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THE UPTAKE OF FERULIC AND *p*-HYDROXYBENZOIC ACIDS BY *CUCUMIS SATIVUS*

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Key Word Index—*Cucumis sativus*; Cucurbitaceae; allelopathy; uptake methods; phenolic acids.

Abstract—The uptake of ferulic acid (FA) and *p*-hydroxybenzoic acid (*p*-HBA) from solutions (0.1–1.0 mM, pH 4.0–7.0), was determined for intact and excised roots of *Cucumis sativus*. Uptake based on depletion of phenolic acids from nutrient solution was compared with uptake of [U-ring-^{14}C] labelled FA and/or *p*-HBA. Although uptake based on radioactive isotopes more accurately reflects actual uptake of the compounds by cucumber seedlings, depletion from nutrient solution may be useful in describing trends over short time periods (< 5 hr). Uptake was modified by the concentration of the phenolic acids and pH of treatment solutions. Greatest uptake occurred at the highest concentration and lowest pH tested. The uptake of FA was 50–75% higher than that of *p*-HBA. While the growth response of cucumber seedlings exposed to mixtures of these two phenolic acids is additive, the uptake was antagonistic. The uptake of FA was unaffected by the presence of *p*-HBA. The uptake of *p*-HBA was reduced by 30% in the presence of FA when compared to the uptake from solutions containing *p*-HBA alone.

INTRODUCTION

Allelopathic [1] or chemical interactions between organisms have been proposed for a variety of species [2–4]. An allelopathic interaction among plants would require: (a) the production and release of an allelochemical compound(s) by a donor plant; (b) uptake of the compound(s) by a recipient plant; and, (c) a detectable recipient plant response [5]. In the past, emphasis has been placed primarily on the first requirement—allelochemical production and release. Research efforts were directed towards the identification and isolation of plant compounds which might have the potential to act as allelochemical agents. Although there has been a more recent interest in determining the physiological responses of plants under allelochemical stresses [6,7], the number of publications specifically addressing uptake of these compounds remain few [8–12].

One of the aims of those investigating allelopathic interactions has been to develop means of predicting plant effects based on the extraction of compounds from field soils. If the uptake of allelopathic compounds is directly related to the growth response (inhibition) of the plant and since uptake might be expected to vary with environmental factors such as soil pH and ionic concentration, descriptive information on the relationship between uptake and these factors becomes critical in the development of predictive tools.

Included among the compounds most often cited as allelopathic agents are the phenolic acids, particularly those derived from benzoic and cinnamic acids. Accurate determinations of phenolic acid uptake by plants is difficult without radioactive ring-labelled compounds. These are unfortunately only available by the costly procedure of custom synthesis. Without a radiotracer, it is difficult to distinguish the absorbed compound from the endogenous pool. Therefore, one of the objectives of this study was to compare the uptake of radioactive compounds with that of solution depletion. High performance liquid chromatographic (HPLC) analysis of solutions in which seedlings were grown was contrasted with methods employing [U-ring-^{14}C]*p*-hydroxybenzoic acid (*p*-HBA) and [U-ring-^{14}C] ferulic acid (FA).

The phenolic acids, FA and *p*-HBA, were selected because they were previously shown to be toxic to a variety of species. When the effects of FA and *p*-HBA on cucumber (*Cucumis sativus* cv Early Green Cluster) leaf expansion are compared [13], FA is a much stronger allelochemical agent than *p*-HBA. A second objective of this study then was to compare the uptake of these two simple phenolic acids, a strong allelochemical agent and a weak one, under varying solution conditions known to affect the degree of seedling response (i.e. concentration of compound and pH). Since the toxic effects of various combined phenolic acids can be antagonistic, additive, or even synergistic, uptake from mixtures of FA and *p*-HBA was also determined.

RESULTS AND DISCUSSION

Method comparison

Two methods of determining the uptake of phenolic acids (FA and *p*-HBA) were compared: (a) the depletion of compound from hydroponic containing cucumber seedlings over time, and (b) the presence of radiotracer in seedling tissues. In depletion studies, the analysis of solution samples was accomplished by either HPLC or

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scintillation spectrophotometry for the [U-ring-^{14}C] phenolic acid. Seedling content studies utilized either whole plants or excised root tissues. In both types of studies, all containers, sampling equipment and treatment solutions were sterilized while seedlings were not. Although the absolute rates of uptake differed between the FA and *p*-HBA, the resulting patterns were the same for both methods. Thus, only data for FA rates are presented (Table 1).

The highest uptake rates were obtained by the solution depletion method based on HPLC analysis. Glucose nutritive agar plates which were inoculated with treatment solutions showed no obvious microbial growth until the five hr sample was taken. This does not, however, eliminate the possibility that the difference between HPLC and ^{14}C data (Table 1) may have been a result of the utilization of FA by a low microbial population either in solution or on the unsterile root surfaces. An effort was made to determine the microbial or physical (primarily photochemical) breakdown of the phenolic acid in solution. Peaks other than FA appearing in the collected solution samples were separated by HPLC and analysed for their radioactivity. With the exception of the FA, the peaks were not identified. The non-FA peaks were found to contain radioactivity (Fig. 1). This suggests that some of the FA in solution was being used as a microbial carbon source and/or was subject to breakdown by other means. However, while assuming the presence of radioactivity in an isolated peak is a result of solution FA breakdown, the possibility of a release of previously absorbed and perhaps altered radiolabelled phenolic compounds from the roots cannot be eliminated. An efflux of the FA itself from the seedling to the solution would appear to be small based on the observed depletion of FA from solution (as determined by HPLC analysis) and would have minimized, rather than increased, the differences the two means of solution analysis (HPLC vs ^{14}C).

Uptake based on the HPLC analysis was specific for FA and excluded the appearance of breakdown products in solution. Thus any observed loss of FA from the treatment solution may have been due to both seedling uptake and microbial/photochemical degradation of the compound. This differed from the ^{14}C-method where radioactivity was monitored and the resulting data included all

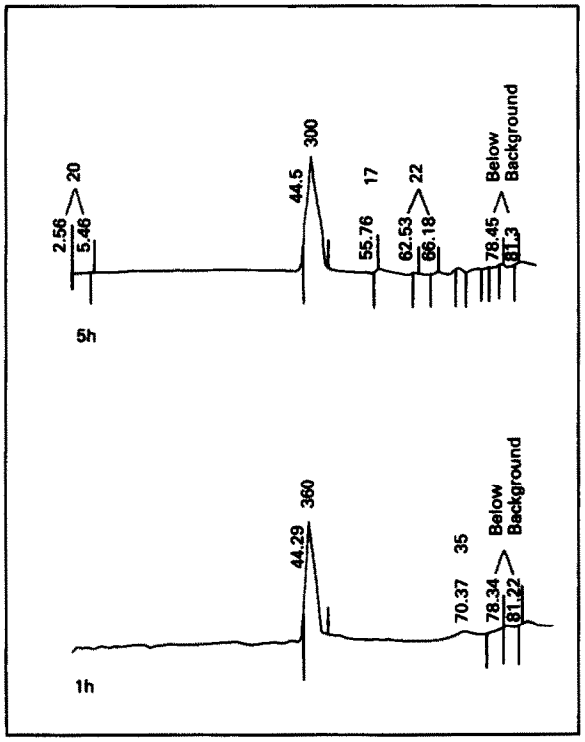

Fig. 1. Peaks separated by HPLC from treatment solutions (0.5 mM FA, labelled with [U-ring-^{14}C]FA to a specific activity of 1800 cpm/ml) containing seedlings. The CPM found in each collected peak is given next to the retention time of that peak. Counts represent a 200 μl sample of the original treatment solution. Solution were sampled at one and five hr.

^{14}C-compounds present in solution, not just FA. Therefore, solution depletion based on analysis of radioactivity may more accurately reflect actual FA uptake by the seedling. The two solution methods, however, did correspond well with each other over the first five hr period suggesting a somewhat constant level of microbial/photochemical degradation.

Table 1. Comparison of uptake by various methods from solutions 0.5 mM FA, pH 5.5

Method	Sample analysis		Rate of uptake mg/g dry wt root/hr
Solution depletion	HPLC		5.50
	^{14}C		2.18
Seedling Content	Excised roots: ^{14}C	Time (hr)	
		0.5	10.01
		3.5	1.46
		5.0	1.02
	Whole plant: ^{14}C	Root weight	
		0.02 g	1.25
		0.08 g	1.47

Unless otherwise noted, mean root dry weight was 0.08 g, and uptake rates were based on five hr exposures.

The ^{14}C solution depletion method compared well with whole plant studies where tissues were analysed for radioactivity. The 0.71 mg discrepancy between the rate estimates of 2.18 and 1.47 mg/g root/hr (Table 1) may have been due to the difference in the counting efficiency for a solution vs seedling tissue. Although the seedling material was intensively processed to maximize counting efficiency, the contained radioactivity was probably still underestimated. Data in Table 1 also suggest that seedling age, expressed by root size, influenced the rate of uptake by whole plants.

Excised root rates differed from rates based on whole plant ^{14}C content. If the rates are converted to actual μg taken up at 0.5, 3.5 and 5.0 hr by the 0.1 g (fr. wt) root samples, values become 20.02, 20.44 and 20.44. Thus, 98% of the total FA absorbed by the excised root samples was taken up within 30 minutes. Later sampling times yielded lower rates because uptake ceased and the same values were being divided by a greater number of hr.

In whole plant studies, uptake increased in a curvelinear or linear manner over time (Fig. 2). Rates calculated at different times for the curvilinear response curve would, of course, also change over time. Rates from a 0.1 mM solution increased from 1.05 (at 2 hr) to 3.3 mg/g root/hr (5 hr). Over the same time period, uptake rate from a 0.5 mM solution remained at a constant 6 mg/g root/h. The initial rate from a 1.0 mM solution was 11.8 mg/g root/h, but decreased with subsequent sampling times. Apparently the time period required to reach the maximum rate of phenolic acid uptake differed substantially with concentration. Given the changes which occurred in the first five hr, uptake rates based on a constant point in time could over or underestimate the maximum rate and complicate comparisons between treatments. To investigate how multiple phenolic acid challenges might affect the uptake of FA, comparisons were made between seedlings given several exposures and those receiving only a single treatment (Fig. 3). Although the uptake response for the single and the triple exposure was linear against time, the rate of multiple challenged seedlings was lower. The lowered rate at which the phenolic acid was taken up by seedlings exposed for an extended (4 days and 5 hr vs 5 hr) period of time may well represent a saturation phase of uptake. Although rates were reduced, seedlings continued to take up appreciable amounts of the phenolic acid over time.

The pH of the solution also modified uptake over time (Fig. 4). Uptake rates calculated from solutions at pH 5.5 or 7.0 remained fairly constant with time (no significant

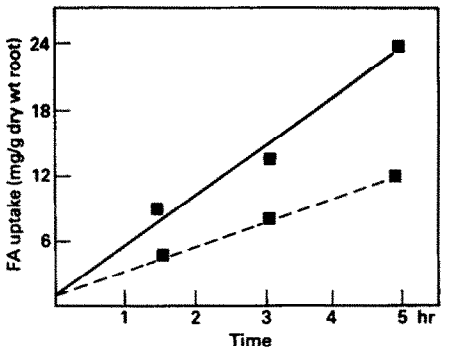

Fig. 3. The uptake (mg/g dry wt) of FA by seedlings given one or three exposures, calculated from ^{14}C analysis of solutions (0.5 mM, pH 5.5), ——: initial exposure (uptake = 4.9 hr + 0.56, $r^2 = 0.93$), ----: three exposures—one every 48 hr (uptake = 2.5 hr + 0.25, $r^2 = 0.99$). Data for initial and third exposures were taken at the same time.

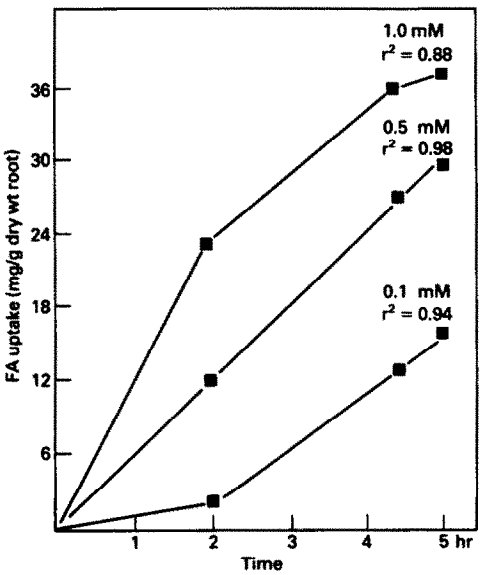

Fig. 2. The uptake (mg/g dry wt) of FA calculated from HPLC analysis of solutions (0.1, 0.5 or 1.0 mM FA, pH 5.5). LSD's for rates (mg/g dry wt/hr) within concentration regressions were: 0.1 mM = 3.4, 0.5 mM = 4.6, 1.0 = 2.7.

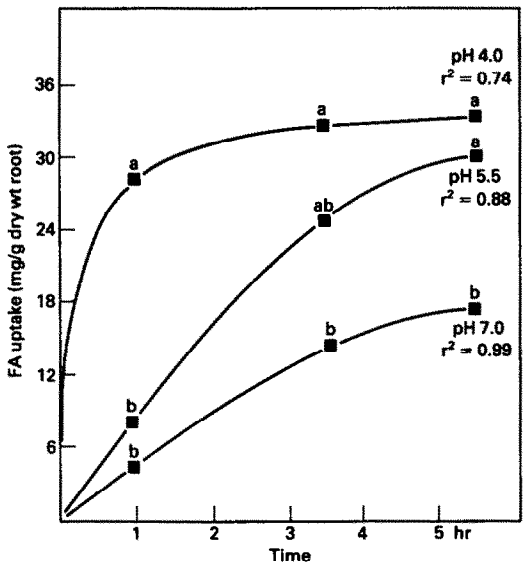

Fig. 4. The uptake (mg/g dry wt) of FA, calculated from HPLC analysis of solutions (0.5 mM FA, pH 4.0, 5.5, or 7.0). LSD's for separation of the rate (mg/g dry wt/hr) of FA uptake within pH regressions were: pH 4.0 = 10.0, pH 5.5 = 2.4, pH 7.0 = 0.5. Mean separation of values at each sampling time are indicated by letters. Mean values at points in time with the same letter are not significantly different at the 0.001 level.

effects). Seedlings exposed to treatment solutions set at pH 4.0 initially took up the phenolic acid rapidly, but rates declined significantly thereafter. Differences in uptake values between pH levels and within sampling times suggested a significant short-term effect of low pH and a merging of the 4.0 and 5.5 values with time. This merging trend may have been, in part, due to the decreased stability of the pH in the 4.0 and 5.5 pH solutions with time. After four hr, and with the initial rapid removal of phenolic acid from solution, the pH differences between treatments originally set at 4.0 and 5.5 became nominal.

The results reported above suggest that in cases where the cost associated with the custom synthesis of radioactive phenolic compounds makes their purchase infeasible, uptake methods which are based on solution depletion by HPLC analysis can be used as an alternative with some limitations. Since the results obtained by the HPLC method are readily affected by microbial and photochemical degradation of the compound in the solution, the system should be sterile and as dark as possible. Although uptake determined from solution depletion (HPLC analysis) were approximately twice those from ^{14}C data, the pattern of the two methods did correspond closely over the first five hrs. Therefore, general trends of uptake can be determined from HPLC analysis of the solution although the absolute values will probably be inflated. The magnitude of this inflation will be determined by the level of microbial activity and degradation of the compound in solution. The validity of this method is further diminished beyond five hrs under unsterile conditions.

Blum *et al.* [14] reported that the inhibitory action of phenolic acids on cucumber was strongly influenced by the pH of the treatment solution. As pH decreased, the inhibition of leaf area, dry weight, and water utilization increased. A similar pH effect on processes such as respiration [15] and ion uptake [10] have been observed for several other species. Harper and Balke [10] found that the uptake of salicylic acid (*o*-hydroxybenzoic acid) was increased by lowering solution pH. They attributed this to the undissociated (permeable) state of the phenolic acid which would predominate at pH levels near the pKa (3) of the compound. The approximate pKa of FA (and *p*-HBA) is 4.85. At pH 4.0, most of the FA and *p*-HBA will be in an undissociated form. As the pH rises, more and more of the phenolic acids are charged (anionic) and, as such, less likely to move across the wall and membrane. Once the FA or *p*-HBA reaches the near neutrality of the cytoplasm, a majority of the molecules will dissociate. Under external acidic conditions, a gradient of undissociated to dissociated phenolic acid would thus be established and maintained. The uptake of other phenolic acids might be expected to respond similarly to pH.

Uptake of FA and p-*HBA*

For comparison purposes, 5 hr depletion rates based on HPLC analysis of FA (Fig. 5) and *p*-HBA (Fig. 6) are shown. The uptake of FA and *p*-HBA by cucumber was determined by both concentration and pH. Greater rates occurred at highest concentration and lowest pH. The effects on rates over time and with concentration or pH were similar for both phenolic acids. The rate of uptake of FA was 50–75% higher than for *p*-HBA.

It is interesting that in cucumber bioassays, the FA is a much more potent inhibitor of growth than the *p*-HBA

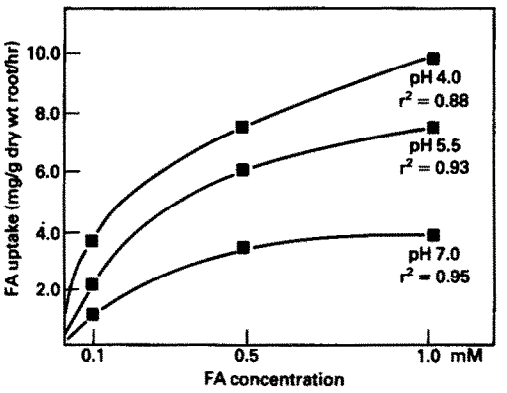

Fig. 5. The rate (mg/g dry wt/hr) of FA uptake from solution (0.1, 0.5, or 1.0 mM FA, pH 4.0, pH 5.5, and pH 7.0). Uptake rates were calculated from HPLC analysis of FA solution depletion after five hrs.

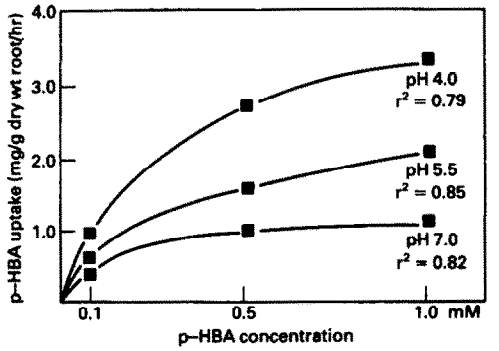

Fig. 6. The rate (mg/g dry wt/hr) of *p*-HBA uptake from solution (0.1, 0.5, or 1.0 mM *p*-HBA, pH 4.0, 5.5, and 7.0). Uptake rates were calculated from HPLC analysis of solution *p*-HBA depletion after five hr.

[13]. At this point, it would be easy to conclude that the greater uptake rates of FA determined the greater level of inhibition. This, however, presumes that the potential toxicity of FA and *p*-HBA and the detoxification mechanisms of cucumber are similar.

Phenolic acids are ubiquitous in the plant kingdom and must certainly occur together in the environment. Plants would not encounter these as isolated compounds, but in complex mixtures. Growth modifications of cucumber suggest that the effects of such mixtures may be additive or antagonistic [13]. The effect of FA and *p*-HBA mixtures on leaf expansion was previously [13] shown to be additive, but only as an initial response. The effect was lost within two days. In this study, the effect of mixtures of FA and *p*-HBA on their respective uptakes by seedlings was tested. The rate of FA uptake was significantly affected only by the initial concentration of FA in solution and the sampling time for which the rate was determined (Table 2). The uptake of *p*-HBA was, however, affected significantly not only by its own initial concentration and time of sampling, but also by the presence of FA in solution (Table 2). The r^2 value of the *p*-HBA uptake model is less

Table 2. Anova table for rates of p-HBA or FA uptake from solutions containing either compound alone or mixtures of both at various concentrations

Dependent variable		Mean square	F	r^2
Rate of pHBA uptake	Model	20.70	11.97	0.68
	Error	1.73		
Variables				
Initial conc. of FA		26.28	15.21*†	
Initial conc. of p-HBA		55.76	32.28*	
Initial conc. of FA × initial conc. of p-HBA		1.78	1.03	
Time		23.20	13.44*	
Rate of FA uptake	Model	101.78	27.43	0.83
	Error	3.71		
Variables				
Initial conc. of FA		370.80	99.91*	
Initial conc. of p-HBA		1.87	0.50	
Initial conc. of FA × initial conc. of p-HBA		6.36	1.71	
Time		58.42	15.74*	

†Significant at an 0.0001 level.

than that of FA due to the 30% drop in p-HBA uptake in the presence of FA regardless of the FA concentration.

While the inhibition of cucumber growth has been shown to be additive, the uptake response here was antagonistic. The short-term additive growth effect must then have been due to an increased toxicity of FA or due to the general increase in millimolar levels of phenolic compounds within the solution rather than an increased general uptake of phenolic acids.

This study demonstrated that the uptake of phenolic compounds can be modified by their concentration and solution conditions such as pH. The difference (decrease) in the rate of uptake by younger seedlings was not studied in detail, but the present data suggests that this may also play a part in determining the susceptibility of plants to the presence of allelochemical compounds in the environment.

The data presented here and in previous studies [14] suggest that under field conditions the pH of the soil solution may vary the plant responses to phenolic allelopathic agents. The pH of the soil solution, would, therefore have to be considered in any evaluation of the allelopathic potential of a given system. In agronomic soils the addition of compounds which would in effect lower the acidity of the soil solution may be used, perhaps at critical stages of crop growth or periods of high phenolic acid input, to 'protect' plants from the inhibitory growth effects of allelopathic agents.

The concentrations of individual plant-available phenolic acids, although not known for the soil solutions of most natural systems [16], are thought to be low. However, if the uptake of phenolic compounds is related to the allelopathic response and these compounds can be taken up in an additive or even antagonistic manner, the potential for inhibition of plant growth by mixtures comprised of several low concentrations of phenolic acids may be possible.

This study contributes needed information on the uptake of allelopathic phenolic acids and the factors which may modify that uptake. That the stronger allelopathic agent (FA) was taken up at a greater rate suggests a possible relationship between uptake and toxicity. Certainly this is an area which needs to be addressed in future research if the impact of allelochemical compounds is ever to be predicted from concentrations in the soil solutions.

EXPERIMENTAL

Plant material. Cucumber seeds were germinated at 27–30° in sterile, moistened vermiculite. After 48 hr, seedlings were transferred to 125 ml, foil-wrapped, autoclaved bottles containing full-strength Hoagland's solution [17]. Seedlings were grown under light banks (2-40W-fluorescent: 1-25W-incandescent) on a 12-hr light/12-hr dark regime. At seedling height, light levels were approximately 176 μmol/m^2/sec. Temperatures within the banks ranged from 21 to 30°. Fresh, aerated nutrient solutions were provided in clean bottles every 48 hr. Nutrient solutions were adjusted to the appropriate pH (4.0, 5.5, 7.0). During experimental periods, treatment solutions contained 5 mM MES [2-(*N*-morpholino)-ethansulphonic acid] buffer to stabilize pH.

Seedlings were used in uptake studies when at least two primary leaves had fully expanded. This state was reached somewhere between 14 to 18 days after germination. Mean dry wt of the root and shoot were 0.08 and 0.40 g, respectively. Each seedling was considered a replicate with 3–5 seedlings per treatment. All experiments were repeated at least three times. Analysis of variance (ANOVA) and general linear modeling (GLM) procedures were carried out using the Statistical Analysis System package of the SAS Institute (Cary, NC, USA). Duncan's mean separations were performed ($\alpha = 0.05$ or less) on discontinuous data with all other significant differences based on the regression analysis of variance. r^2 values, where presented, represent significant linear or curvilinear models.

Uptake studies. The uptake of FA or p-HBA was based on depletion of the compound from the treatment solutions or on the analysis of seedling materials for the radiotracer. Uptake calculations were expressed on a root (dry) wt basis. That the phenolic acids themselves were actually absorbed by roots was established by extracting roots and shoots treated with labelled compound. Labelled FA and p-HBA were isolated and identified by HPLC analysis. Additional evidence for uptake came from a

saponification procedure (reported elsewhere) which releases phenolic acid esters linked to the cell wall. When FA treated seedlings were subjected to this extraction, no FA was removed from their tissues. As esterification is believed to be a major form of phenolic acid association with primary walls, this suggests that the radiolabel found in the tissue was not attached to the wall but was taken up by the symplast of the seedling.

Radioactive [U-ring-^{14}C]FA was prepared from [U-ring-^{14}C]vanillin (Surplus radiochemicals, Amersham, Arlington Heights, IL., USA) using a procedure of ref. [18], and verified by HPLC ^{1}H NMR comparisons to authentic FA. The specific activity of the stock solution was 253 μ Ci/mM. The [U-ring-^{14}C]*p*-HBA was purchased (surplus, Amersham) with a specific activity of 33 mCi/mM.

Solution depletion studies. Prior to treatment, all glassware and equipment were autoclaved and the solutions sterile filtered through a 0.22 μm membrane. All solution preparation was carried out under low light (non-fluorescent) conditions to minimize breakdown of the phenolic compounds. Bulk treatment solutions; 0.00, 0.10, 0.25, 0.50, or 1.00 mM FA or *p*-HBA were adjusted to the appropriate pH. In some experiments, a radiochemical was then added as a tracer. In radiolabelled experiments, enough [U-ring-^{14}C]p-HBA was added to bring 1 ml of treatment solution within the range of 15 000–20 000 cpm (approximately 0.01 μCi/ml). Labelled FA solutions ranged from 1000 to 2000 cpm/ml (approximately 0.001 μCi/ml). The contribution of radiochemical to the treatment concentration of phenolic acid was considered insignificant.

Bulk treatment solutions (100 ml) were added to autoclaved bottles and an initial sample of the individual solutions was taken. One seedling was transferred into each prepared bottle. Solutions were sampled over a 6 hr period beginning with 0.5 hr. At each sampling time, 1.0 or 0.2 ml was removed. These samples were either passed through an 0.22 μm filter and stored in a freezer (1 ml samples) for later HPLC (Waters, Milford, MA, USA) analyses or placed into vials (0.2 ml samples) for liquid scintillation spectrophotometry (Beckman, Fullerton, CA, USA). The counting efficiency for radioactive samples was always within the range of 82–87%.

After the last sampling, seedlings were removed and the final pH and volume of treatment solutions noted. Seedlings were desorbed in 100 ml of nutrient solution or, for radioactive experiments, in unlabelled solutions of the same pH and phenolic acid concentration as the treatment. Desorption solutions were sampled after 20 min. Less than 1% of the absorbed phenolic acid was found in the desorption solutions of unlabelled experiments as determined by HPLC. Samples from the desorption solutions of seedlings exposed to radiochemicals were equal to or below background levels of radioactivity even when 5–10 ml samples of solution were concentrated and analysed.

Analysis of whole plant material for radiotracers. Seedlings used in radioisotope depletion studies were separated into root, stem, cotyledons and leaves, and freeze-dried. Dry seedlings were weighed and ground. Subsamples of approximately 50 mg were placed in scintillation vials with 0.5 ml of peroxide (30%) and left to bleach in the light until dry. EtOH (2 ml, 95%) was added to each, and the capped vials incubated at 40–50° overnight [19]. Scintiverse (Fisher, Springfield, NJ, USA) cocktail was added and the radioactivity determined by liquid scintillation spectrophotometry.

Excised root studies. Root segments (0.1 g) were excised and placed on ice in cheesecloth bags. Bags were then submerged in bulk treatment solutions which were prepared as in the depletion studies. At each sampling time, three bags per treatment were removed and rinsed for 20 sec in unlabelled solutions. Bags were then desorbed for 20 min in a fresh, unlabelled solution. The excised roots were removed, macerated in scintillation vials with cocktail, and assayed for radioactivity. Other untreated root segments were weighed fresh, oven (65°) or freeze-dried (−55°), and reweighed to obtain a fr./dry wt conversion factor.

Mixture experiment. Seedlings were placed in solutions containing 0.25, 0.50, or 1.00 mM FA alone, 0.25 or 0.50 mM *p*-HBA alone, and all combinations of the two at those concentrations. Radiotracers of both phenolic compounds (at the before defined specific activities) were utilized in separate experiments. Uptake was determined by the previously described whole plant methods.

Multiple exposure experiment. One set of seedlings was exposed twice (at 48 hr intervals) to solutions of 0.5 mM FA, pH 5.5. These exposed seedlings along with another set which had not received any FA treatment were then placed in 0.5 mM FA solutions labelled with [U-ring-^{14}C]FA. Uptake was calculated from ^{14}C-analysis of the treatment solution.

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