each allelochemical. In fact, root systems are composed of several root types, each characterized by different anatomical, morphological, and physiological features that make it able to differentially respond under stress conditions (Waisel and Eshel, 2002). Abenavoli *et al.* (2008) demonstrated a selective and species-specific effect of coumarin on single root types: in *Arabidopsis* coumarin inhibited the primary root length whereas stimulated the lateral root formation. By contrast, in *Zea mays* coumarin inhibited the seminal and nodal root growth and left unchanged the primary root (Abenavoli *et al.*, 2004). Maize represents a useful model plant to study the effects of allelochemicals on root system, since its complex root system is characterized by

radicle, seminal, and nodal roots, differing in origin, development, physiology, and anatomy (McCully, 1999). To better understand the effects of *trans*-cinnamic acid, ferulic acid and coumarin on maize root system, morphological responses of different root types (*i.e.* radicle, seminal, nodal roots together with their laterals) were monitored. A 192 h exposure to *trans*-cinnamic acid was long enough to significantly inhibit the elongation process of radicle, lateral radicle and seminal roots; whereas the formation of nodal and the seminal lateral roots was completely inhibited (Fig. 5). Conversely, amendment with ferulic acid determined an even reduction (ranging from 50 to 70% inhibition *vs* control) in the elongation of all root types (Fig. 5). Finally, in coumarin-treated microcosms the radicle root elongation was markedly inhibited whereas no other root types were found after 192 h exposure. Root length readings confirmed the different sensitivity of maize root types to allelochemicals suggesting the following order of response: nodal > seminal > radicle roots, as previously reported by Abenavoli *et al.* (2004).

Since root length is known to be dependent on both biomass allocation (dry weight) and structural parameters (fineness and tissue density), they were also evaluated. In fact, plants can produce longer roots either by increasing the allocation of biomass towards the root or by increasing the fineness (Fr) and/or by reducing the density of tissue (TMDr). After 192 h of exposure, *trans*-cinnamic acid significantly reduced the root structural parameters: that is dry weight of the radicle and both fineness and tissue density (Table 3). In addition, the dry weight of the seminal root was affected, whereas structural parameters remained unchanged. Ferulic acid inhibited the fineness of radicle whereas the biomass allocation (dry weight and tissue density) was not affected (Table 3). Conversely, biomass allocation of seminal roots was reduced whereas the two structural parameters were not modified. On the other hand, the reduction of nodal roots may be attributable to the variation of the tissue density. In lateral roots of the seminal, fineness was sensitive to ferulic acid showing a significant inhibition. Finally, coumarin showed a reduction of biomass allocation (dry weight), but not in the structural parameters (Table 3). Therefore, plants may produce longer roots either by increasing biomass allocation to the roots, as also demonstrated under a low supply of nitrogen (Ryser and Lambers, 1995; Sorgonà *et al.*, 2005), or by increasing the root fineness and/or reducing root tissue density, thus leaving the biomass allocation unchanged (Ryser, 1998).

After 192 h of exposure, *trans*-cinnamic acid significantly reduced the radicle dry weight, and both fineness and tissue density, structural parameters.

In addition, the dry weight of the seminal root was affected, whereas structural parameters were left unaffected (Table 3). Ferulic acid inhibited the fineness of radicle leaving unchanged the biomass allocation (dry weight) and tissue density. Conversely, biomass allocation of seminal roots was reduced whereas the two structural parameters were not modified. On the other hand, the reduction of nodal roots after contact with ferulic acid may be attributable to tissue density variation. In lateral seminal roots, fineness was sensitive to ferulic acid showing a significant inhibition (Table 3). Finally, coumarin showed a reduction of biomass allocation (dry weight), but not in the structural parameters (Table 3).

Taken together these results indicate that each root type may adopt a different strategy in response to allelochemicals both in terms of biomass allocation and/or fineness and tissue density. Hence, the question of which 'morphological component' among biomass allocation and/or structural parameters mainly contributes to the variation in root length of each root order in response to allelochemicals still remains open.

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