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Humic Substances Can Modulate the Allelopathic Potential of Caffeic, Ferulic, and Salicylic Acids for Seedlings of Lettuce (*Lactuca sativa* L.) and Tomato (*Lycopersicon esculentum* Mill.)

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The capacity of a leonardite humic acid (LHA), a soil humic acid (SHA), and a soil fulvic acid (SFA) in modulating the allelopathic potential of caffeic acid (CA), ferulic acid (FA), and salicylic acid (SA) on seedlings of lettuce (*Lactuca sativa* L.) and tomato (*Lycopersicon esculentum* Mill.) was investigated. Lettuce showed a sensitivity greater than that of tomato to CA, FA, and SA phytotoxicity, which was significantly reduced or even suppressed in the presence of SHA or SFA, especially at the highest dose, but not LHA. In general, SFA was slightly more active than SHA, and the efficiency of the action depended on their concentration, the plant species and the organ examined, and the allelochemical. The daily measured residual concentration of CA and FA decreased drastically and that of SA slightly in the presence of germinating seeds of lettuce, which were thus able to absorb and/or enhance the degradation of CA and FA. The adsorption capacity of SHA for the three allelochemicals was small and decreased in the order FA > CA > SA, thus suggesting that adsorption could be a relevant mechanism, but not the only one, involved in the "antiallelopathic" action.

KEYWORDS: Humic acid; fulvic acid; allelopathic potential; allelochemicals; caffeic acid; ferulic acid; salicylic acid; lettuce; *Lactuca sativa* L.; tomato; *Lycopersicon esculentum* Mill.; seed germination; plant early growth; adsorption kinetics; adsorption isotherm

INTRODUCTION

Humic substances (HS), which include two main fractions, humic acids and fulvic acids, are the most important components of nonliving soil organic matter and are known to exert, among several others, important morphological, physiological, and biochemical effects on seed germination and plant growth (*1*−*6*), including a genetic action (*7*). Ample evidence exists that low molecular weight HS can be taken up by plants and incorporated in plant tissues, where they can interfere directly with several metabolic processes (*3*−*6*). The stimulating or inhibiting effects of HS on plant growth are ascertained to depend on their origin, nature, and concentration and the possible interactions with several chemical species of different nature and biological function, such as pesticides or plant mutagens, present in the germination and growth medium (*7*−*9*). Despite efforts made to relate HS structure, functionalities, and molecular weight to their biological effects on higher plants (*6*, *7*, *10*), the mechanisms by which HS exert these effects are still scarcely known. Apparently, HS molecules or fractions rich in carboxylic groups and of small molecular size are the most biologically active (*6*, *7*, *10*).

"Allelopathy" is a phenomenon that occurs in the vicinity of plant roots, that is, the rhizosphere, and consists of the stimulation or inhibition of growth, development, and reproduction of plants, microorganisms, and animals. The allelopathic activity is principally ascribed to secondary products of plant metabolism of wide chemical nature, including phenolic acids, called "allelochemicals" (*11*, *12*), which can be identified in plant extracts, root exudates and litter leachates of wild and cultivated tree and herbaceous species. Nowadays, worldwide interest is increasing on the possible use of allelochemical compounds as selective biopesticides and/or as leads for the formulation of new synthetic herbicides and agrochemicals (*12*). Allelochemicals can act in plant organisms through a variety of physiological and biochemical mechanisms, including inhibition of mitotic activity in roots and shoots, ion uptake, photosynthesis, respiration, and protein formation and decrease of cell membrane permeability (*11*). In particular, some derivatives of benzoic and cinnamic acids have been shown to alter ion uptake, leaf stomatal conductance and transpiration, and net assimilation rate and to induce oxidative cell damage (*13*, *14*). Among cinnamic acid derivatives, caffeic acid and ferulic acid identified in plant tissues and root exudates of tomato (*15*), cucumber (*16*), alfalfa (*17*), rice (*18*), and wild oat (*19*) can induce phytotoxic effects on tomato (*14*), lettuce, and cucumber (*16*). Among benzoic acid derivatives, salicylic acid, which is present in the roots and green tissues of several plants and has

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been identified in root exudates of cucumber (*16*), alfalfa (*17*), and rice (*18*), behaves as a phytotoxin for lettuce and cucumber (*16*).

Allelochemicals released into the rhizosphere compartment are expected to interact to various extents with soil mineral and organic components, which may thus influence directly and/or indirectly their fate in the soil−plant system, including adsorption and desorption, degradation, and bioavailability. Sorption of various allelopathic phenolic acids onto soil particles is shown to be related to the sorbate structure and to increase with increasing allelochemical concentration following a Freundlich model, whereas the amount sorbed is correlated to different soil properties (*20*−*22*). In particular, soil HS are able to adsorb a wide variety of organic chemicals by various molecular mechanisms (*23*) and to exert a protection activity on seed germination and plant growth from various phytotoxic compounds (*7*−*9*). On these bases, a role of HS can be feasibly hypothesized in the regulation of plant allelopathy. However, no information is available in the current literature on this topic.

The main objectives of this study were thus to evaluate (i) the capacity of three HS samples used at two concentrations in modulating the effects of three ascertained allelopathic compounds on the germination of lettuce and tomato and (ii) adsorption as the possible mechanism responsible for HS activity on the allelopathic effects of the three allelochemicals.

MATERIALS AND METHODS

Humic Substances, Allelochemicals, and Plant Species. The three HS samples used in this work belong to the Standard and Reference Collection of Humic and Fulvic Acids of the International Humic Substances Society (IHSS). These are (a) the leonardite humic acid (LHA) isolated from a leonardite originated from the Gascoyne Mine in Bowman County, North Dakota, and (b) the soil standard humic acid (SHA) and fulvic acid (SFA) isolated from a soil near Joliet, IL. The HS samples were obtained by IHSS in homogenized, freeze-dried H^+ form (*24*, *25*).

The three allelochemicals used in this work, caffeic acid (CA) (3,4-dihydroxycinnamic acid; purity ≥ 97%), ferulic acid (FA) (4-hydroxy-3-methoxycinnamic acid; purity ≥ 98%), and salicylic acid (SA) (2-hydroxybenzoic acid; purity ≥ 99%), were obtained from Sigma-Aldrich srl, Milano, Italy.

Lettuce (*Lactuca sativa* L.) and tomato (*Lycopersicon esculentum* Mill.) seeds used in this work are often used in general bioassays and as dicotyledon target species in standard phytotoxic bioassay for allelochemicals (*26*).

Bioassays. Sets of 10 seeds of lettuce, cv. Romana, or 7 seeds of tomato, cv. S. Marzano Vesuvio 2, were placed on filter paper in Petri dishes (5-cm diameter), and 1.4 mL of the following aqueous solutions or suspensions was added: (i) distilled H_2O, used as the positive control; (ii) LHA, SHA, or SFA at concentrations of 20 and 200 mg/L; (iii) CA, FA, and SA at a concentration of 20 mg/L, used as negative controls; (iv) mixtures of LHA, SHA, or SFA and CA, FA, or SA at the above-mentioned concentrations, which were mechanically shaken for 12 h at room temperature (20 ± 2 °C) before addition to the seeds. In a preliminary experiment, CA, FA, or SA at a concentration of 10 or 50 mg/L was tested on lettuce seedlings to select the most appropriate concentration to be used in the subsequent experiments. The pH of all solutions ranged from 4 to 6. Seed germination was achieved in a Phytotron growth chamber at 25 ± 1 °C in the dark.

The effects of HS, CA, FA, SA, and their mixtures on seed germination and early growth of lettuce and tomato were evaluated by measuring the root and shoot lengths of seedlings obtained after a period of 4 days for lettuce and 6 days for tomato. All experiments were replicated four times. All data were statistically analyzed by one-way analysis of variance (ANOVA) at 95, 99, and 99.9% confidence levels. The mean values were separated by using the least significance difference test (LSD). The mean values obtained for LHA, SHA, SFA, CA, FA, and SA treatments were statistically compared with the positive control (H_2O) values, whereas the mean values obtained for the HS−allelochemical mixtures were compared with the corresponding negative control (CA, FA, and SA alone) value.

Residual Concentration of Allelochemicals during Germination. To determine the residual concentration of the allelochemical during the germination period, aliquots of CA, FA, and SA solutions at a concentration of 20 mg/L were added, in the presence or absence of lettuce seeds, to Petri dishes, which were placed in a Phytotron chamber at 25 ± 1 °C in the dark. The concentration of each allelochemical was measured every 24 h for 4 days. In another experiment, 10 seeds of lettuce were germinated for 3 days with H_2O in the conditions described above. Successively, the germination medium (~0.8 mL) containing lettuce seedling exudates was collected in two glass vials, and CA or FA solutions were added to obtain a final concentration of 20 mg/L. The vials were kept in the same conditions of germination, and the residual concentration of CA or FA was quantified every 24 h for 4 successive days. All experiments were triplicated.

In each experiment, the allelochemical concentration was measured by high-performance liquid chromatography (HPLC) analysis using a Spectra System pump [Thermo Separation Products (California) Inc., now Thermo Electron Corporation, San Jose, CA] equipped with a Rheodyne 7125 injection valve fitted with a 20-μL loop and connected to Supelcosil LC-18 chromatographic column (250 mm × 4.5 mm × 5 μm). The mobile phase used was water/acetonitrile/acetic acid at a ratio of 70:29:1 (v/v/v) for CA and FA analysis and 67:32:1 (v/v/v) for SA analysis. At the flow rate of 1 mL/min, retention times were 4.8, 7.1, and 11.6 min for CA, FA, and SA, respectively. The three allelochemicals were quantified with UV detection at a 280-nm wavelength by using a Spectromonitor 3200 (Thermo Separation Products (California), Inc.) and an external standard.

Adsorption Experiments. Adsorption kinetics of CA, FA, and SA onto SHA were measured by shaking for eight time periods aliquots of 10 mg of SHA suspended in 5 mL of aqueous solutions of each allelochemical at a concentration of 5 mg/L. All experiments were conducted in triplicate at a temperature of 25 ± 2 °C and in the dark to avoid possible photodegradation of the allelochemicals. At the end of each time period, the suspensions were centrifuged at 15000*g* for 15 min, and CA, FA, and SA concentrations in the supernatant solutions were determined by HPLC using the same apparatus and analytical conditions described above.

Adsorption data of CA, FA, and SA onto SHA were obtained using the slurry-type method by equilibrating for 8 h at 25 ± 2 °C in the dark 10 mg of each HA with 5 mL of aqueous solutions of each allelochemical at concentrations of 0 (blank) 1, 2, 4, 10, 20, and 50 mg/L. All experiments were conducted in triplicate. The suspensions were then centrifuged at 15000*g* for 15 min, and the allelochemical concentration in the supernatant solutions was determined by HPLC as described above.

The adsorption isotherms were then constructed by fitting the experimental adsorption data in a linear model, $x/m = KC$, the nonlinear Freundlich equation, $x/m = KC^{1/n}$, and the Langmuir equation, $x/m = (KbC)/(1 + KC)$, where x/m is the amount (mg) of sorbate adsorbed by 1 kg of substrate, C is the equilibrium concentration (mg/L) of sorbate in solution, the constant K is a measure of the adsorption capacity of the substrate, $1/n$ indicates the degree of nonlinearity between the solution concentration and the amount adsorbed, and b represents the Langmuir adsorption maximum. Also, the distribution coefficient, K_d, which is defined as the mean value of the amount of sorbate adsorbed at each equilibrium concentration, was calculated for the three allelochemicals.

RESULTS AND DISCUSSION

Effect of HS, Allelochemicals, and Their Combinations on Lettuce and Tomato Germination. The results of preliminary experiments on the effects of CA, FA, and SA at concentrations of 10 and 50 mg/L on root and shoot lengths of 4-day-old lettuce seedlings are shown in **Table 1**. The LSD test indicates the existence of a highly significant difference for root length between each allelochemical and the control

Table 1. Effects of CA, FA, and SA at Two Concentrations on Root and Shoot Lengths of 4-Day-Old Lettuce Seedlings and LSD Test Values[a]

treatment	root length (mm)	shoot length (mm)
H_2O	22.6 ± 1.4	16.9 ± 0.3
CA, 10 mg/L	19.0 ± 1.2 **	15.9 ± 0.8 ns
CA, 50 mg/L	15.6 ± 1.0 ***	14.6 ± 1.0 **
FA, 10 mg/L	16.1 ± 0.5 ***	15.5 ± 1.4 ns
FA, 50 mg/L	15.2 ± 0.9 ***	13.0 ± 1.0 ***
SA, 10 mg/L	18.4 ± 2.2 **	16.8 ± 1.1 ns
SA, 50 mg/L	13.8 ± 1.8 ***	15.0 ± 0.4 *
	LSD (0.01*P*): 3.2	LSD (0.05*P*): 1.5
	LSD (0.001*P*): 4.3	LSD (0.01*P*): 2.0
		LSD (0.001*P*): 2.7

[a] ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; ns, nonsignificant, according to the LSD test. Standard deviations ($n = 4$) are also indicated.

Table 2. Significance Level (*F* Value) Obtained by One-Way ANOVA of All Data Obtained for Root and Shoot Lengths of Lettuce and Tomato Seedlings[a]

		lettuce		tomato	
treatment	df[b]	root length	shoot length	root length	shoot length
HS[c]	6	2.26 ns	5.68 ***	9.09 ***	10.22 ***
allelochemicals[d]	3	13.17 ***	7.92 **	1.16 ns	3.91 *
CA + HS[e]	6	6.50 ***	8.17 ***	6.56 ***	2.16 ns
FA + HS[f]	6	8.66 ***	7.83 ***	0.48 ns	3.59 **
SA + HS[g]	6	5.80 ***	4.33 **	4.64 **	7.27 ***

[a] ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; ns, nonsignificant. [b] Degree of freedom. [c] LHA, SHA, and SFA at 20 and 200 mg/L and H_2O. [d] CA, FA, and SA at 20 mg/L and H_2O. [e] All combinations CA + HS at 20 and 200 mg/L and CA. [f] All combinations FA + HS at 20 and 200 mg/L and FA. [g] All combinations SA + HS at 20 and 200 mg/L and SA.

(H_2O), whereas either a significant or a nonsignificant difference exists for shoot length. As expected, the inhibitory effect of any allelochemical is more evident at the higher concentration (50 mg/L) and for roots more than for shoots. The maximum inhibition is exerted at the higher concentration by SA (~40% reduction) for roots and by FA (~23% reduction) for shoots. These results are in agreement with the previously observed increasing toxicity on lettuce germination of FA and SA at increasing doses and the more severe damage on roots than on shoots of several plant species in various experimental conditions (*16*, *27*−*29*). On the basis of these preliminary results, an intermediate concentration of 20 mg/L was used for each allelochemical in the subsequent bioassays for lettuce and tomato.

Experimental data obtained for root and shoot lengths of lettuce and tomato, expressed as percentages of the corresponding positive control (H_2O) data, and results of statistical analysis are referred in **Tables 2**−**6**. The *F* values obtained by ANOVA analysis of data of lettuce seedlings (**Table 2**) show a highly significant (99.9 and 99% confidence level) difference between the mean values of both root and shoot lengths, as a function of the presence of HS (only for shoots), allelochemicals, and allelochemical plus HS combinations. In general, this is true also for tomato, but with the exceptions of the treatments with allelochemicals and with FA plus HS on roots and CA plus HS on shoots, the results of which are nonsignificant.

Results shown in **Table 3** indicate that HS do not influence significantly root and shoot growth during the 4-day germination of lettuce, with the exception of SFA at the higher dose, which decreases shoot elongation. In the case of tomato, the growth of both root and shoot is enhanced by LHA at 20 mg/L and depressed at 200 mg/L, whereas SFA at both concentrations reduces shoot length. In general, HS of different origin and nature are known to affect germination and early growth of plants as a function of the concentration applied, the plant species tested, and the environmental conditions of germination (*3*).

The results of treatments with CA, alone and in combination with each HS, on the growth of lettuce and tomato seedlings are shown in **Table 4**. The presence of CA reduces significantly shoot length and, especially, root length (~35%) of lettuce seedlings, whereas CA phytotoxicity on root growth is reduced significantly in the presence of SHA at 200 mg/L and SFA at both concentrations. Furthermore, both SHA and SFA at 200 mg/L reduce significantly CA phytotoxicity on shoot growth. No statistically significant effect is shown on CA allelopathy by the simultaneous presence of LHA at either concentration and SHA at 20 mg/L.

In particular, the allelopathic action of CA (**Table 4**) and the depressing action exerted by SFA at high concentration (**Table 3**) on shoot and root lengths of lettuce disappear when both compounds are present simultaneously. This result suggests the occurrence of a negative synergism that produces positive effects on lettuce germination. Recently, the allelopathic potential of chloroform- and methanol-extracted fractions of tomato root exudates was shown to be suppressed or reduced when these fractions are applied in combination with a soil HA (*30*). The modulation effects exerted by HS were tentatively ascribed, at

Table 3. Effects of HS at Two Concentrations on Root and Shoot Lengths of 4-Day-Old Lettuce and 6-Day-Old Tomato Seedlings Relative to the Positive Control and LSD Test Values[a]

	lettuce		tomato	
treatment	root length (mm)	shoot length (mm)	root length (mm)	shoot length (mm)
H_2O[b]	100.0 ± 2.0	100.0 ± 7.0	100.0 ± 6.9	100.0 ± 7.8
LHA, 20 mg/L	100.7 ± 18.2	100.5 ± 11.5 ns	118.7 ± 14.5 **	117.5 ± 10.6 **
LHA, 200 mg/L	99.4 ± 10.3	97.5 ± 6.6 ns	74.5 ± 4.4 ***	77.9 ± 5.5 **
SHA, 20 mg/L	100.0 ± 9.1	102.1 ± 8.0 ns	100.1 ± 6.3 ns	87.1 ± 5.2 ns
SHA, 200 mg/L	113.4 ± 11.7	103.9 ± 4.5 ns	89.7 ± 8.1 ns	95.0 ± 10.7 ns
SFA, 20 mg/L	87.2 ± 2.0	90.9 ± 4.3 ns	91.8 ± 5.5 ns	84.7 ± 6.1 *
SFA, 200 mg/L	90.2 ± 2.8	76.7 ± 2.2 ***	105.6 ± 5.6 ns	80.5 ± 2.1 **
		LSD (0.001*P*): 21.5	LSD (0.01*P*): 18.4	LSD (0.05*P*): 12.6
			LSD (0.001*P*): 24.8	LSD (0.01*P*): 17.20

[a] ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; ns, nonsignificant, according to the LSD test. Standard deviations ($n = 4$) are also indicated. [b] Positive control.

Table 4. Effects of CA and Combinations CA plus HS on Root and Shoot Lengths of 4-Day-Old Lettuce Seedlings and 6-Day-Old Tomato Seedlings Relative to the Positive Control Treatment and LSD Test Values[a]

	lettuce		tomato	
treatment	root length (mm)	shoot length (mm)	root length (mm)	shoot length (mm)
H_2O[b]	100.0 ± 2.0	100.0 ± 7.0	100.0 ± 6.9	100.0 ± 7.8
CA, 20 mg/L[c]	64.9 ± 6.1 ***[d]	84.3 ± 6.4 **[d]	94.6 ± 4.6 ns[d]	82.8 ± 8.0 *[d]
CA + LHA 20 mg/L	75.0 ± 11.1 ns[e]	83.4 ± 2.8 ns	99.0 ± 4.1 ns	90.1 ± 9.6 ns
CA + LHA 200 mg/L	74.2 ± 2.7 ns	80.9 ± 9.1 ns	71.4 ± 6.8 **	79.1 ± 7.5 ns
CA + SHA 20 mg/L	76.6 ± 5.4 ns	74.8 ± 8.0 ns	107.7 ± 11.8 ns	90.7 ± 7.1 ns
CA + SHA 200 mg/L	82.6 ± 9.8 **	97.0 ± 4.8 *	82.0 ± 13.2 ns	92.6 ± 8.3 ns
CA + SFA 20 mg/L	83.5 ± 9.5 **	93.4 ± 5.0 ns	98.0 ± 9.3 ns	89.9 ± 6.6 ns
CA + SFA 200 mg/L	93.4 ± 8.9 ***	104.3 ± 4.9 ***	109.2 ± 7.8 *	95.0 ± 6.8 ns
	LSD (0.05*P*): 12.8	LSD (0.05*P*): 10.6	LSD (0.05*P*): 14.5	LSD (0.05*P*): 13.1
	LSD (0.01*P*): 17.5	LSD (0.01*P*): 14.4	LSD (0.01*P*): 19.7	
	LSD (0.001*P*): 23.4	LSD (0.001*P*): 19.2		

[a] ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; ns, nonsignificant, according to the LSD test. Standard deviations ($n = 4$) are also indicated. [b] Positive control. [c] Negative control. [d] Statistically compared to the positive control. [e] All of the mean values of the combinations CA + HS are statistically compared to the negative control.

Table 5. Effects of FA and Combinations FA plus HS on Root and Shoot Lengths of 4-Day-Old Lettuce Seedlings and 6-Day-Old Tomato Seedlings Relative to the Positive Control Treatment and LSD Test Values[a]

	lettuce		tomato	
treatment	root lengtn (mm)	shoot lengtn (mm)	root lengtn (mm)	shoot lengtn (mm)
H_2O[b]	100.0 ± 2.0	100.0 ± 7.0	100.0 ± 6.9	100.0 ± 7.8
FA, 20 mg/L[c]	72.8 ± 7.7 ***[d]	75.0 ± 5.5 ***[d]	100.8 ± 12.3	85.3 ± 10.6 *[d]
FA + LHA, 20 mg/L	68.5 ± 4.3 ns[e]	73.7 ± 4.7 ns	103.1 ± 15.1	93.7 ± 9.2 ns
FA + LHA, 200 mg/L	73.9 ± 1.7 ns	76.9 ± 3.9 ns	90.7 ± 16.1	84.4 ± 12.7 ns
FA + SHA, 20 mg/L	81.5 ± 7.2 ns	79.5 ± 12.0 ns	96.8 ± 4.2	83.7 ± 7.4 ns
FA + SHA, 200 mg/L	86.3 ± 2.4 *	94.3 ± 5.8 **	98.8 ± 9.7	92.3 ± 5.1 ns
FA + SFA, 20 mg/L	69.1 ± 4.2 ns	72.4 ± 4.3 ns	98.4 ± 7.2	83.7 ± 3.6 ns
FA + SFA, 200 mg/L	90.2 ± 13.9 **	75.8 ± 3.7 ns	105.8 ± 12.4	109.5 ± 8.5 **
	LSD (0.05*P*): 11.1	LSD (0.05*P*): 10.7		LSD (0.05*P*): 14.4
	LSD (0.01*P*): 15.1	LSD (0.01*P*): 14.6		LSD (0.01*P*): 19.6
	LSD (0.001*P*): 20.3	LSD (0.001*P*): 19.6		

[a] ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; ns, nonsignificant, according to the LSD test. Standard deviations ($n = 4$) are also indicated. [b] Positive control. [c] Negative control. [d] Statistically compared to the positive control. [e] All of the mean values of the combinations FA + HS are statistically compared to the negative control.

least partially, to some kind of interaction, possibly adsorption, which could decrease the bioavailability of allelochemicals present in these exudate fractions. Furthermore, HS of different origin and nature were shown to exert an antimutagenic and antitoxic effect in germinating seeds of leguminous plants treated with the mutagen maleic hydrazide, which was only in minor part attributable to adsorption of maleic hydrazide onto HS (*7*).

The allelopathic effect of CA on tomato seeds is significant only for shoots, and only the combinations with LHA or SFA at 200 mg/L are able to reduce or promote, respectively, tomato root length with respect to the negative control (**Table 4**). Differences measured are not statistically relevant for all other CA plus HS combinations tested.

The results of treatments with FA, alone and in combination with each HS, on the growth of lettuce and tomato seedlings are shown in **Table 5**. The FA alone reduces significantly ($P \leq 0.001$) the length of roots and shoots of lettuce, whereas the simultaneous presence of SHA at 200 mg/L attenuates significantly the FA negative effect on both root and shoot lengths of lettuce, and that of SFA at 200 mg/L has a significant positive effect only on root length. Similar to CA, also in the case of FA the HS more efficient in contrasting the allelopathic potential are those originated from soil.

In experiments with tomato seedlings FA is toxic only for shoots, and only its combination with SFA at 200 mg/L significantly reduces its toxicity by enhancing shoot growth even more than the positive control (**Table 5**). Similar to CA in combination with SFA, also for the FA plus SFA combination, a negative synergism apparently occurs, which leads to the complete suppression of growth inhibition exerted by either FA or SFA alone. For all other FA plus HS combinations, differences measured for either lettuce or tomato growth are not significant with respect to the negative control.

The results of treatments with SA, either alone or in combination with each HS, on seedlings of lettuce and tomato are shown in **Table 6**. The SA alone reduces the elongation of both roots and shoots of lettuce, with respect to the H_2O control. A highly significant antiallelopathic effect is measured only when SHA at 200 mg/L is present simultaneously. No significant variations are observed for the other SA plus HS combinations.

In the case of tomato SA affects negatively only shoots, whereas significant variations are observed for both root and shoot lengths in some SA plus HS combinations with respect to SA alone (**Table 6**). In particular, the presence of LHA or SFA at 200 mg/L produces, respectively, a significant decrease or increase of tomato shoot length, with respect to SA alone. Furthermore, tomato root length increases even above the value of the control (H_2O) for the combinations of SA with LHA and SHA at 20 mg/L and SFA at both concentrations. Finally, an evident negative synergism can be observed for shoot growth in the combination of SA plus SFA at 200 mg/L (**Table 6**). These results would thus indicate that allelopathy is a dynamic process strictly related to the surrounding medium conditions, and the interaction of allelochemicals with humic substances

Table 6. Effects of SA and Combinations SA plus HS on Root and Shoot Lengths of 4-Day-Old Lettuce Seedlings and 6-Day-Old Tomato Seedlings Relative to the Positive Control Treatment and LSD Test Values[a]

	lettuce		tomato	
treatment	root length (mm)	shoot length (mm)	root length (mm)	shoot length (mm)
H_2O[b]	100.0 ± 2.0	100.0 ± 7.0	100.0 ± 6.9	100.0 ± 7.8
SA, 20 mg/L[c]	70.0 ± 11.2 ***[d]	87.0 ± 6.4 *[d]	90.5 ± 4.6 ns[d]	87.6 ± 9.3 *[d]
SA + LHA, 20 mg/L	65.8 ± 11.6 ns[e]	80.3 ± 8.0 ns	113.3 ± 10.4 **	77.9 ± 7.3 ns
SA + LHA, 200 mg/L	65.5 ± 8.1 ns	75.3 ± 6.9 ns	87.7 ± 7.9 ns	69.9 ± 5.8 **
SA + SHA, 20 mg/L	66.6 ± 11.8 ns	75.8 ± 8.4 ns	108.0 ± 9.6 *	84.7 ± 6.8 ns
SA + SHA, 200 mg/L	92.4 ± 11.0 **	91.9 ± 9.4 ns	89.1 ± 11.5 ns	83.2 ± 7.6 ns
SA + SFA, 20 mg/L	67.4 ± 3.9 ns	76.7 ± 6.5 ns	118.1 ± 10.9 **	95.4 ± 5.5 ns
SA + SFA, 200 mg/L	78.2 ± 11.9 ns	90.5 ± 7.4 ns	109.8 ± 12.2 *	102.9 ± 7.0 *
	LSD (0.05*P*): 16.2 LSD (0.01*P*): 22.1 LSD (0.001*P*): 29.6	LSD (0.05*P*): 12.7	LSD (0.05*P*): 16.1 LSD (0.01*P*): 21.8	LSD (0.05*P*): 12.1 LSD (0.01*P*): 16.5

[a] ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; ns, nonsignificant, according to the LSD test. Standard deviations ($n = 4$) are also indicated. [b] Positive control. [c] Negative control. [d] Statistically compared to the positive control. [e] All of the mean values of the combinations CA + HS are statistically compared to the negative control.

represents one of the multiple factors, apparently of crucial importance, in the general modulation effects of allelopathy in plants.

Residual Concentration of Allelochemicals during Germination. Figure 1 shows the residual concentrations of CA, FA, and SA measured every 24 h during a 4-day period, in the presence or absence of lettuce seedlings, or in the presence of aqueous solution containing lettuce seedling exudates obtained after a 3-day germination (only CA and FA). In the absence of seeds, the concentration of CA, FA, and SA in the germination medium decreases from 20 to 15.1, 15.4, and 17 mg/L, respectively. In the presence of lettuce seedlings, the CA concentration rapidly decreases to disappear after 72 h, and the FA concentration reaches a value of <1 mg/L after 96 h, whereas the SA concentration decreases only by ~25% of the initial dose. These results indicate the active involvement of lettuce germinating seedlings in reducing to different extents the concentrations of allelochemicals. The concentration of CA and FA treated with lettuce seedling exudates increases during the first 2 days, which is possibly due to the presence of these allelochemicals in the seedling exudates, and then decreases markedly, especially for FA, with respect to that measured in H_2O only. These results would suggest that both absorption of the allelochemical by seedlings and its enhanced degradation may occur simultaneously.

Adsorption Experiments. Adsorption kinetics curves of CA, FA, and SA at 5 mg/L onto SHA (**Figure 2**) indicate that in any case adsorption is rapid and almost completed in the first few hours, then reaching a steady state. On the basis of these results, an equilibration time of 8 h was adopted for the three allelochemicals in the adsorption isotherm study.

Correlation coefficients (**Table 7**) calculated over the concentration range tested for adsorption of CA, FA, and SA onto SHA indicate that in any case the experimental data better fit (higher correlation coefficients) a linear model (**Figure 3**) rather than nonlinear Freundlich or Langmuir models (correlation coefficients not shown). This result implies that a constant partition of the allelochemical occurs between the solution and the substrate, that is, adsorption is directly proportional to the sorbate concentration in solution, and that no saturation occurs over the whole range of concentrations tested.

The adsorption constants, K, and distribution coefficients, K_d, of CA, FA, and SA onto SHA (**Table 7**) exhibit the same trend, which indicates a decrease of the adsorption degree in the order FA > CA > SA. In particular, the K values of CA and FA are, respectively, 3 and 6 times greater than the K value of SA. These

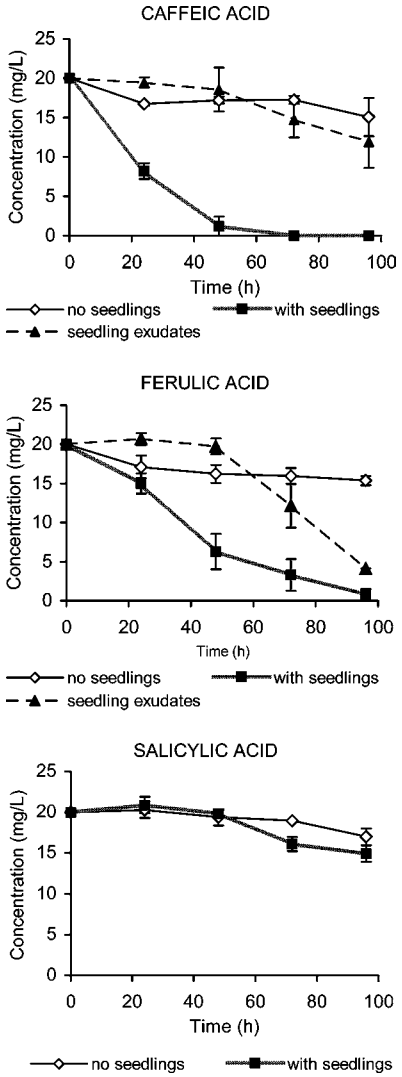

Figure 1. Residual concentrations of the three allelochemicals measured during 4-day germination in the presence or absence of lettuce seedlings or seedling exudates.

results are in agreement with those of Dalton et al. (*20*), who found that cinnamic acid derivatives generally sorb more than benzoic acid derivatives to soils. The relatively limited adsorption capacity measured for the studied allelochemicals onto SHA can be related to their high water solubility (*31*). However, no

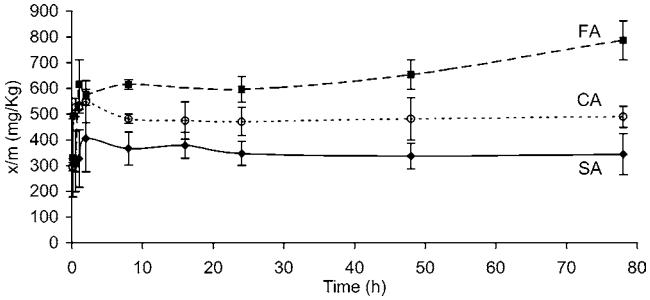

Figure 2. Adsorption kinetics curves of CA, FA, and SA at 5 mg/L onto SHA. The vertical line on each marker point indicates the standard deviation for three replicates.

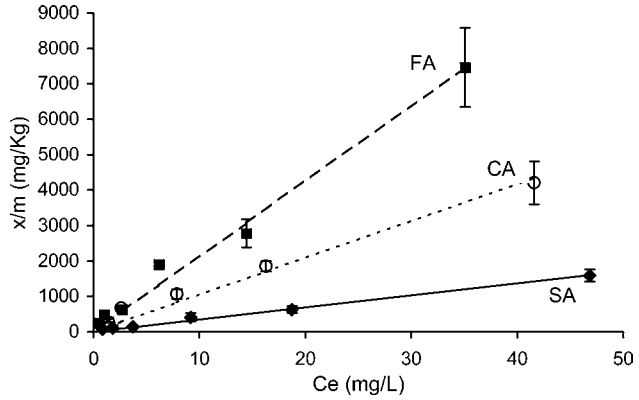

Figure 3. Linear adsorption isotherms of CA, FA, and SA onto SHA. The vertical line on each marker point indicates the standard deviation for three replicates.

Table 7. Correlation Coefficients (r), Adsorption Constants (K), and Distribution Coefficients (K_d) for Linear Adsorption Isotherms of Caffeic, Ferulic, and Salicylic Acids onto SHA

sample	r	K (L kg^{-1})	K_d (L kg^{-1})
caffeic acid	0.986	104.3	158.6
ferulic acid	0.993	212.9	318.0
salicylic acid	0.996	34.3	50.0

data are available in the literature on the adsorption capacity of HAs for these compounds.

Furthermore, data in **Figure 3** show that when the allelochemical at a concentration of 20 mg/L is interacted with SHA, the equilibrium concentrations measured are 16.3, 14.4, and 18.7 mg/L, respectively, for CA, FA, and SA. Results of bioassays on lettuce show that at these concentrations the allelochemicals are likely to be still toxic, causing a significant reduction of root growth. In other words, the limited adsorption of the allelochemicals studied onto SHA cannot be considered as the only mechanism responsible for the reduced allelopathic action observed in the combinations of CA, FA, and SA with SHA at 200 mg/L on lettuce. Thus, one or more mechanism(s) still unknown, other than adsorption, should be responsible for the action of HS in controlling plant allelopathy.

Finally, the different capacity of HS examined to modulate the allelopathic action of CA, FA, and SA could be reasonably related to some differences in their compositional, structural, and functional properties. In general, samples SHA and SFA feature smaller C and phenolic group contents, larger H, O, carboxyl group, and total acidity contents, and much larger N content than LHA (**Table 8**) (*21*). However, in agreement with previous findings (*3*, *6*, *7*, *10*), the great capacity of SHA and, especially, SFA to modulate the allelopathic potential of the allelochemicals tested appears to be related positively only to their large carboxylic group content and/or to the typical small molecular weight in the case of fulvic acids.

Table 8. Elemental and Acidic Functional Group Composition (on a Moisture- and Ash-free Basis) of Humic and Fulvic Acids Examined (*21*)

	% (w/w)					mequiv/g of C		
sample	C	H	N	S	O	carboxyl	phenolic	total acidity
LHA	63.81	3.70	1.23	0.76	31.27	7.46	2.31	9.77
SHA	58.13	3.68	4.14	0.44	34.08	8.28	1.87	10.15
SFA	50.12	4.28	3.75	0.89	42.61	13.24	2.27	15.51

In conclusion, the three allelochemicals tested appear to exert a similar, highly toxic effect on lettuce, especially on roots, whereas their toxic action on tomato is apparent only on shoots. These effects appear to be modulated markedly by the presence of soil humic and fulvic acids on lettuce and of soil fulvic acid on tomato, whereas leonardite humic acid appears to stimulate only at low concentration the growth of tomato seedlings. The efficiency of HS in controlling the allelopathic action of the allelochemicals tested is apparently related not only to their origin and nature, that is, their structural and functional properties, and concentration, but also to the plant species, the organ considered, and the kind of allelochemical. In any case, the concentration of the allelochemical decreases during germination, and for CA and FA it appears to be influenced markedly by the presence of germinating seedlings that probably absorb the compound and/or enhance its degradation during germination. Adsorption of any allelochemical onto SHA is rapid and follows a linear model. In general, the adsorption capacity of SHA is small for the three allelochemicals and follows the order FA > CA > SA. Adsorption can be suggested to be, at least partly, responsible for the antiallelochemical action of HS.

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