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Characterization and localization of plant phenolics likely involved in the pathogen resistance expressed by endomycorrhizal roots

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Abstract. Phytochemical characterization of the major phenolic compounds and their ultrastructural localization were carried out on onion roots (*Allium cepa* L.) colonized by two vesicular-arbuscular mycorrhizal (VAM) fungi: *Glomus intraradix* Schenck & Smith and *G. versiforme* (Karst.) Berch. Free and wall-bound forms of phenolic components were quantified in relation to the duration of symbiosis. Both ferulic and *p*-coumaric acids, as well as *N*-feruloyltyramine were identified as the major phenolic metabolites bound to the cell walls of VAM onion roots. Results from mycorrhized and control plants suggest the presence of a mechanism leading to the oxidative condensation of phenols, the latter process depending on the presence or absence of symbiosis. Bioassays reveal that *N*-feruloyltyramine induces the branching of hyphae and reduces total fungal development. The overall results lead us to suggest that the progressive binding of phenolic compounds in VAM roots is directly involved in the control of VAM endophytic establishment and development, as it gradually reduces the plasticity and elasticity of the symbiotic matrix. Phenolic compounds bound to cell walls could also be indirectly responsible for the resistance of VAM roots to pathogenic fungi, since they result in increased resistance by the cell wall to the action of digestive enzymes.

Key words: Vesicular-arbuscular mycorrhizae – Phenolic metabolism – Cinnamoyl amides – Ultrastructural localization – Disease resistance

Introduction

Phenylpropanoid compounds are secondary metabolites of ubiquitous occurrence in the plant kingdom (Harborne 1980). They are derived from phenylalanine by enzymatic deamination and substitution of the aromatic ring, giving rise to a family of cinnamic acids and related derivatives such as the cinnamoyl amides found in several plant families (Martin-Tanguy et al. 1978). Accumulation of phenylpropanoid compounds in plant cells contributes to a number of different metabolic functions, the nature of which depends on the molecular structure and compartmentation of these compounds (Hahlbrock and Scheel 1989). In fact, phenolic compounds may act as antimicrobial agents (Bailey and Mansfield 1982), signaling molecules modulating plant-microorganism interactions (Lynn and Chang 1990), or polymeric constituents of plant cell walls which affect their digestibility and plasticity (Fry 1986). Despite their constitutive expression, which is concomitant with cell ontogenesis, the relative location and concentration of phenylpropanoid derivatives may vary in response to cell invasion by microorganisms. Such variation results from enzymatic activities of phenylpropanoid biosynthesis, including group substitution, oxidation, condensation and/or polymerization, and may lead to increased resistance of plants to pathogens. Previous studies on vesicular-arbuscular mycorrhizal (VAM) fungi have dealt with the effect of the symbiotic association on phenolic metabolism of the host roots, with the aim of investigating the possible role of phenolics in protecting VAM roots against pathogenic fungi. However, the reported results on this specific topic are very inconsistent. For instance, both oxidation and polymerization of tomato root phenols have been reported to increase following VAM association and were accompanied by increased lignification of VAM roots (Dehne and Schönbeck 1979). Krishna and Bagyaraj (1984) reported a continuous increase of total soluble phenols in VAM roots of *Arachis hypogaea*, whereas Codignola et al. (1989) reported no variation of phenylalanine ammonia lyase activity, the first enzyme in phenolic biosynthesis, and that wall-bound phenols in leek roots changed neither quantitatively nor qualitatively after the establishment of symbiosis.

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Therefore, the present work was aimed at investigating the nature of the major phenolic compounds of onion roots as well as their relative concentration and localization in relation to the process of mycorrhization. The results lead us to suggest a model which depicts the alteration of phenolic metabolism in roots as induced by VAM fungi. In addition, this model is discussed in relation to the control of VAM endophytic establishment and the increased resistance of onion roots to pathogenic fungi.

Materials and methods

Mycorrhized plant material

Seeds of onion (*Allium cepa* L. cv. Improved Autumn Spice No. 202), obtained from Stokes Seeds Ltd., St. Catharines, Ontario, were surface sterilized for 2 min in 2% sodium hypochlorite. They were sown in a row, 5 cm from 1-year-old leek plants previously mycorrhized with either *Glomus intraradix* (DAOM no. 181602) Schenck & Smith or *G. versiforme* (Karst.) Berch (DAOM no. 196673). Onion seedlings were fertilized weekly with 4 l m^{-2} of Long Ashton solution, and their growth was maintained at day/night temperatures of 21/16° C, a light intensity of 330 μmol s^{-1} m^{-2}, a 16-h photoperiod and 70% relative humidity. After 21 weeks of growth, root colonization of the seedlings was estimated to be 80% using standard staining and counting procedures (Plenchette et al. 1982). Uninoculated control plants were treated following the same protocol.

Reagents

Bovine serum albumin, molecular mass markers for SDS-PAGE, naringinase, *p*-nitrophenol-α-L-rhamnopyranoside and polyethylene glycol 20000 were purchased from Sigma Chemical Co., St. Louis, Mo. *p*-Coumaric, ferulic, and sinapic acids and tyramine were from Aldrich Chemical Co., Milwaukee, Wis. β-Glucosidase was purchased from Apin Chemicals Ltd., South Wales. [2-^{14}C]-cinnamic acid (specific activity of 3 mCi $mmol^{-1}$) was obtained from ICN Chemicals, Irvine, Calif., and tetrachloroauric acid from BDH Chemicals, Montreal, Québec.

Preparative extraction and purification of soluble phenolics

Ten kilograms of 4-month-old onion roots was washed under tap water. Aliquots of 1 kg were then homogenized in a blender with 1 l 90% aqueous methanol and the homogenate was extracted for 12 h in the dark at 4° C. After filtration, the methanolic extract was evaporated under reduced pressure (40° C) to about 200 ml and the aqueous residue was designated the "total soluble phenols". The latter was defatted with 3 × 50 ml chloroform and then extracted with 2 × 100 ml ethyl acetate. The organic layer was evaporated, stored at −80° C and designated the "total soluble phenol-ethyl acetate extract" (TSPE). The aqueous residue from the ethyl acetate extractions was lyophilized, kept at −80° C and designated the "aqueous residual fraction" (AR).

HPLC methods

Aliquots from the TSPE and AR fractions were solubilized in 15% aqueous methanol containing 0.5% acetic acid, filtered through 0.45-μm membrane filters, and chromatographed on a RP Nova-Pak C_{18}, 4 μm, Radial-Pak cartridge (8 mm × 100 mm, Waters) with an aqueous methanolic gradient containing 0.5% acetic acid as eluent. The gradient was as follows: 0 to 3 min, 15% methanol, isocratic; 15 to 70% methanol over 20 min; 70 to 100% methanol over 1 min; 100% methanol, isocratic for 4 min; followed by 15% methanol in 5 min which was further maintained for 7 min. Sample elution was driven by a pump system Model 510 (Waters) equipped with a 2-ml super-loop injection valve connected in series to an absorbance detector Model 440 and an LC Spectrophotometer Lambda Max Model 481 (Waters). Detection of peaks was carried out simultaneously at 254 and 340 nm. Subfractions corresponding to the major peaks were collected and further purified in the appropriate isocratic modes.

Hydrolysis of root phenolics

Each HPLC-purified fraction was lyophilized and an aliquot was solubilized in 4 μl of dimethylsulfoxide to which 100 μl of distilled water was added. Each sample was subjected to acid, alkaline and enzymatic (naringinase) hydrolysis. Acid hydrolysis was performed by adding 950 μl 1 N HCl to 50 μl of the eluate and heating at 60° C under nitrogen for 30 min. Alkaline hydrolysis was assayed by adding 950 μl 1 N NaOH to 50 μl of eluate and standing at 60° C under nitrogen in the dark for 30 min. Purified eluates in 50-μl aliquots were incubated with 950 μl of the purified enzyme (naringinase, 1000 U in phosphate buffer, pH 7) for 1 h at room temperature. Assays were performed in triplicate and samples with boiled naringinase served as controls. Following acid and enzymatic hydrolyses, the mixtures were frozen, lyophilized, dissolved in a minimum amount of methanol, and brought to 15% aqueous methanol containing 0.5% acetic acid. Samples hydrolyzed under alkaline conditions were acidified and directly extracted with ethyl acetate. After evaporation of the organic solvent, the residue was solubilized in methanol and chromatographed by HPLC.

Determination of molecular structures

The HPLC-purified compounds were subjected to NMR analysis using a Bruker instrument AM-300 at 300.13 MHz for proton (^{1}H) and 75.47 MHz for ^{13}C spectra at 298° K in deuteriated methanol. ^{1}H/^{19}F probes, 5 and 10 mm, were used for ^{1}H and ^{13}C analyses, respectively. References for the chemical shifts (solvent lines) were 3.3 ppm for ^{1}H and 49 ppm for ^{13}C, respectively. Thus, ^{1}H, ^{1}H two dimensions (^{1}H × ^{1}H), ^{13}C multiplicity, ^{13}C-^{1}H direct correlation and long-range correlation spectra were obtained and used as the main criteria for the confirmation of molecular structures as compared with those of standard spectra. The mass spectrum of *N*-feruloyltyramine, solubilized in acetonitrile, was determined with a gas chromatograph-mass spectrometer (GC-MS) Model 5970 (Hewlett-Packard). The gas chromatograph was equipped with an apolar capillary column (Hewlett-Packard High Performance No. 1, phase thickness of 0.33 μm, internal diameter of 0.2 mm, 25 m long). The optimum chromatographic parameters were as follows: initial temperature 75° C; increment rate 10° C min^{-1}; final temperature 325° C; total time 35 min. The quadrupole mass spectrometer was equipped for molecular fragmentation by electron impact (EIM). Mass spectral data between 70 and 800 Da were collected at a scanning frequency of 1.17 s. UV spectra of the identified native compounds and of authentic analogs were recorded in methanol with a UV-visible spectrophotometer Model DMS 100 (VARIAN) using 1-cm-path quartz cells.

Chemical synthesis of the cinnamoyl amide

N-feruloyltyramine was synthesized by refluxing a mixture of 1.08 g ferulic acid, 7 ml of thionyl chloride ($SOCl_2$) and a drop of dimethylformamide for 45 min. The mixture was cooled in an ice bath to 15° C before adding, in several aliquots, 35 ml of a 20% NaOH solution containing 1 g tyramine. The mixture was vigorously shaken for 5 min then poured in a separating funnel and extracted with ethyl acetate. The organic layer was washed with a saturated aqueous NaCl solution and then dried over anhydrous Na_2SO_4. The ethyl acetate extract was evaporated and the cinnamoyl amide residue was dissolved in 15% aqueous methanol for further preparative chromatography on an RP C_{18} HPLC column following the elution procedures previously described. The synthetic molecule was subjected to ^1H NMR and UV spectral analyses.

Incorporation of [2-^{14}C]cinnamic acid into root phenolics

Segments (ca. 500 mg) of 12 to 24-week-old roots were incubated with 2 μCi labeled cinnamic acid for 2 h. After washing, the root segments were ground in a mortar with 80% methanol and extracted for 60 min at room temperature. The methanolic extract was reduced by evaporation and brought to 1 ml for further chromatographic analyses. Aliquots of the latter extracts were subjected to HPLC in the gradient mode, and eluates were recovered every 30 s in separate vials, for 20 min. Liquid scintillation cocktail (Ready-Protein, Beckman) was added to 1 ml fractions of the eluate and measured for radioactivity.

Quantification of soluble and wall-bound root phenolics

Onion roots were collected from 5-, 12-, and 21-week-old mycorrhized and nonmycorrhized plants. Root samples were harvested from 15 plants each in order to give three replicates according to age and symbiotic status. Statistical analysis of the results was carried out using Duncan's multiple range test.

Quantification of soluble phenols. Root samples were frozen, lyophilized and ground to a powder. Aliquots (35 mg) of this material were extracted in methanol for 60 min, filtered and the residues successively washed with 50 ml methanol and 50 ml acetone. The combined filtrates were reduced in a rotary evaporator at 40° C and the residue brought to a final volume of 3.5 ml with 15% aqueous methanol containing 0.5% acetic acid. After solvent-solvent extraction of this solution with 25 ml chloroform, an aliquot of the aqueous phase was analysed by HPLC. The concentration of identified compounds was determined from a multipoint calibration curve calculated on the basis of peak area compared to the concentration of standards.

Quantification of bound phenols. The dry residue remaining after extraction of soluble phenolics was suspended in 3.5 ml of 1 N NaOH and allowed to react for 40 min at 60° C in the dark under nitrogen, according to Harris and Hartley (1980). The mixture was acidified with 3.7 ml of 1 N HCl and extracted with ethyl acetate. The organic layer identified as "total wall-bound phenols" was evaporated and the residue dissolved in 3.5 ml of a 15% aqueous methanolic solution containing 0.5% acetic acid. An aliquot of this hydrolysate was analysed for phenols by HPLC. Identification of peaks of both soluble and bound phenols was confirmed by co-elution with the appropriate reference compounds in both gradient and isocratic modes and by comparison of their UV spectra.

In situ localization of root phenols

Purification of naringinase. Commercial naringinase was purified by gel filtration followed by anion-exchange chromatography. The protein fractions were collected for naringinase assay using *p*-nitrophenyl-α-rhamnopyranoside as the chromogenic substrate (Romero et al. 1985), and the active fraction was subjected to SDS-PAGE (Laemmli 1970) using 12% acrylamide gels. The molecular mass of the purified polypeptide was determined to be 68 kDa.

Naringinase specificity towards phenolic extracts. In order to characterize the specificity of naringinase towards onion root phenolics, the AR fraction was incubated in the presence of the purified enzyme for 60 min at room temperature. The reaction mixture was lyophilized and the products dissolved in a minimal amount of methanol. The methanol extract was then subjected to HPLC in the gradient mode.

Preparation of the naringinase-gold complex. The colloidal gold suspension was prepared according to the method of Frens (1973) with 15±2 nm gold particles. Accordingly, 5 ml of 0.2% aqueous tetrachloroauric acid was added to 95 ml of distilled water and allowed to boil for 2 min. Optimal pH and the minimal amount of protein were determined according to standard methods (Bendayan 1987) and found to be pH 6 and 10 μg of protein/ml of gold sol, respectively. Consequently, 50 μg naringinase was mixed with 5 ml of the colloidal gold at pH 6. The mixture was centrifuged at 25000 rpm using a SS34 rotor for 60 min at 4° C. The resulting red pellet was resuspended in 0.5 ml phosphate-buffered saline (PBS) pH 7, containing 0.02% polyethylene glycol 20000 (PBS-PEG) and used as the stock solution.

Cytochemical labeling

Root segments (1 mm) were fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 for 2 h at 4° C, then carefully rinsed with the same buffer. After dehydration in an ethanol series, samples were embedded in Epon 812, and ultrathin sections (60 nm) were mounted on Formvar-coated nickel grids. Sections were first floated on a drop of PBS-PEG, pH 7 for 10 min, then incubated for 30 min with a drop of naringinase-gold complex (diluted in 0.1 M PBS, pH 7 to give an OD of 0.5 at 525 nm). Grids were washed with PBS, pH 7 rinsed with distilled water, and then contrasted with uranyl acetate and lead citrate before being examined in a Siemens Elmiskope 102 electron microscope at 80 kV. Final plates were reproduced with TMAX 100 Kodak film. Specificity of labeling was assessed using the following control tests: (i) incubation with naringinase-gold complex that was previously incubated with its substrate, naringin (1 mg ml^{-1} in PBS); (ii) incubation with naringinase followed by naringinase-gold complex; (iii) incubation with stabilized gold suspension alone; (iv) incubation with bovine serum albumin-gold complex. Labeling and control experiments were repeated on 20 ultrathin sections.

Spore germination assays

Ri T-DNA carrot roots, transformed by *Agrobacterium rhizogenes* (Riker) Conn. and mycorrhized by *G. intraradix* according to Bécard and Fortin (1988), were obtained from Mrs Sylvie Fortier, Centre de recherche en biologie forestière, Université Laval, Québec. Synchronized clusters of spores, each containing 5 to 10 units, were transferred on minimal media containing 1.5% Noble agar in distilled water, 200 μg ml^{-1} apple pectin, pH 6.5 (control medium A), which was supplemented with 44 μg ml^{-1} *N*-feruloyltyramine (medium B). Each assay was performed using 10 re-

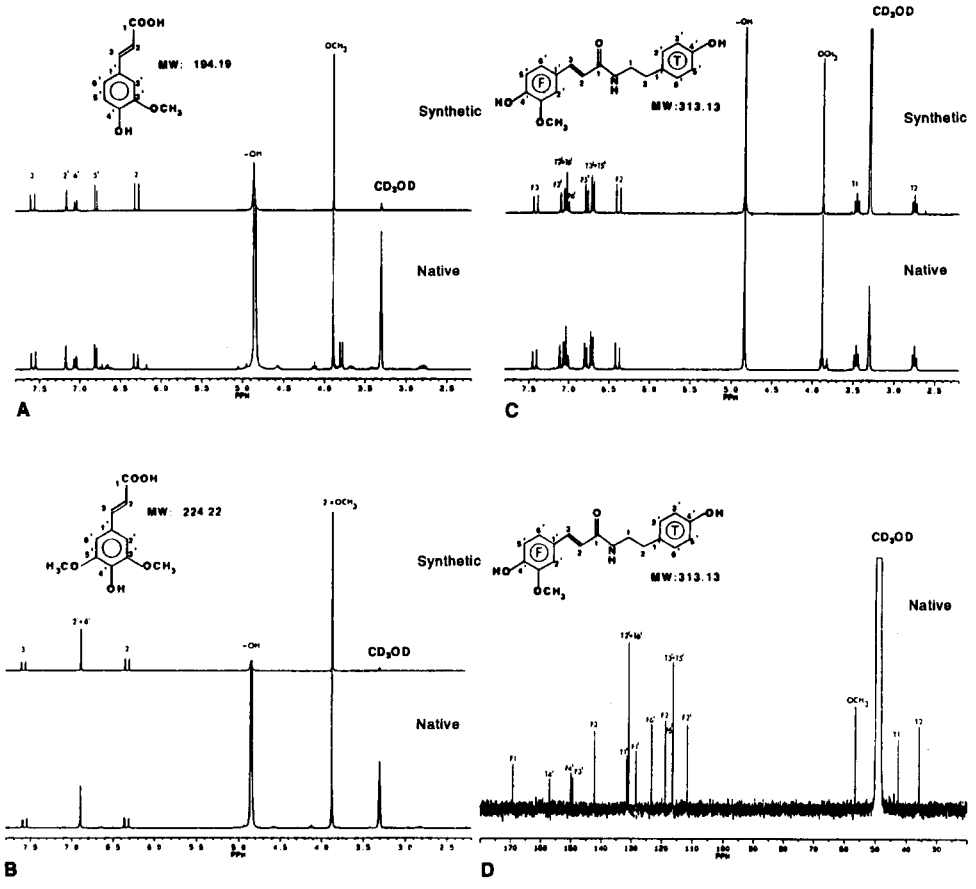

Fig. 1. Proton (^{1}H) NMR spectra of synthetic and native ferulic acid (**A**), sinapic acid (**B**), and *N*-feruloyltyramine (**C**). **D** (^{13}C) NMR spectrum of *N*-feruloyltyramine as purified from onion roots

plicates for 30 days at room temperature in the dark. Spore germination and mycelial growth were observed under a stereomicroscope. Mycelium growth was drawn manually on the lid of the Petri dishes and photographed at different magnifications. Photographs of the hand-drawn mycelial growth were taken with Kodalith orthofilm type 3.

Results

Identification of the major phenolics of onion roots

The major soluble compounds extracted from onion roots and further purified by HPLC were identified as ferulic and sinapic acids, their respective glycosides, *N*-feruloyltyramine and an unidentified *N*-feruloyltyramine glycoside. Confirmation of the molecular structures of the aglycones was obtained by NMR analysis which was performed on the hydrolyzed and synthetic compounds (Fig. 1). EIM spectral data for *N*-feruloyltyramine were as follows: M^+ *m/z*: 313.13; MS *m/z* (relative abundance $\times 10^{-2}$): 313 (M^+, 31), 194 (43), 193 (75), 192 (74), 177 (feruloyl moiety, 100), 145 (45), 120 (26), 77 (20), 62 (25). UV spectra λ_{max}(EtOH) nm ($\log\epsilon$) for *N*-feruloyltyramine were 219 (4.29), 228 (4.21), 295 (4.13) and 321 (4.22). HPLC retention times of the compounds obtained before and after hydrolysis are shown in Table 1.

The extraction of total wall-bound phenolics from the residual root tissues yielded two of the aglycones previously identified as soluble phenols, namely ferulic acid and *N*-feruloyltyramine. In addition, co-elution of the native *p*-coumaric acid in both the gradient and the isocratic modes and comparison of the UV spectra with a synthetic sample confirmed the presence of this compound as bound to the cell walls. Moreover, sinapic acid was not found as a bound phenolic, and naringin and naringenin were found in neither the native nor the hydrolyzed extracts. Furthermore, there were no qualitative differences between the phenolic patterns of mycorrhized and nonmycorrhized roots.

Table 1. Retention times (min) of native phenolics and their aglycones recovered after hydrolysis. *S*, Sinapic acid; *F*, ferulic acid; *FT*, *N*-feruloyltyramine; *ND*, not determined

Peak	Native		Hydrolysate			
	Gradient	Isocratic	HCl	KOH	Naringinase	Identification
I	10.5	5.7[a]	14.9[c]	14.9[c]		S
		6.2[a]	14.9[c]	14.9[c]		S
II	12.2	10.9[a]	14.6[c]		14.6[c]	F
		11.7[a]	14.9[c]		14.9[c]	S
III	15.7	7.4[b]	9.3[d]		15.7[c]	ND
			10.7[d]		18.6[c]	FT
IV	18.6	10.7[b]				FT

[a] Acetic acetonitrile/water 90/10, resolving into two peaks
[b] Acetic acetonitrile/water 78/22
[c] Gradient as described in Materials and methods
[d] Isocratic as in [b]

Incorporation of [2-^{14}C]cinnamic acid into soluble phenolics of onion roots

[^{14}C]Cinnamic acid was actively metabolized by 12-week-old mycorrhized and nonmycorrhized onion roots to ferulic acid, sinapic acid, and *N*-feruloyltyramine, among other unidentified compounds (Table 2). The qualitative pattern of label incorporation was quite similar to that of the 21-week-old roots. In contrast to nonmycorrhized roots, the incorporation of cinnamic acid label into all soluble phenolics was much lower in mycorrhized roots of corresponding ages, except for the *N*-feruloyltyramine, in which levels of radioactivity were equivalent to those found in control roots. These results clearly indicate that mycorrhization does not stimulate the biosynthesis of these metabolites but may instead accelerate their biochemical turnover.

Quantification of phenolic compounds

Both soluble and bound forms of *N*-feruloyltyramine were quantified at different growth stages of both mycorrhized and control roots (Fig. 2A, B), whereas only the bound forms of ferulic and *p*-coumaric acids were evaluated (Fig. 2C, D). The results indicate that whereas the soluble *N*-feruloyltyramine increases with time in nonmycorrhized roots, its concentration remains relatively low in the mycorrhized tissue (Fig. 2A). In contrast, the concentration of wall-bound *N*-feruloyltyramine remains constant in control roots, whereas it increases significantly with time in mycorrhized roots (Fig. 2B). The concentration of wall-bound ferulic acid increased with time in mycorrhized roots, but remained consistently low in control roots (Fig. 2C). Moreover, the concentration of wall-bound *p*-coumaric acid exhibited a slight increase with age in control roots, when compared to the strong augmentation observed in mycorrhized roots. In both types of symbiosis, *p*-coumaric acid concentration reach a maximal value at the 21st week, the highest values being observed for the roots colonized by *G. intraradix* (Fig. 2D).

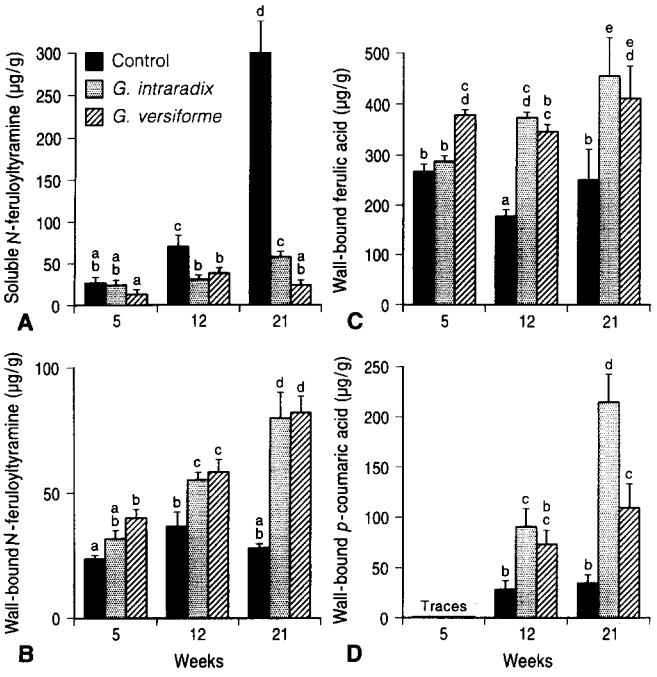

Fig. 2. Concentration of soluble (**A**) and wall-bound (**B**) *N*-feruloyltyramine, and wall-bound ferulic (**C**) and *p*-coumaric acids (**D**) measured in onion roots on a dry weight basis either nonmycorrhized or endomycorrhized by *Glomus intraradix* or *G. versiforme*, 5, 12, and 21 weeks after sowing. Columns with different letters represent means of triplicates which are different at the 5% level of probability using Duncan's multiple range test. *Vertical bars* represented standard deviations

Specificity of naringinase towards phenolic glycosides

Naringinase is known for its specificity toward phenol-α-glycoside linkages such as naringin (naringin 7-*O*-rhamnoglucoside). It sequentially cleaves the rhamnose and glucose moieties to successively yield prunin and finally the aglycone naringenin (Romero et al. 1985). Incubation of the "phenolic-glycoside" fraction (AR) with naringinase yielded a hydrolysate containing high levels of ferulic acid, sinapic acid and *N*-feru-

Table 2. Incorporation of [2-^{14}C]cinnamic acid into phenolic compounds by control and endomycorrhized onion roots. Fractions were collected at 30-s intervals. Fractions not corresponding to an identified compound and with less than 1000 dpm are not reported here. The mean background was 30 dpm. *g*, Glycoside; other abbreviations as in Table 1

Fraction number	Phenolic compound	Radioactivity (dpm)			
		Control		Endomycorrhized	
		12 weeks	21 weeks	12 weeks	21 weeks
19	Sg	409	1697	66	125
23	Fg, Sg	130	287	76	64
28	F, S	4736	5815	146	1025
30	FTg	1019	684	49	125
35	FT	5386	6996	6539	4993
% of total		47	46	64.6	57.8
Total (dpm)		24836	33490	10650	10949

Table 3. Relative amounts of phenolic aglycones released after naringinase hydrolysis. Abbreviations as in Table 1

Retention time[a] (min)	Aglycone	Relative amount[b] (%)
14.50	F	100
14.76	ND	50.17
14.93	S	192.35
18.60	FT	102.67
18.88	ND	187.55

[a] Peaks obtained in gradient mode, which show an increased surface area after hydrolysis

[b] Calculated as % increase of peak surface area after hydrolysis

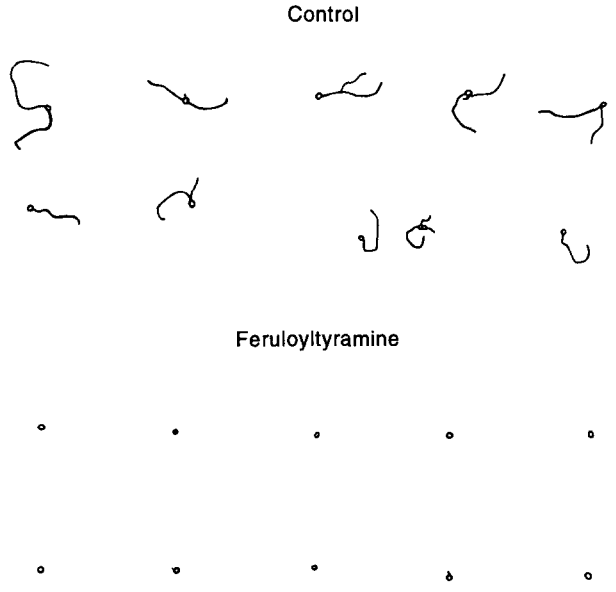

Fig. 3. Tracing of the mycelial growth from *G. intraradix* spores after 1 month of culture in Petri dishes in the absence (*control*) or presence of *N*-feruloyltyramine (44 μg ml^{-1}). The 10 replicates of each treatment shown indicate that *N*-feruloyltyramine almost completely inhibits the linear hyphal growth that is otherwise consistently observed in control replicates. The illustration is 25% of the actual size

loyltyramine, as well as two unidentified compounds, (Table 3). Moreover, naringinase hydrolysis did not liberate any of the compounds whose retention times exceeded 20 min in the gradient mode. The bound forms of the two unknown glycosides were not detected in the extracts of wall-bound phenolics.

Characterization of the developmental response of a VAM fungus to N-feruloyltyramine

The cinnamoyl amide *N*-feruloyltyramine strongly reduced growth of the endomycorrhizal fungus *G. intraradix* when added at twice the concentration found in 5-week-old mycorrhized roots (Fig. 3). The reduced hyphal development observed on media containing *N*-feruloyltyramine was consistently characterized by an intensely branched hyphal growth, which contrasts with the extended linear development of hyphae observed on the control media (Fig. 4).

Localization of onion root phenolics

The cytochemical labeling of mycorrhized roots was performed in order to determine the subcellular locali-

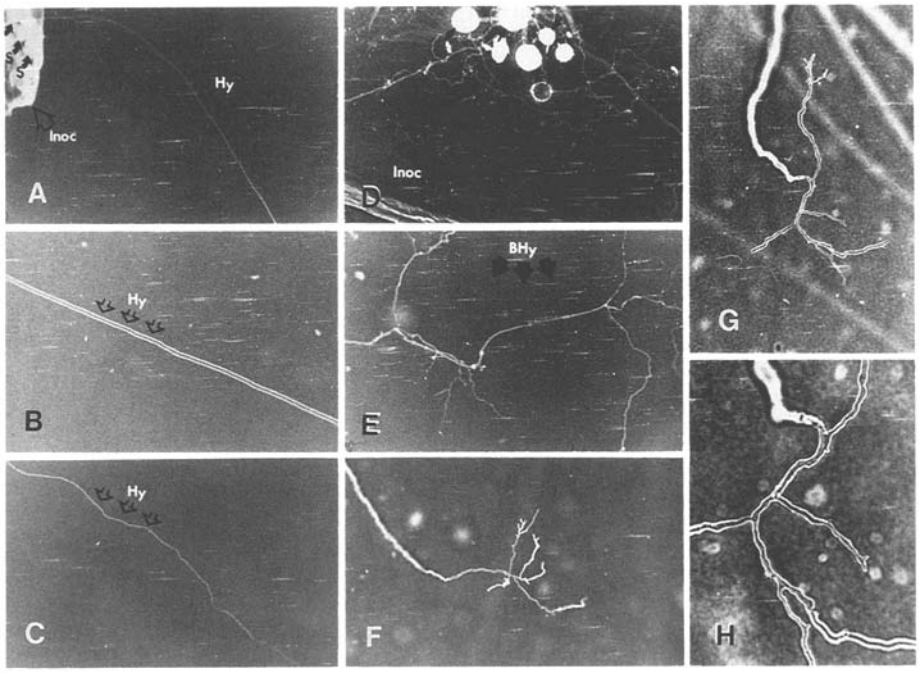

Fig. 4A–H. Photomicrographs of fungal development resulting from the presence or absence of *N*-feruloyltyramine. **A**, **B**, **C** Fungal development on control medium. **A** Part of the inoculum (*Inoc*) consisting of agar containing synchronized spores of *G. intraradix* (derived from a Ri T-DNA system) is visible in the upper-left corner. Because of the photographic procedure, spores (*s*) and hyphae (*Hy*) appear white. Note the extended linear growth of the hyphae. ×40. **B** This photomicrograph clearly reveals the coenocytic feature of the hypha (*Hy*) which is characteristic of zygomycetes such as vesicular arbuscular endomycorrhizal fungi. ×100. **C** The end section of the same hypha reveals a less linear pattern of hyphal growth before development ceases. ×40. **D–H** Fungal development on medium containing *N*-feruloyltyramine (44 μg ml^{-1}). **D** shows the inoculum (piece of agar containing a cluster of synchronized spores) with seven spores (*arrows*) surrounded by a network of branched hyphae whose development was posterior to the inoculation. ×60. **E** shows one of the rare hyphae reaching the experimental medium outside of the inoculum (*Inoc*). ×60. Hyphal development in all 10 replicates did not extend more than 2 mm from the inoculum and the hyphae were always intensely branched (*BHy*), as in the figure. **F–H** A terminally branched hypha at increasing magnification. Note the coenocytic feature of the terminal branches. **F** ×100; **G** ×200; **H** ×400

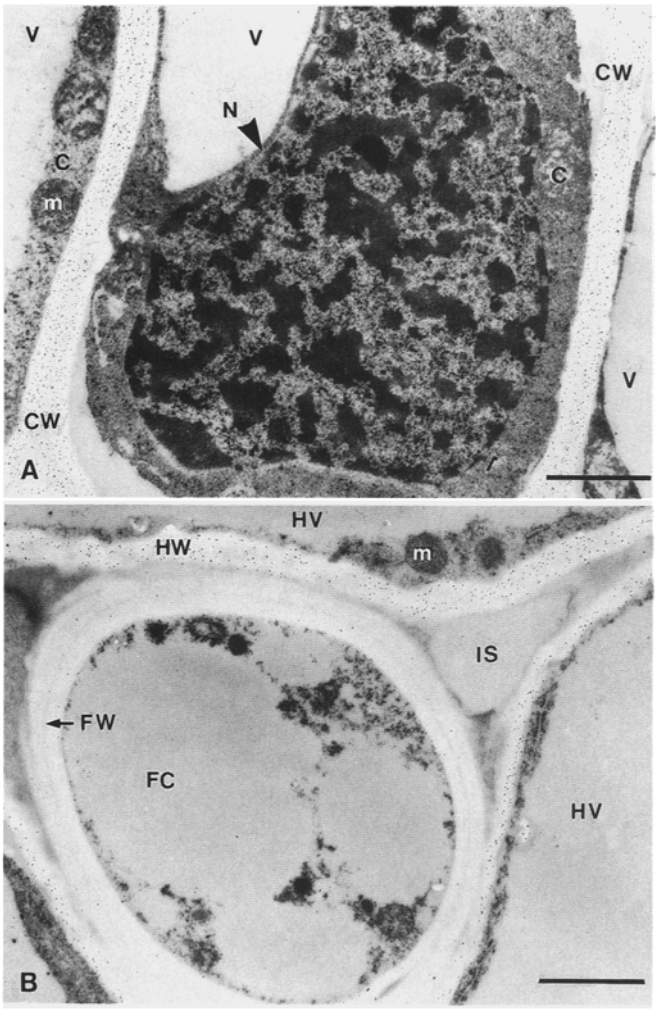

Fig. 5A, B. Transmission electron micrograph of endomycorrhized onion root. **A** A cell of the stele (*St*). The cell wall (*CW*) is strongly labeled, whereas cytoplasm (*C*), vacuoles (*V*) and the nucleus (*N*) are devoid of gold particles. ×20000. **B** Intercellular hypha surrounded by cell walls of cortical cells (*HW*). Note the regular labeling of the host wall (*HW*) structure, whereas the fungal wall (*FW*) is not significantly labeled. Almost no gold particles can be detected over plant and fungal cytoplasms (*HC*, *FC*), host vacuoles (*V*) or intercellular spaces (*IS*). ×20000. *Bars* 1 μm

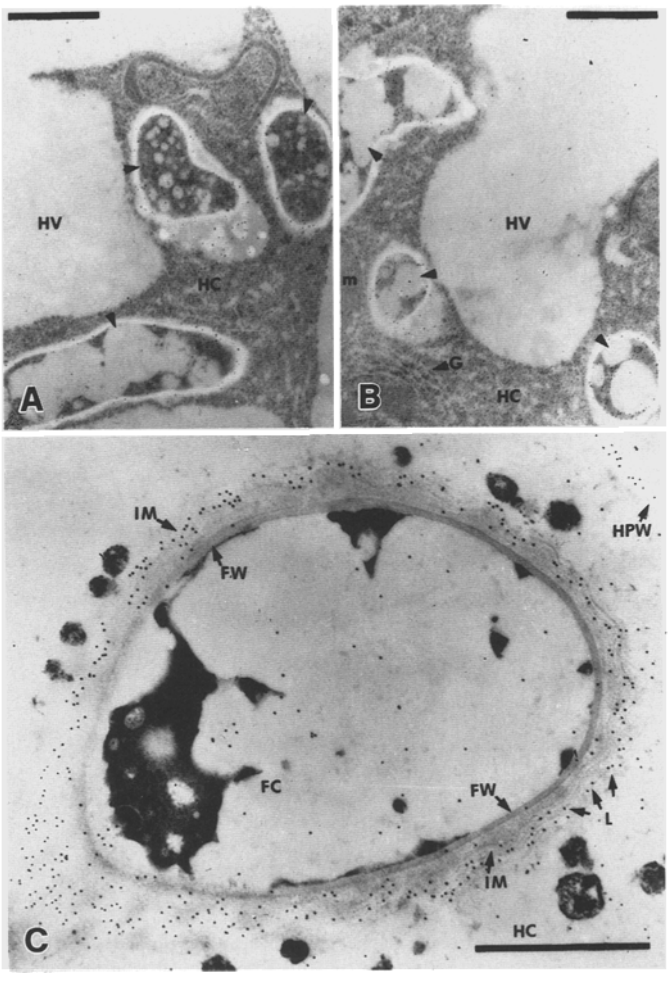

Fig. 6A–C. Transmission electron micrographs of cortical cells of endomycorrhized onion root containing fungal arbuscular structures. **A**, **B** Portions of the host cytoplasm (*HC*) containing young and biologically active arbuscular structures (*arrows*) with a granular cytoplasmic content, surrounded by an electron-light layer of material apparently derived from the host cytoplasm (*HC*). Mitochondria (*m*) and Golgi apparatus (*G*) can be observed in the host cytoplasm (*HC*) near the arbuscular structures. Gold particles are sparsely distributed over the host cytoplasm (*HC*) surrounding the arbuscule. The labeling is more intense over the electron-light material accumulated around the arbuscule and is at a maximum over the fungal cytoplasm (*FC*). ×20000. **C** Photomicrograph of a transversely sectioned old arbuscular trunk located in a cortical cell. A segment of the host primary wall (*HPW*) is visible at the upper-right corner of the photomicrograph. The rest of the figure shows an elliptical structure (the arbuscular trunk) composed of an internal homogeneous layer which is the fungal wall (*FW*). The numerous laminations (*L*) which seem successively deposited and encapsulate the fungal wall (*FW*) correspond to the interfacial material (*IM*) synthesized by the host. Gold particles are specifically located over the host primary wall (*HPW*) covering the interfacial material (*IM*), in perfect alignment with the irregular feature of the layered deposit. The residual host (*HC*) and fungal (*FC*) cytoplasm are not significantly labeled. ×37500. *Bars* 1 μm

zation of the compound(s) which exhibited positive naringinase hydrolysis. The results obtained using the naringinase-gold probe revealed a consistent association of gold particles with the walls of stelar (Fig. 5A) and cortical (Fig. 5B) cells, whereas the cytoplasm and vacuolar contents of these cells were not significantly labeled. No significant labeling was observed in the cell wall or cytoplasm of the intercellular hyphae (Fig. 5B). Among the symbiotic structures observed, young arbuscules exhibited an intense labeling over their cytoplasm (Fig. 6A, B). Moreover, the host cytoplasm surrounding these hyphae showed a diffuse labeling which was more intense over the electron-light material accumulated around the hyphal structures. This material seems to be synthesized by the host and may, therefore, correspond to the building units of the interfacial matrix. Mitochondria and Golgi bodies were usually observed near the arbuscular structures in the host cytoplasm, suggesting the presence of high biosynthetic

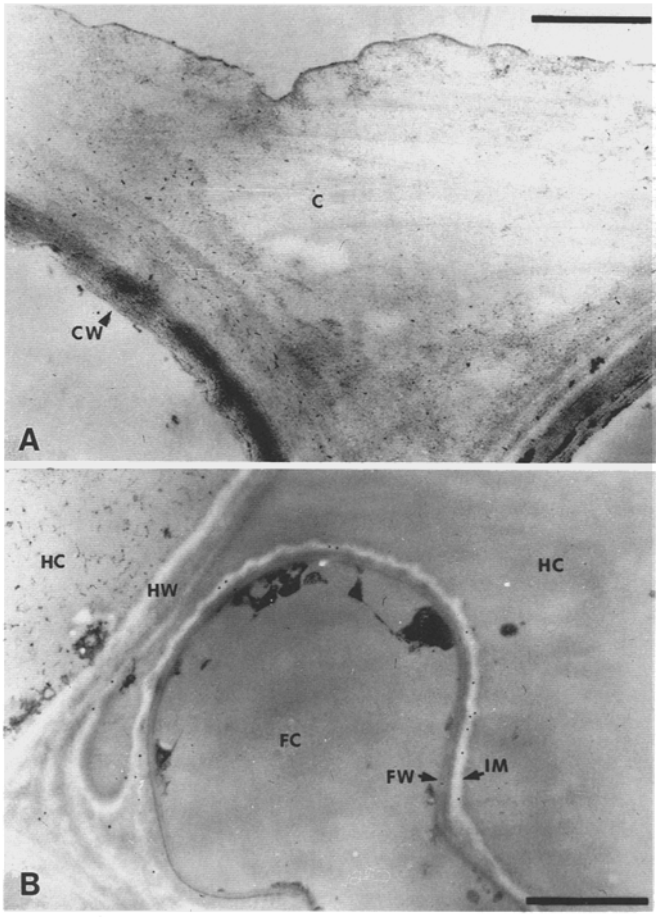

Fig. 7. A Transmission electron micrograph of epidermal cells of an endomycorrhized onion root showing the wall of epidermal cells (*CW*) and their outer cuticle (*c*). These structures were consistently devoid of gold particles. ×25000. **B** Transverse electron micrograph of a control sample performed on an arbuscular structure located inside a cortical cell. The fungal wall (*FW*) and the interfacial material (*IM*) are clearly visible. Moreover, at the lower-left corner, a structural continuity is visible between the cell wall (*CW*) and the interfacial material (*IM*) suggesting the host origin of the latter. The intense labeling usually observed over these two structures is strongly reduced by the control treatments; only few gold particles are still detectable. ×25000

activity. Old arbuscular trunks (Fig. 6C) were surrounded by a thick, laminated interfacial material. The latter is a result of a cumulative and chronological process of deposition of pseudo-parietal material derived from the host and containing cellulose and pectin (Bonfante-Fasolo et al. 1990). This structure was always intensely labeled by the naringinase-gold probe, whereas the hyphal wall was devoid of gold particles. Furthermore, the cell walls of the epidermis as well as the outer cuticle were consistently devoid of gold particles (Fig. 7A). Control tests confirmed the specificity of the probe for some phenol-glycoside linkages similar to naringin (5,7,4′-trihydroxyflavanone 7-α-L-rhamnoglucoside), as indicated by the significant reduction in gold labeling over the interfacial material and cortical cell walls (Fig. 7B).

Discussion

The whole set of experiments designed to quantify and localize the root phenolics was repeated twice on *A. cepa* L., and once on *A. porrum* L. and *A. sativa* L. In all cases, the qualitative and quantitative results showed general patterns equivalent to those presented in this report.

Comparison of the soluble and bound phenolics isolated from nonmycorrhized *A. cepa* L. roots with those from symbiotic roots revealed no qualitative differences. Similar results were previously reported by Codignola et al. (1989). The identity of these compounds has already been reported, and the presence of *p*-coumaric and ferulic acids as major root phenolics is in agreement with the results of Harris and Hartley (1980) on the phenolic constituents in monocotyledonous roots. However, to our knowledge, this is the first report on the occurrence of *N*-feruloyltyramine in the roots of the genus *Allium*, although it has recently been reported in the bulbs of *Allium chinense* (Goda et al. 1986). Moreover, several reports have revealed the ubiquity and the role of cinnamoyl amides in aerial parts of plants (Martin-Tanguy et al. 1978; Ponchet et al. 1982). Whereas *p*-coumaric and ferulic acids are known as constituents of the root cell walls implicated in the lignification and/or regulation of cell wall plasticity and its digestibility (Fry 1986), hydroxycinnamoyl amides are believed to interact with specific biological events. The latter include floral induction (Cabanne et al. 1977), seed dormancy (Jassey et al. 1982), the responses of potato tissues to fungal attack (Clarke 1982) and of tobacco leaves to viral attack (Negrel and Jeandet 1987), as well as the inhibitory effect of hydroxycinnamoyl amides on the in vitro multiplication of tobacco mosaic virus (Martin-Tanguy et al. 1976).

Hydrolysis of the phenolic glycoside fraction with naringinase released three known aglycone moities, namely ferulic acid, sinapic acid and *N*-feruloyltyramine. Two of these, ferulic acid and *N*-feruloyltyramine, were found to accumulate as ester-bound phenolics and correlate with the naringinase-gold labeling. Immunocytochemical localization reveals that gold particles are predominantly localized over the host cell walls, as well as the arbuscular interfacial material, both of which are known for their carbohydrate and glycoprotein composition (Bonfante-Fasolo 1988). In contrast, the walls and cytoplasm of intercellular hyphae were predominantly devoid of labeling. Such observations indicate that naringinase is probing the phenolic molecules that are derived from plant cell metabolism. These results differ from those previously reported by Codignola et al. (1989) who observed no differences in concentration or localization of wall-bound phenols in endomycorrhized and nonmycorrhized roots of *A. porrum*. Furthermore, the naringinase labeling of arbuscular structures observed in this study supports the view that endomycorrhization plays a role in the overall increase of phenolic condensation in colonized root cells. Consequently, it is reasonable to suggest that ferulic acid and *N*-feruloyltyramine released

after alkaline hydrolysis are, in fact, bound to the cell-wall material.

In the [^{14}C]cinnamic acid labeling experiments, phenolic synthesis was not positively stimulated by endomycorrhization; however, the high incorporation of label into *N*-feruloyltyramine suggests that the latter acts as an important sink in the phenylpropanoid pathway. It may be suggested, therefore, that the high concentration of wall-bound phenolics found in endomycorrhized roots is the result of increased oxidative activity triggered by the symbiotic interaction, which in turn catalyses the binding of phenolics from the soluble pool to the cell wall. The present knowledge of the effect of endomycorrhization on peroxidase activity is ambiguous. Spanu and Bonfante-Fasolo (1988) reported a transient, early peak of peroxidase activity following colonization and concluded that VAM establishment does not significantly induce this enzyme activity. On the other hand, R. S. Pacovsky 1990 (personal communication) demonstrated the presence of new isoenzymes of host origin (endomycorrhizins) induced by mycorrhization which exhibit 50% of the total peroxidase activity found in mycorrhized tissue. Such conflicting results call for further investigation of the induction/stimulation of peroxidase activity by VAM endophytic establishment.

The increased binding of phenolics such as ferulic acid and *N*-feruloyltyramine, cross-linked to polysaccharides or hydroxyproline-rich glycoprotein in plant cell walls, has been reported to result in an important decrease in wall plasticity and digestibility (Clarke 1982; Friend 1981; Fry 1983; Hahlbrock and Scheel 1989). Furthermore, these authors postulated that such a cellular alteration would lead to an increased resistance towards pathogenic invasion. Therefore, the increase in phenolic binding to cell walls described in our study may be associated with the control of VAM endophytic establishment and development as it gradually reduces the plasticity and elasticity of the symbiotic matrix. Indeed, it is evident that condensation of phenolics during synthesis of the successive layers of interfacial matrix around the hyphal wall would restrict the plasticity of the interfacial material, confining the development of the arbuscule to a limited space. Moreover, bioassays conducted in this study reveal that *N*-feruloyltyramine strongly affects the physiology of the VAM fungus *G. intraradix*, most likely by a process of toxification. However, we can not speculate on the biochemical mechanism involving *N*-feruloyltyramine in the strong reduction of the growth of VAM fungi and in the induction of intense branching. The binding of phenolics could also be directly responsible for the resistance of VAM roots to pathogenic fungi, since it results in a significant increase of the cell-wall resistance to digestive enzymes (Fry 1983). Such a biochemical reaction suggests that the resistance may proceed by a cell-to-cell reaction to VAM colonization instead of a systemic one. This hypothesis is also supported by the fact that the resistance induced by VAM association against pathogenic fungi is mainly restricted to endomycorrhized root segments (Becker 1976), and that the degree of resistance is proportional to the level of root colonization (Dehne 1982). The induced resistance observed in VAM roots is ubiquitous in the plant kingdom. Such a low specificity is probably linked to a very general plant biochemical reaction, similar to the way in which oxidative condensation of common phenolics in plants reacts to invading microorganisms. Moreover, the spatial restriction of the reaction (Becker 1976) reveals the undiffusible nature of the metabolic alteration leading to resistance, suggesting that structural alterations are more likely to be involved in the process. In conclusion, we do not claim that phenol binding to a cell wall component is the sole factor leading to increased resistance of VAM roots to pathogens, but it should be considered as a very important element among a diversified set of reactions not yet fully understood.

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