

Soybean (*Glycine max*) Root Lignification Induced by Ferulic Acid. The Possible Mode of Action

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Received: 7 April 2008 / Revised: 17 June 2008 / Accepted: 19 June 2008 / Published online: 15 July 2008
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Abstract Ferulic acid, in the form of feruloyl CoA, occupies a central position as an intermediate in the phenylpropanoid pathway. Due to the allelopathic function, its effects were tested on root growth, H₂O₂ and lignin contents, and activities of cinnamyl alcohol dehydrogenase (CAD, EC 1.1.1.195) and peroxidase (POD, EC 1.11.1.7) from soybean (*Glycine max* (L.) Merr.) root seedlings. Three-day-old seedlings were cultivated in half-strength Hoagland's solution (pH 6.0), with or without 1.0 mM ferulic acid in a growth chamber (25°C, 12/12 hr light/dark photoperiod, irradiance of 280 μmol m⁻² s⁻¹) for 24 or 48 hr. Exogenously supplied ferulic acid induced premature cessation of root growth, with disintegration of the root cap, compression of cells in the quiescent center, increase of the vascular cylinder diameter, and earlier lignification of the metaxylem. Moreover, the allelochemical decreased CAD activity and H₂O₂ level and increased the anionic isoform PODa5 activity and lignin content. The lignin monomer composition of ferulic acid-exposed roots revealed a significant increase of guaiacyl (G) units. When applied jointly with piperonylic acid (an inhibitor of the cinnamate 4-hydroxylase, C4H), ferulic acid increased lignin content. By contrast, the application of 3,4-(methylenedioxy) cinnamic acid (an inhibitor of the 4-coumarate:CoA ligase, 4CL) with ferulic acid did not. Taken together, these results suggest that ferulic acid may be channeled into the phenylpropanoid pathway (by the 4CL reaction) and, further, may increase the lignin monomer amount solidifying the cell wall and restricting the root growth.

Keywords Allelopathy · Cinnamyl alcohol dehydrogenase · Lignin · Phenylpropanoid pathway · Root growth · Soybean

Introduction

The phenylpropanoid pathway is an important metabolic pathway involved in the synthesis of phenolic compounds and a wide range of secondary products in plants, including lignin. The first rate-limiting enzyme of this pathway is phenylalanine ammonia-lyase (PAL), which in association with cinnamate 4-hydroxylase (C4H), 4-coumarate:CoA ligase (4CL), and cinnamoyl-CoA reductase (CCR) among others, leads to the synthesis of *p*-coumaral-, coniferyl-, and sinapaldehydes. These metabolites are converted into their corresponding alcohols by cinnamyl alcohol dehydrogenase (CAD). In the last step of the pathway, peroxidase (POD) catalyzes the monolignol polymerization that leads to lignin synthesis. Thus, lignin is a complex heteropolymer of hydroxylated and methoxylated phenylpropane units, derived from the oxidative polymerization of different hydroxycinnamyl alcohols (*p*-coumaryl, coniferyl, and sinapyl) connected by labile ether bonds and/or resistant carbon-carbon linkages (Boerjan et al. 2003). As the main structural component of secondarily thickened plant cell walls, lignin contributes to the compression strength of stems imparting mechanical support, and to the efficient conduction of water and solutes over long distances within the vascular systems (Donaldson 2001).

In general, cell walls become lignified when cell expansion decreases, either when the cell is under stress or when it differentiates to a particular specialization, notably the xylem (Christensen et al. 1998). Ferulic acid, a cinnamic acid derivative, is an abundant compound in soil frequently mentioned in the literature (Einhellig 1995; Inderjit and Duke 2003; Weir et al. 2004). Stress of plant roots from ferulic acid reduces water utilization, inhibits foliar expansion and

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root elongation, reduces rates of photosynthesis, and inhibits nutrient uptake. At the cellular level, the allelochemical induces lipid peroxidation, affects certain enzymatic activities, and rapidly depolarizes the root cell membrane causing a generalized increase in membrane permeability, thus blocking plant nutrient uptake (Weir et al. 2004). At the same time, ferulic acid may be esterified with cell wall polysaccharides, incorporated into the lignin structure, or form bridges that connect lignin with wall polysaccharides, thus making cell walls rigid and restricting cell growth (Iiyama et al. 1990; Sánchez et al. 1996; Lam et al. 2001).

More recently, dos Santos et al. (2004) reported that ferulic acid reduction of soybean growth might be due to premature lignification of root tissues associated with increases in enzyme activities of the phenylpropanoid pathway, such as phenylalanine ammonia lyase (PAL) and POD. The aim of the present report was to investigate what the mode of action of ferulic acid is on the lignification process. For this, light and electron microscopy studies and determinations of CAD and POD activities, H_2O_2 level, and lignin content and composition were carried out after treatment of soybean roots with ferulic acid and inhibitors of the phenylpropanoid pathway.

Methods and Materials

General Procedures Soybean (*Glycine max* L. Merrill) seeds, surface-sterilized with 2% sodium hypochlorite for 5 min and rinsed extensively with deionized water, were dark-germinated (at 25°C) on two sheets of moistened filter paper. Twenty-five 3-d-old seedlings of uniform size were supported on an adjustable acrylic plate and transferred into a glass container (10 × 16 cm) filled with 200 ml of half-strength Hoagland's solution (pH 6.0), with or without 1.0 mM ferulic acid. Additional experiments with 0.1 mM of piperonylic acid (PIP) or 2.0 mM of 3,4-(methylenedioxy) cinnamic acid (MDCA) were made as indicated in the figure legends. The container was kept in a growth chamber (25°C, 12/12 hr L/D photoperiod, irradiance of 280 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Roots were measured at the beginning and at the end of experiments (24 or 48 hr). When indicated, the fresh root weight was determined immediately after incubation, and the dry weight was estimated after oven-drying at 80°C until it reached a constant weight. CAD and POD activities and lignin contents were determined after the incubation period of 24 hr, while light and electron microscopy analyzes were carried out after 48 hr of incubation. Ferulic acid, PIP, and MDCA were purchased from Sigma-Aldrich Chemical Co (St. Louis, MO, USA), and all other reagents used were of the purest grade available or chromatographic grade.

Light Microscopy Studies Samples of material for morphological and anatomical studies were fixed in FAA 50

(Johansen 1940). The material was conserved in ethanol 70% (Jensen 1962). The anatomical description was made from the analysis of permanent slides obtained of longitudinal and transversal sections of the roots. In the preparation of permanent slides, plant material was embedded in glycol methacrylate according to the technique described by Gerrits (1991). These slides were stained with toluidine blue O (O'Brien et al. 1964) and mounted in Permount. Additionally, fresh transverse cross-sections of roots were immersed in a freshly prepared solution of phloroglucinol-HCl (Berlyn and Miksche 1976). Micrographs were photographed with an Olympus[®] photomicroscope. Scales were calculated using a decimal ruler and a micrometer under the same optical conditions used for each case.

Electron Microscopy Studies For scanning electron microscopy, fresh root segments were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. Tissues were postfixed in a solution containing 1% osmium tetroxide, 0.8% potassium ferrocyanide, and 5 mM calcium chloride in 0.1 M cacodylate buffer, and then in a 1% tannic acid solution. Further, the samples were dehydrated in graded ethanol solutions, critical-point-dried in CO_2 , sputter-coated with gold, and examined on a Jeol-JSM-5310[®] field emission scanning electron microscope. For transmission electron microscopy, fresh root segments were washed in 0.01 M phosphate-buffered saline and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. Tissues were postfixed in a solution containing 1% osmium tetroxide and 0.8% potassium ferrocyanide in 0.1 M cacodylate buffer, washed in the same buffer, dehydrated in acetone, and embedded in Spurr resin. Ultrathin sections obtained in a Reichert Ultracut E ultramicrotome were stained with uranyl acetate and lead citrate, and examined in a Zeiss EM900[®] transmission electron microscope.

Cinnamyl Alcohol Dehydrogenase Assay Cinnamyl alcohol dehydrogenase was extracted from fresh roots (2 g) with 3 ml of an extraction medium containing 40 mM of β -mercaptoethanol and 100 mM potassium phosphate buffer (pH 7.3). The homogenate was centrifuged at 2,200 × *g* for 15 min, and the supernatant was used as enzyme preparation (dos Santos et al. 2006). CAD was assayed chromatographically by the reaction of reduction of sinapaldehyde to sinapyl alcohol. The assay was carried out, at 30°C, in 1.0 ml of reaction mixture containing 200 μl of crude enzyme preparation (≤ 0.35 mg of protein), 104 nmol NADPH, and 150 nmol Tris-HCl buffer (pH 8.0). At the start, 50 nmol of sinapaldehyde were added, and the reaction was stopped after 3 min of incubation by adding 50 μl of 5 N HCl. Parallel controls with sinapaldehyde added in the reaction mixture (without NADPH) were made. All samples were filtered through a 0.45- μm disposable syringe filter (Hamilton[®] Co., NV, USA) and analyzed (20 μl) with a Shimadzu[®] Liquid Chromatograph (Tokyo,

Japan) equipped with an LC-10AD pump, a Rheodyne® injector, an SPD-10A UV detector, a CBM-101 Communications Bus Module, and a Class-CR10 workstation system. A reversed-phase Shimpack® CLC-ODS (M) column (150×4.6 mm, 5 μm) was used at room temperature together with the same type of pre-column (10×4.6 mm). The mobile phase was methanol/acetic acid 4% in water (20:80, v/v) with a flow rate of 1.2 ml min⁻¹ for an isocratic run of 20 min. Absorption was detected at 345 nm. Data collection and integration were performed with Class-CR10 software (Shimadzu®, Tokyo, Japan). Sinapyl alcohol was identified by comparing its retention time with standard values. CAD activity was expressed as nanomole sinapaldehyde consumed per minute per milligram protein. Protein was determined spectrophotometrically at 595 nm (Bradford 1976), with bovine serum albumin as a standard.

Peroxidase Assay Root (0.8 g) was homogenized in Eppendorf tubes with 0.12 ml of cold extraction medium containing 0.886 M sodium potassium buffer pH 7.0, 1.0 mM EDTA, 1.0 mM sodium metabisulfite, 9.96 mM sodium borate, 5% PVP-40 (polyvinylpyrrolidone), 0.5% β-mercaptoethanol, 10% glycerol, 2% ascorbic acid, and 4% polyethylene glycol (Pereira et al. 2001). The homogenates were centrifuged (21,900 ×g, 30 min, 4°C), and the supernatant was used as soluble POD extract. For cell wall-bound POD isolation, fresh roots (5 g) were macerated with 67 mM phosphate buffer (50 ml, pH 7.0) containing 0.5 g PVP (dos Santos et al. 2004). The extract was centrifuged (2,200 ×g, 5 min, 4°C). The pellet was washed with deionized water until no soluble POD activity was detected in the supernatant. The pellet was incubated in 10 ml of 1 M NaCl (prepared in 50 mM phosphate buffer, pH 7.0) for 1 hr. The homogenate was centrifuged (2,200 ×g, 5 min, 4°C) and the supernatant obtained. Cold acetone (30 ml) was slowly added in the supernatant under constant stirring. After centrifugation (10,000 ×g, 30 min, 4°C), the pellet was resuspended with 0.15 ml of 1 M NaCl and considered as cell wall-(ionically)-bound POD.

For polyacrylamide gel electrophoresis (PAGE), samples (50 μl) of the enzyme extract were applied in 12% gel prepared in 0.375 M Tris-HCl pH 8.8 buffer. Electrophoresis was performed for 5 hr at 200 V. The running buffer used was 0.1 M Tris-glycine pH 8.3 (Pereira et al. 2001). This gel was incubated at 37°C for 15 min with 50 ml of 1 M sodium citrate buffer, pH 4.7, adjusted with acetic acid, 50 ml of methanol, and 0.05 g of benzidine. Then, 5 ml of 30% H₂O₂ were added to the staining mixture and the preparation was maintained at 37°C until the time of isozyme detection (Mangolin et al. 1994).

H₂O₂ Quantification Fresh roots (2 g) were homogenized in 3 ml of 50 mM phosphate buffer, pH 6.8 (Hsu and Kao 2007). The homogenate was centrifuged at 2,200 ×g for 20 min.

Further, 1.5 ml of extracted solution, mixed with 0.5 ml of 0.1% titanium chloride in 20% (v/v) H₂SO₄, was then centrifuged at 2,200 ×g for 15 min. Absorbance was measured at 410 nm, and H₂O₂ was quantified with a calibration curve of known standard concentrations. Whereas the blank consisted of a reaction mixture without tissue extract, its absorbance was subtracted from the mixture with H₂O₂ extract. Results were expressed as nanomole H₂O₂ per gram fresh weight.

Quantification of Lignin Content and Composition After the incubation period, dry roots (0.3 g) were homogenized in 50 mM potassium phosphate buffer (7 ml, pH 7.0) with a mortar and pestle and transferred into a centrifuge tube (Ferrarese et al. 2002). The pellet was centrifuged (1,400 ×g, 4 min) and washed by successive stirring and centrifugation as follows: twice with phosphate buffer pH 7.0 (7 ml); 3× with 1% (v/v) Triton® X-100 in pH 7.0 buffer (7 ml); 2× with 1 M NaCl in pH 7.0 buffer (7 ml); 2× with distilled water (7 ml); and 2× with acetone (5 ml). The pellet was dried in an oven (60°C, 24 hr) and cooled in a vacuum desiccator. The dry matter was defined as a protein-free cell wall fraction. Further, all dry protein-free tissue was placed into a screw-cap centrifuge tube containing the reaction mixture (1.2 ml of thioglycolic acid plus 6 ml of 2 M HCl) and heated (95°C, 4 hr). After cooling at room temperature, the sample was centrifuged (1,400 ×g, 5 min), and the supernatant was discarded. The pellet contained the complex lignin–thioglycolic acid (LTGA). The pellet was washed three times with distilled water (7 ml) and the LTGA extracted by shaking (30°C, 18 hr, 115 oscillations per minute) in 0.5 M NaOH (6 ml). After centrifugation (1,400 ×g, 5 min), the supernatant was stored. The pellet was washed again with 0.5 M NaOH (3 ml) and mixed with the supernatant obtained earlier. The combined alkali extracts were acidified with concentrated HCl (1.8 ml). After precipitation (0°C, 4 hr), LTGA was recovered by centrifugation (1,400 ×g, 5 min) and washed two times with distilled water (7 ml). The pellet was dried at 60°C, dissolved in 0.5 M NaOH, and diluted to yield an appropriate absorbance for spectrophotometric determination at 280 nm. Lignin was expressed as milligram LTGA per gram dry weight.

Alkaline cupric oxidation was used to determine lignin monomer composition (Chen and McClure 2000). Protein-free cell wall fraction (25 mg) was sealed in a Pyrex® ampule containing 1 ml of 2 M NaOH plus 0.2 g of CuO and heated to 170°C for 2 hr, while shaking the sample occasionally during the reaction. After oxidation, the sample was cooled at room temperature, acidified to pH 2 with 2 M HCl, and extracted twice with anhydrous ethyl ether. The organic extracts were combined, dried, and resuspended in methanol/acetic acid 4% in water (20:80, v/v). All samples were filtered through a 0.45-μm disposable syringe filter (Hamilton® Co., NV, USA) and analyzed by high-performance liquid chroma-

tography (HPLC), as described earlier. The mobile phase was methanol/acetic acid 4% in water (20:80, v/v), with a flow rate of 0.8 ml min^{-1} for an isocratic run of 20 min. Quantification of *p*-hydroxybenzaldehyde, vanillin, and syringaldehyde was performed at 290 nm by corresponding standards. Results were expressed as microgram monomer per milligram cell wall.

Additionally, cell wall-bound ferulic acid was extracted after alkaline hydrolysis (de Ascensao and Dubery 2003). Dry roots (0.2 g) were homogenized in 4 ml of 50% methanol with a mortar and pestle and transferred into a centrifuge tube, heated to 80°C for 1.5 hr. Supernatant was discarded after centrifugation ($2,200 \times g$, 10 min). The pellet was washed twice with 2 ml of 50% methanol and dried in an oven (60°C , 24 hr). Dry material (0.1 g) was resuspended in 10 ml of 0.5 M NaOH and heated to 96°C for 2 hr. The sample was acidified to pH 2 with HCl, centrifuged ($2,200 \times g$, 10 min), and the supernatant was extracted twice with 10 ml of anhydrous ethyl ether. The organic extracts were combined, dried, and resuspended in methanol/acetic acid 4% in water (30/70, v/v). All samples were filtered through a $0.45\text{-}\mu\text{m}$ disposable syringe filter (Hamilton® Co., NV, USA) and analyzed by HPLC, as described earlier. The mobile phase was methanol/water (30:70, v/v) with a flow rate of 0.8 ml min^{-1} for an isocratic run of 20 min. Cell wall-released ferulic acid was identified at 330 nm by co-injection with authentic standard.

Statistical Design The experimental design was completely randomized, and each plot was represented by one glass container with 25 seedlings. Data are expressed as the mean of three to five independent experiments \pm S.E. The one-way variance analysis to test the significance of the observed differences was performed by Sisvar® package (Version 4.6, UFPA, Brazil). The difference among parameters was evaluated by the Scott–Knott test, and *P* values <0.05 were considered as statistically significant.

Results

Effects of Ferulic Acid on Root Growth As may be judged visually, differences in the roots were apparent (Fig. 1). Primary root elongation of treated seedlings was inhibited

by the compound. Roots became brown, thicker, and less flexible. After 24 and 48 hr, the allelochemical prompted significant decreases in the root lengths when compared to the control conditions (Table 1).

Light and Electron Microscopy Analyses Subsequent experiments were carried out to evaluate anatomical changes in roots by light microscopy (Table 1). The effects of ferulic acid on diameter (root and vascular cylinder) measures of medial and basal sections were examined after root treatments. After 24 hr, the vascular cylinder diameters increased in both medial (40.9%) and basal (14.3%) sections in comparison to controls. Significant effects of ferulic acid were evident at 48 hr; the root diameter decreased (11.3%) while the vascular cylinder diameter increased (19%) in the medial section. Cross sections of the medial region in untreated roots showed a normal pattern of growth (Fig. 2A,C). Results of the two histochemical methods used to verify lignification gave results that agreed closely. When the ferulic acid-exposed roots (Fig. 2B) were subjected to phloroglucinol-HCl reagent, an intense coloration indicating lignified cell walls developed when compared to control (Fig. 2A). Moreover, the number of differentiated xylem elements formed was greater in ferulic acid-treated roots than in control roots. In ferulic acid-treated roots stained with toluidine blue O (Fig. 2D), the metaxylem also showed lignification when compared to control (Fig. 2C). In addition, longitudinal sections of the transition zone in treated roots indicated initiation of lateral roots and lignification of the primary xylem (Fig. 2E,F). Similar sections revealed a smaller distance between the quiescent center and the differentiated cells in ferulic acid-treated roots when compared to the untreated roots (Fig. 2G,H).

Transmission electron microscopy observations of ferulic acid-treated root caps showed a reduced number of starch granules in comparison to control (Fig. 3A,B). Micrographs of the quiescent center in treated roots revealed that the cells were smaller and compressed, and contained irregular and enlarged nucleoli and many lipid globules in comparison to the untreated roots (Fig. 3C,D). Finally, scanning electron microscopy photomicrographs showed disintegration of the root cap in the ferulic acid treated roots in comparison to control (Fig. 3E,F).

Fig. 1 Effects of ferulic acid on soybean root length. Control (A) and treated (B) roots with 1.0 mM ferulic acid after 48 hr

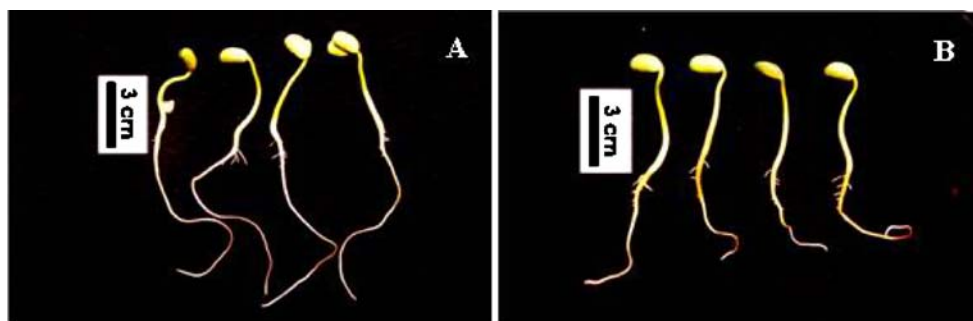


Table 1 Anatomical characteristics of ferulic acid treated soybean roots after 24 and 48 hr

		Medial region			Basal region	
		Root length (cm)	Root diameter (μm)	Cylinder diameter (μm)	Root diameter (μm)	Cylinder diameter (μm)
24 hr	C	3.3 \pm 0.21	5.8 \pm 0.37	2.2 \pm 0.07	7.7 \pm 0.50	2.8 \pm 0.10
	T	2.2 \pm 0.09 ^a	7.5 \pm 0.59	3.1 \pm 0.06 ^a	8.2 \pm 0.22	3.2 \pm 0.09*
	%	-33.3	ns	+40.9	ns	+14.3
48 hr	C	7.0 \pm 0.14	7.1 \pm 0.05	2.1 \pm 0.02	8.0 \pm 0.32	3.0 \pm 0.11
	T	2.7 \pm 0.16 ^a	6.3 \pm 0.44 ^a	2.5 \pm 0.07 ^a	7.2 \pm 0.26	2.9 \pm 0.11
	%	-61.4	-11.3	+19.0	ns	ns

The symbol % represents inhibition (-) or activation (+) of statistically significant means after treatment in comparison to control. C control, T treatment, ns not significant.

^a Means ($N=4\pm\text{SE}$) significantly ($P\leq 0.05$) smaller than the control experiment (Scott-Knott's test)

Effects of Ferulic Acid on CAD and POD Activities and H_2O_2 Content Ferulic acid-affected CAD activity was significantly different from control (Fig. 4). The allelochemical decreased the enzymatic activity by 42.8% at 1.0 mM treatment. In addition to this earlier finding, electrophoretic patterns of POD isozymes were determined in soybean roots after allelochemical treatment (Fig. 5). PAGE zimograms of POD in ferulic acid treated roots revealed that only the anionic form PODa5 increased compared to the control condition. Moreover, the allelochemical decreased the H_2O_2 content 30% when compared to control (Fig. 6).

Effects of Ferulic Acid on Lignin Content and Composition Lignin content in soybean roots increased following 1.0 mM ferulic acid treatment by about 46% at 24 hr (Figs. 7 and 8). Figure 7 reveals that PIP, a potent quasi-irreversible inhibitor of cinnamate 4-hydroxylase (C4H), reduced lignin content of soybean roots 37.6% compared to control. Similar to ferulic acid, treatment of roots with allelochemical plus PIP increased lignin content 26.5% compared to the control condition. Experiments with MDCA, a competitive inhibitor of 4-coumarate:CoA ligase (4CL), are seen in Fig. 8. Lignin content was not affected by MDCA, alone or jointly with ferulic acid (FA plus MDCA), in comparison with the control experiment. The analysis of alkaline cupric oxidation products (Fig. 9) revealed that lignin monomer content (*p*-hydroxybenzaldehyde+guaiacyl+syringyl; H+G+S) increased 3.8-fold compared to that in untreated roots. The allelochemical mainly increased the guaiacyl (G) monomer. When subjected to alkaline hydrolysis, cell walls of allelochemical-exposed roots released significant amount of ferulic acid identified chromatographically (Fig. 10).

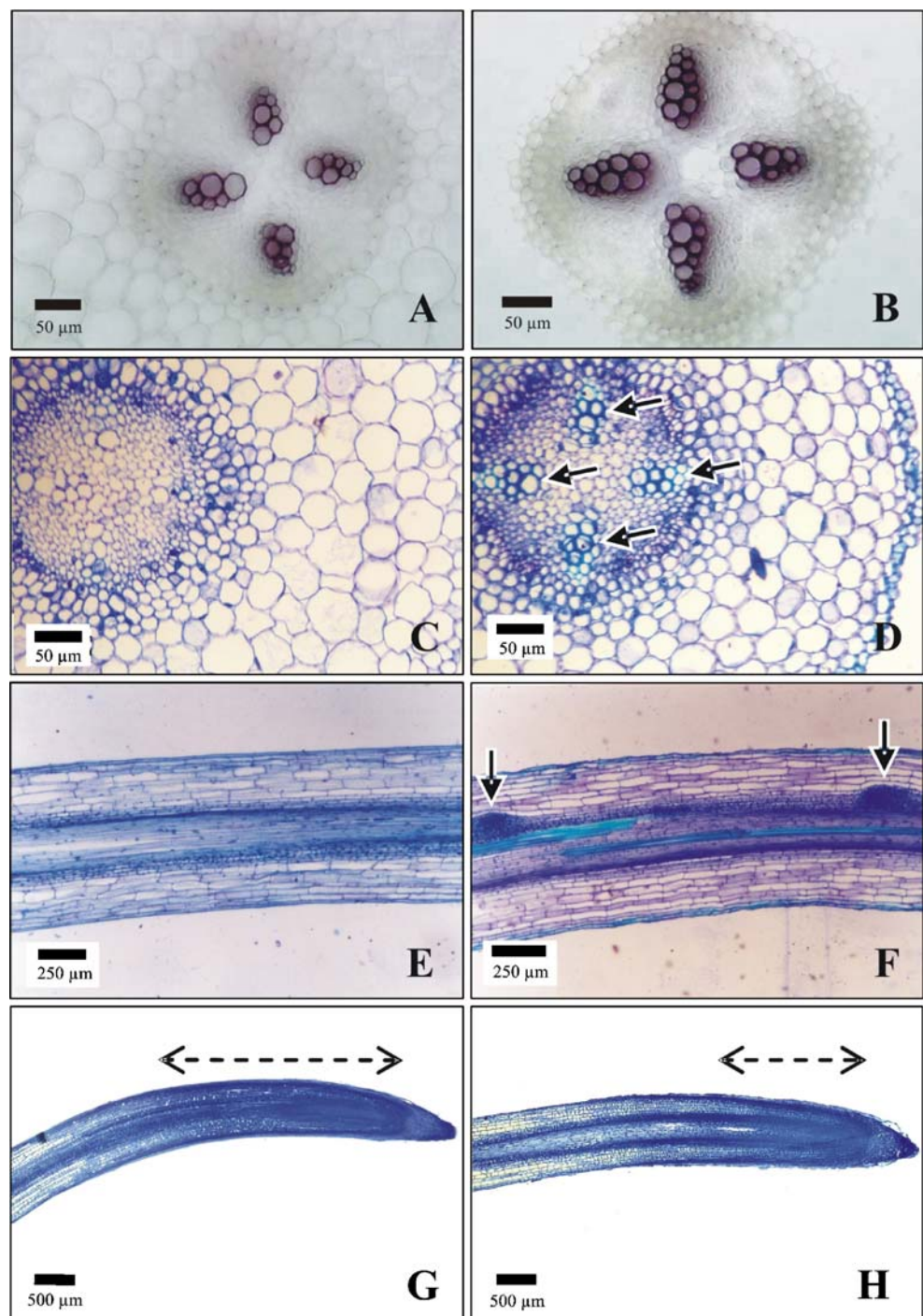
Discussion

Root growth is characterized by high metabolic rates, and for this reason roots are highly susceptible to environmental stresses, such as allelochemicals in soils (Cruz-Ortega et al.

1998). The experimental conditions used in this work were chosen because the net uptake of ferulic acid by the root system is high (Shann and Blum 1987a), and lignification begins during the early stages of seedling growth (dos Santos et al. 2004; Passardi et al. 2005). At the final stages of xylem cell differentiation, lignin is deposited within the carbohydrate matrix of the cell wall by infilling of interlamellar voids and, at the same time, by the formation of chemical bonds with the non-cellulosic carbohydrates (Donaldson 2001). An important fact revealed in the present work is that the reduction of soybean root length (Table 1) by ferulic acid is associated with an increase in the lignin contents (Figs. 7 and 8).

Structural changes of root cells have been associated with root growth inhibition induced by stress (Cruz-Ortega et al. 1998). The increase in root diameter, especially of vessel cylinder, may be attributed to the early differentiation of vessel tissues verified by the lignification of metaxylem (Fig. 2D). As reported earlier, cell walls may be lignified when stressed or when they differentiate to xylem (Christensen et al. 1998). Anatomical observations here showed that the diameter of the vascular cylinder in the medial and basal regions was enhanced after 24 and 48 hr of treatments, compared with controls (Table 1). Ferulic acid-treated roots showed a greater number of differentiated xylem elements and lignification of cell walls (Fig. 2B), induction of lateral roots (Fig. 2F) with lignification of the metaxylem (Fig. 2D), and an increase of the central cylinder (Fig. 2D), all consistent with increased lignin production (Figs. 7 and 8). Furthermore, a smaller distance between the quiescent center and the first differentiated cells together with clustered cells in the longitudinal section of treated roots (Fig. 2H) indicate premature cessation of root growth. There is evidence to corroborate these results (Chon et al. 2002). For example, coumarin-treated alfalfa (*Medicago sativa*) roots revealed a significant increase in diameter due to an expanding of the vascular cylinder and cortex cell layers. Moreover, this allelochemical inhibited root elongation and cell division, indicating that the thickness of roots was enlarged due to inhibition of root longitudinal

Fig. 2 Light microscopy photomicrographs of control (A, C, E, G) and treated (B, D, F, H) soybean roots with 1.0 mM ferulic acid for 48 hr. A and B: phloroglucinol-HCl staining method indicating lignified cell walls in cross sections of the medial zone. C and D: toluidine blue O staining method in cross sections of the medial zone (*arrows* indicate lignification of metaxylem). E and F: longitudinal sections of the central cylinder in the basal region (*arrows* indicate induction of lateral roots and lignification). G and H: longitudinal sections (*dotted lines* indicate the distance between the quiescent center and first differentiated cells)

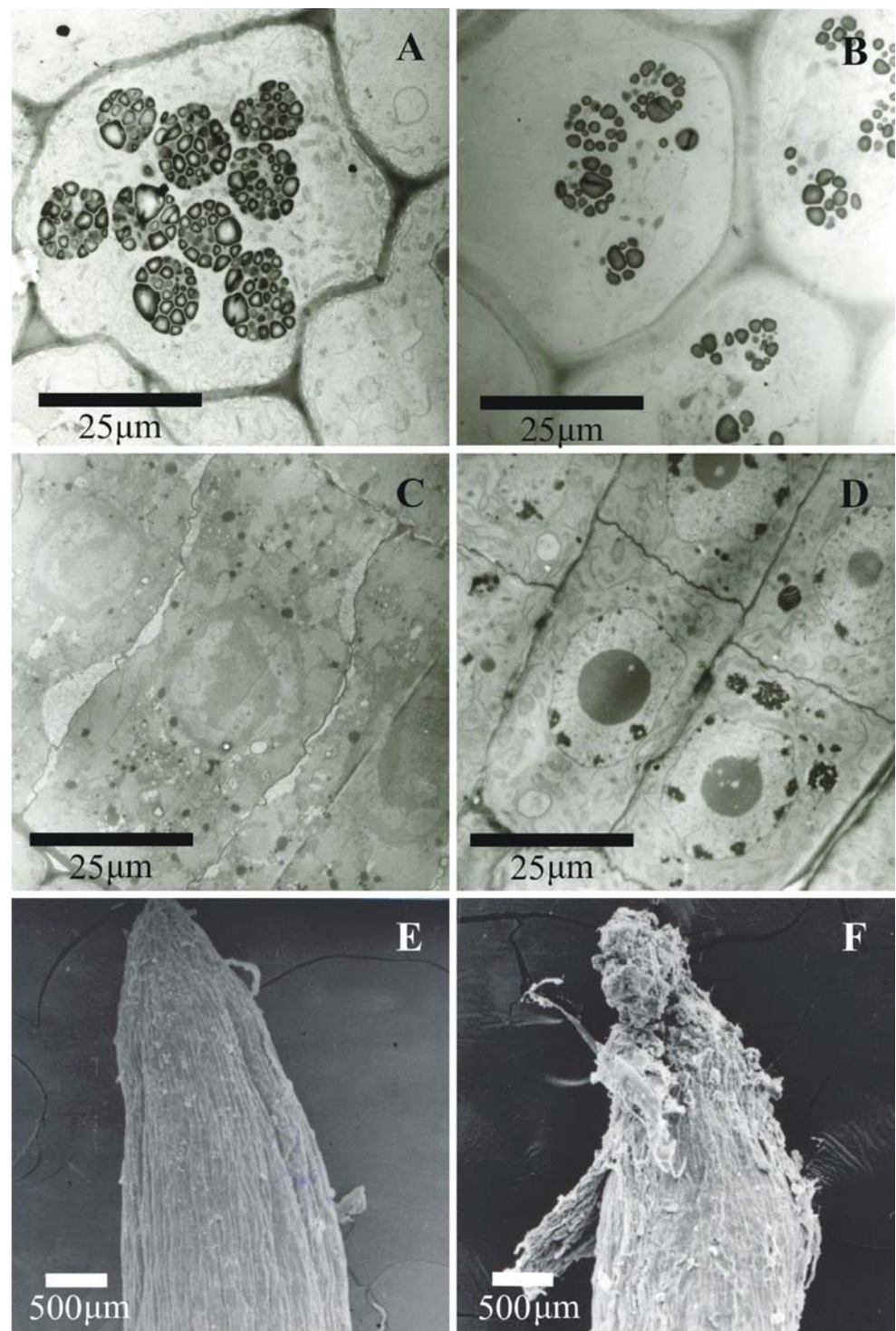


growth. The water-soluble extract of alfalfa leaves, which contains mainly ferulic acid among other allelochemicals, showed similar effects (Chon et al. 2002).

At the ultrastructural level, treatment with ferulic acid adversely affected soybean root cells. Electron micrographs showed a reduction in the number of starch granules (Fig. 3B), an increase in lipid globules, and compression of cells with enlarged nucleoli (Fig. 3D) in ferulic acid treated roots. Disintegration of the root cap in treated roots (Fig. 3F) suggests

death of cells after direct contact with the allelochemical. In simplest terms, ferulic acid inhibited the root growth and led to cellular ultrastructural abnormalities. Studies have shown that allelochemicals can affect the cellular structure of growing roots. White mustard (*Sinapis alba*) radicle treated with gramine and hordenine showed vacuolation, disorganization of organelles, and damage to cells walls (Liu and Lovett 1993). Root cell ultrastructure of cucumber exposed to 2-benzoxazolinone presented cytoplasmic vacuolation and

Fig. 3 Electron microscopy photomicrographs of control (A, C, E) and treated (B, D, F) soybean roots with 1.0 mM ferulic acid for 48 hr. A and B: transmission electron microscopy of root cap showing starch granules. C and D: transmission electron microscopy of quiescent center cells showing compression with enlarged nucleoli and lipid globules in treated roots. E and F: scanning electron microscopy of root cap showing epidermic tissue disintegrated in treated roots



reduced number of starch granules (Burgos et al. 2004). Mustard (*Brassica juncea*) seedlings treated with benzoic acid showed cellular disorganization (Kaur et al. 2005). According to these authors, inhibition of root growth was due to reduced lipid catabolism, protein synthesis, and adverse effects on cell division and mineral uptake. In agreement, ferulic acid inhibits protein synthesis, affects membrane permeability, and decreases uptake of nutrients (Politycka 1996; Baziramakenga

et al. 1997). Moreover, it reduces lipid mobilization followed by accumulation of unsaturated fatty acids in germinating canola (*Brassica napus*) seeds (Baleroni et al. 2000). It also increases the contents of saturated and unsaturated fatty acids, xylose, fructose, and sucrose in soybean root (Ferrarese et al. 2001). Thus, the cellular structure changes reported here appear to be, at least partially, associated with changes in the lipid and carbohydrate metabolism (Ho 1988).

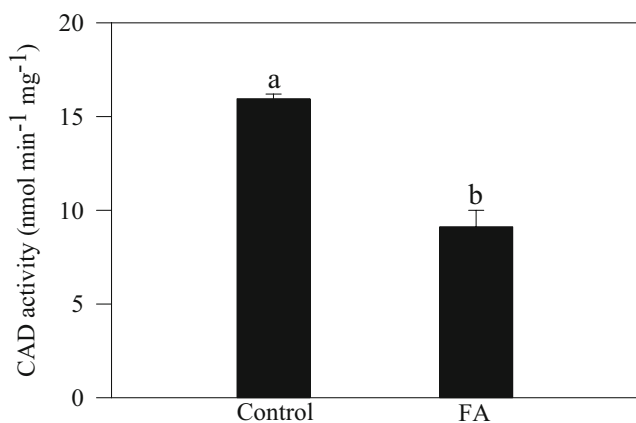


Fig. 4 Effects of ferulic acid (FA) on CAD activity in soybean roots. Mean±SE values (N=4) followed by the different letter are significantly different according to the Scott–Knott test ($P \leq 0.05$)

There is evidence that the effects of ferulic acid on soybean are related to premature lignification of roots, since enzyme (PAL and POD) activities of the phenylpropanoid pathway increased jointly with lignin production (dos Santos et al. 2004). Direct incorporation of exogenous ferulic acid, independent of phenylpropanoid metabolism, was initially hypothesized. This possibility should not be discounted. The radiotracer [U-ring-¹⁴C]ferulic acid] was found in residues of lignin isolated from cucumber seedlings treated with the allelochemical (Shann and Blum 1987b). To test the hypothesis that exogenous ferulic acid induces the lignification process, CAD activity was determined in treated soybean roots. Surprisingly, this showed that CAD activity decreases in ferulic acid treated roots (Fig. 4) despite lignin production (Figs. 7 and 8). Increase in CAD activity would be anticipated, since it is considered to be a lignification marker (Boerjan et al. 2003). Increased lignification under reduced CAD activity might, at least in principle, strengthen the hypothesis of direct incorporation of exogenous ferulic acid into lignin polymer. However, plants are able to circumvent the block in CAD activity by shipping substrates, the cinnamaldehydes, to the cell wall for polymerization (Boerjan et al. 2003). This indicates that the impact of CAD on lignin biosynthesis may not be critical. Cross-coupling of hydroxycinnamyl aldehydes into lignin compensates for the reduced availability of monolignols in CAD-deficient plants (Kim et al. 2000; Li et al. 2001).

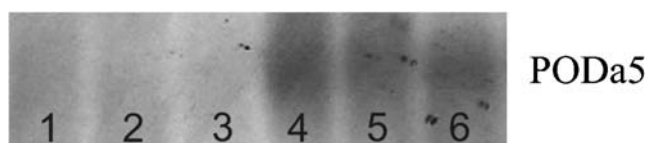


Fig. 5 PAGE isozyme patterns of POD in soybean roots. 4 to 6, ferulic acid-treated roots showing increase of the anionic isoform of soluble enzyme (PODa5) in comparison to control (1 to 3). POD activity was stained using H₂O₂ as the substrate

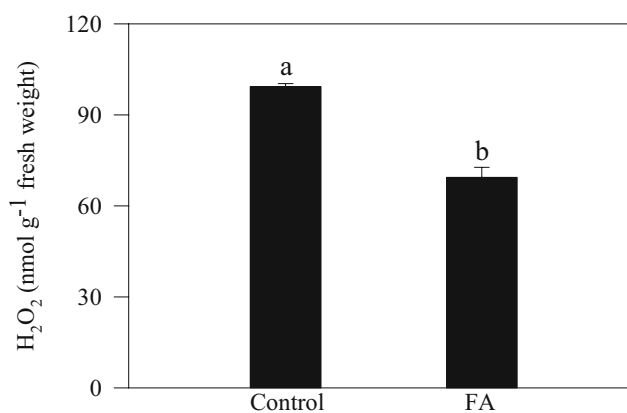


Fig. 6 Effects of ferulic acid (FA) on H₂O₂ content in soybean roots. Mean±SE values (N=3) followed by the different letter are significantly different according to the Scott–Knott test ($P \leq 0.05$)

Ferulic acid increased the activity of anionic isoform PODa5 in treated roots (Fig. 5). Anionic isoperoxidases are often held to be those most directly involved in lignification of xylem cells (Wallace and Fry 1999; Passardi et al. 2005). POD is able to dehydrogenate monolignols, to induce lignification after addition of H₂O₂ in tissue sections, and to reveal specific colocalization of isoforms in lignifying tissues (Ros Barceló et al. 2004; Passardi et al. 2005). H₂O₂, produced by the pH-dependent POD and NADPH oxidase complex, is a necessary substrate for the cell wall’s lignifying process catalyzed by POD, thus causing a rapid cross-linking of cell-wall polymers (Wojtaszek 1997). Ferulic acid exposure reduced the H₂O₂ content of roots (Fig. 6). It is feasible that these facts may, in part, explain the increase in anionic isoform PODa5 in treated roots (Fig. 5), consistent with increased lignin production (Figs. 7 and 8).

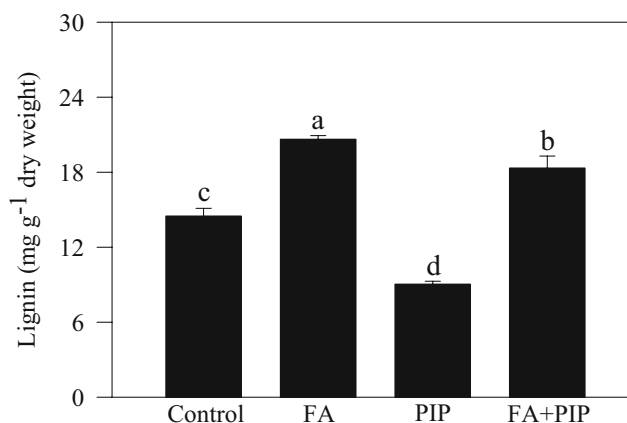


Fig. 7 Changes in the lignin contents of soybean roots untreated (Control) or treated with 1.0 mM ferulic acid (FA), 0.1 mM piperonylic acid (PIP) and 1.0 mM ferulic acid plus 0.1 mM piperonylic acid (FA+PIP) for 24 hr. Mean±SE value (N=5) followed by different letters are significantly different according to the Scott–Knott test ($P < 0.05$)

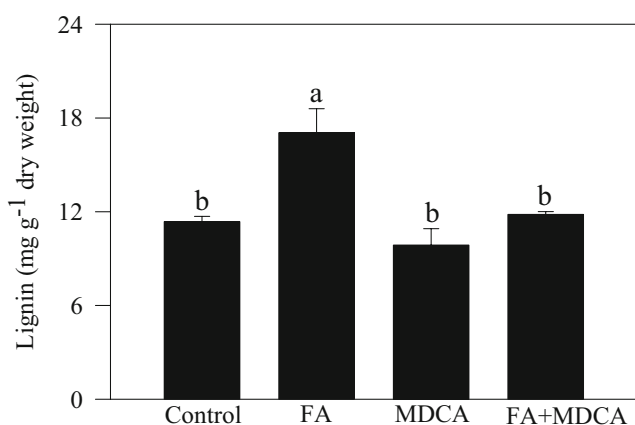


Fig. 8 Changes in the lignin contents of soybean roots untreated (*Control*) or treated with 1.0 mM ferulic acid (*FA*), 2.0 mM methylene dioxocinnamic acid (*MDCA*) and 1.0 mM ferulic acid plus 2.0 mM methylene dioxocinnamic acid (*FA+MDCA*) for 24 hr. Mean \pm SE values ($N=5$) followed by same letter are not significantly different according to the Scott–Knott test ($P<0.05$)

Hamada et al. (2003) demonstrated that exogenously supplied ferulic acid was converted to feruloyl and then to coniferyl and sinapyl alcohols, in poplar (*Populus alba*) callus. Since feruloyl CoA is an intermediate of phenylpropanoid metabolism (Fig. 10), a possible entry of free ferulic acid into the pathway, by the 4CL reaction, must be considered. To elucidate, subsequent experiments were made by growing roots with two inhibitors of the pathway enzymes: PIP, a quasi-irreversible inhibitor of cinnamate 4-hydroxylase (C4H), and MDCA, a competitive inhibitor 4-coumarate:CoA ligase (4CL). Figure 7 shows that roots grown under ferulic acid treatment produce more lignin, while PIP-treated roots synthesize less lignin, compared to standard conditions. This is in

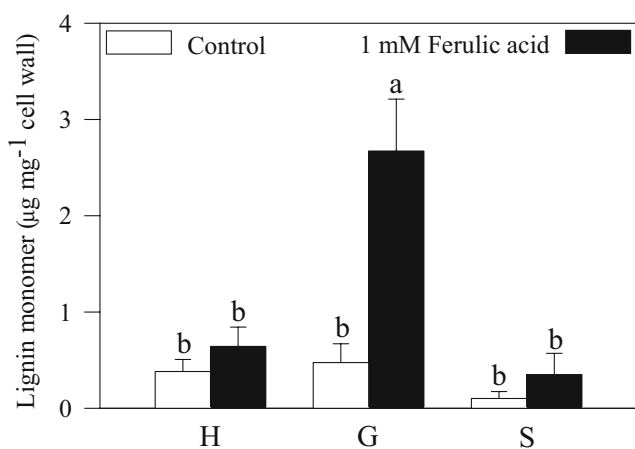


Fig. 9 Effects of ferulic acid on lignin monomer composition. *H*, *p*-hydroxybenzaldehyde; *G*, guaiacyl, and *S*, syringyl monomers. Mean \pm SE values ($N=3$) followed by the different letters are significantly different according to the Scott–Knott test ($P\leq 0.05$)

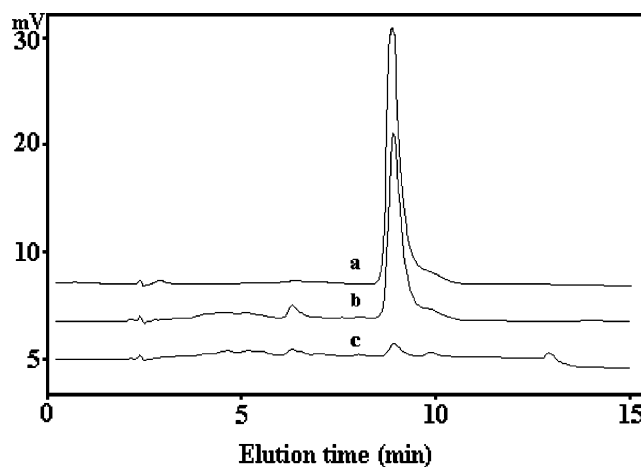


Fig. 10 HPLC elution profiles of ferulic acid standard (*a*, retention time=8.83 min), cell wall-released ferulic acid in the treated roots (*b*, retention time=8.87 min), and cell wall-released ferulic acid in the control roots (*c*, retention time=8.85 min)

agreement with the fact that PIP is an effective inhibitor of C4H, and acts before the entry point of ferulic acid in the pathway (Schoch et al. 2002). When applied jointly with PIP, ferulic acid prompted an increase in lignin content, suggesting its entrance into pathway by the 4CL reaction. To strengthen this assumption, roots were incubated with MDCA, an inhibitor of 4CL (Schoch et al. 2002). Lignin content did not change after MDCA or MDCA plus ferulic acid treatments, compared to controls (Fig. 8), thus indicating that the access of exogenous allelochemical has been blocked at this metabolic point.

Since exogenous ferulic acid may be incorporated into lignin structure by means of the phenylpropanoid pathway, changes in the lignin monomer content should not be discounted. In order to ascertain lignin composition, cell walls isolated from ferulic acid exposed roots were subjected to alkaline cupric oxidation. Results shown herein reveal a striking feature. The lignin monomer contents increased in treated roots (Fig. 9). Root lignin from ferulic acid exposed plants was mainly composed of guaiacyl (*G*) unit in a 18:72:10 (*H/G/S*) ratio, compared to control plants (40:50:10 ratio). It is well-known that in dicotyledonous angiosperms, lignin is derived from three monomer types; *p*-hydroxyphenyl (*H*), guaiacyl (*G*), and syringyl (*S*) units, derived from *p*-coumaryl, coniferyl, and sinapyl alcohol, respectively (Boerjan et al. 2003). One of the important precursors of *p*-coumaryl alcohol in the phenylpropanoid pathway is ferulic acid (Fig. 11). Moreover, lignin polymerization is regulated by the cell through the supply of available monomers (Boerjan et al. 2003). In robinia (*Robinia pseudoacacia*), labeled ferulic acid was incorporated into guaiacyl (*G*) and syringyl (*S*) lignin, and these incorporations increased as cell-wall lignification proceeded (Yamauchi and Fukushima 2004). Based on results reported herein (Figs. 6, 7, 8, 9 and 10), it is

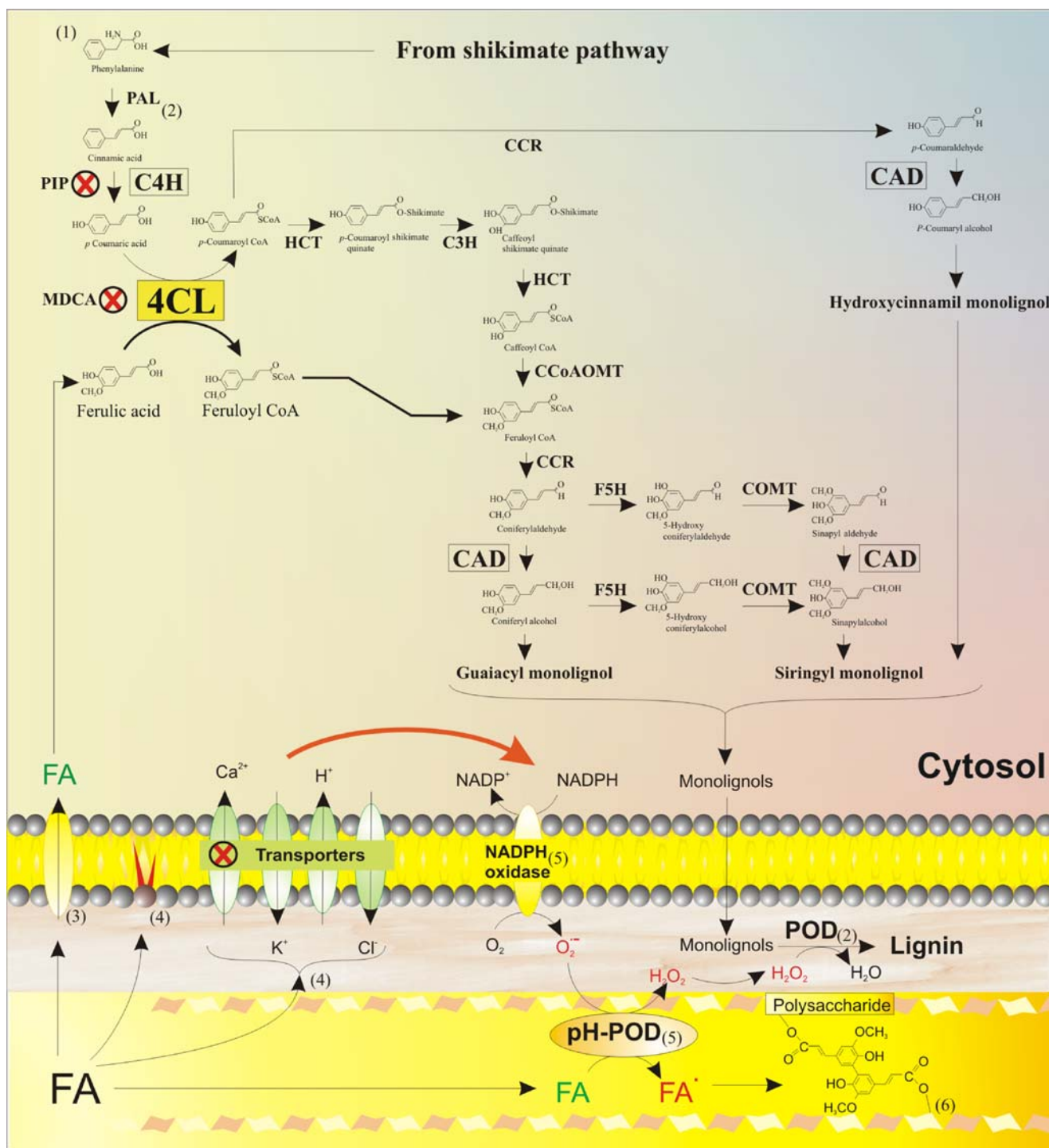


Fig. 11 Proposed mode of action for ferulic acid on lignification of soybean roots. PAL phenylalanine ammonia lyase, C4H cinnamate 4-hydroxylase, 4CL 4-coumarate:CoA ligase, HCT hydroxycinnamoyl-CoA:quinat/shikimate hydroxycinnamoyltransferase, C3H p-coumarate 3-hydroxylase, CCoAOMT caffeoyl-CoA O-methyltransferase, CCR cinnamoyl-CoA reductase, F5H ferulate 5-hydroxylase, COMT caffeic

acid/5-hydroxy ferulic acid O-methyltransferase, CAD cinnamyl alcohol dehydrogenase, POD peroxidase, FA ferulic acid, PIP piperonylic acid, MDCA 3,4-(methylenedioxy) cinnamic acid. (1) Chen et al. (2006), (2) dos Santos et al. (2004), (3) Shann and Blum (1987a), (4) Baziramakenga et al. (1995), (5) Wojtaszek (1997), (6) Boerjan et al. (2003)

plausible to suggest that exogenous ferulic acid was channeled into the phenylpropanoid pathway, and later on, caused an increase in lignin. Consistent with these findings, cell walls of allelochemical-exposed roots released significant amount of ferulic acid (Fig. 10).

The focus of the present work was to investigate how the mode of action of ferulic acid is related to the lignification process. Light and electron microscopy studies, combined with biochemical assays, suggest a possible mechanism of soybean response to ferulic acid (Fig. 10). Exogenously applied ferulic acid induces premature cessation of soybean root growth, with disintegration of the root cap, and cellular modifications, such as compression of cells in the quiescent center, early lignification of the metaxylem and cell wall, and increase of the vascular cylinder diameter. At the metabolic level, ferulic acid is channeled into the phenylpropanoid pathway and converted to feruloyl CoA by the 4CL reaction. Catalyzed by subsequent enzymatic reactions, feruloyl CoA is then converted to coniferal- and sinapaldehydes. As an endwise enzyme of the pathway, CAD might not be a limiting step. So, either these metabolites are converted into the respective alcohols by CAD or, eventually circumvent the inhibited CAD reaction (Kim et al. 2000; Li et al. 2001; Boerjan et al. 2003) by polymerizing with lignin in the cell wall. Lignin polymerization requires a sufficient supply of H₂O₂, which is produced by the pH-dependent POD and NADPH oxidase complex, after changes in the membrane permeability (Baziramakenga et al. 1995; Wojtaszek 1997). Whether or not this is the actual process, it seems plausible to assume that ferulic acid induced inhibition in root growth of soybean may be due to excessive production of monolignol from exogenously applied ferulic acid. Monolignol polymerization forms a complex network that solidifies the plant cell wall and restricts plant growth.

Acknowledgements Research was financially supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). O. Ferrarese-Filho and M.L.L. Ferrarese are research fellows of CNPq. W.D. dos Santos is the recipient of a CNPq fellowship. The authors thank Dr. Wanderley de Souza and Maria de Fátima P. S. Machado for cooperation in extending instrumental facilities. The authors thank Aparecida M.D. Ramos and Gisele A. Bubna for their technical assistance.

References

- BALERONI, C. R. S., FERRARESE, M. L. L., BRACCINI, A. L., SCAPIM, C. A., and FERRARESE-FILHO, O. 2000. Effects of ferulic and *p*-coumaric acids on canola (*Brassica napus* L. cv. Hyola 401) seed germination. *Seed Sci. Technol.* 28:201–207.
- BAZIRAMAKENGA, R., LEROUX, G. D., and SIMARD, R. R. 1995. Effects of benzoic and cinnamic acids on membrane permeability of soybean roots. *J. Chem. Ecol.* 21:1271–1285.
- BAZIRAMAKENGA, R., LEROUX, G. D., and SIMARD, R. R. 1997. Allelopathic effects of phenolic acids on nucleic acid and protein levels in soybean seedlings. *Can. J. Bot.* 75:445–450.
- BERLYN, G. P., and MIKSCH, J. P. 1976. Botanical microtechnique and cytochemistry. The Iowa State University Press, Ames, Iowa.
- BOERJAN, W., RALPH, J., and BAUCHER, M. 2003. Lignin biosynthesis. *Annu. Rev. Plant Biol.* 54:519–546.
- BRADFORD, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.
- BURGOS, N. R., TALBERT, R. E., KIM, K. S., and KUK, Y. I. 2004. Growth inhibition and root ultrastructure of cucumber seedlings exposed to allelochemical from rye (*Secale cereale*). *J. Chem. Ecol.* 30:671–689.
- CHEN, M., and MCCLURE, J. W. 2000. Altered lignin composition in phenylalanine ammonia-lyase-inhibited radish seedlings: implications for seed-derived sinapoyl esters as lignin precursors. *Phytochemistry.* 53:365–370.
- CHEN, F., REDDY, M. S. S., TEMPLE, S., JACKSON, L., SHADLE, G., and DIXON, R. A. 2006. Multi-site genetic modulation of monolignol biosynthesis suggests new routes for formation of syringyl lignin and wall-bound ferulic acid in alfalfa (*Medicago sativa* L.). *Plant J.* 48:113–124.
- CHON, S.-U., CHOI, S.-K., JUNG, S., JANG, H.-G., PYO, B.-S., and KIM, S.-M. 2002. Effects of alfalfa leaf extracts and phenolic allelochemicals on early seedling growth and root morphology of alfalfa and barnyard grass. *Crop Protection.* 21:1077–1082.
- CHRISTENSEN, J. H., BAUW, G., WELINDER, K. G., VAN MONTAGU, M., and BOERJAN, W. 1998. Purification and characterization of peroxidases correlated with lignification in poplar xylem. *Plant Physiol.* 118:125–135.
- CRUZ-ORTEGA, R., ANAYA, A. L., HERNÁNDEZ-BAUTISTA, B. E., and LAGUNA-HERNÁNDEZ, G. 1998. Effects of allelochemical stress produced by *Sicyos deppei* on seedling root ultrastructure of *Phaseolus vulgaris* and *Cucurbita ficifolia*. *J. Chem. Ecol.* 24:2039–2057.
- DONALDSON, L. A. 2001. Lignification and lignin topochemistry—an ultrastructural view. *Phytochemistry.* 57:859–876.
- DE ASCENSAO, A. R. F. D. C., and DUBERY, I. A. 2003. Soluble and wall-bound phenolics and phenolics polymers in *Musa acuminata* roots exposed to elicitors from *Fusarium oxysporum* f. sp. *cubense*. *Phytochemistry.* 63:679–686.
- DOS SANTOS, W. D., FERRARESE, M. L. L., FINGER, A., TEIXEIRA, A. C. N., and FERRARESE-FILHO, O. 2004. Lignification and related enzymes in soybean root growth-inhibition by ferulic acid. *J. Chem. Ecol.* 30:1199–1208.
- DOS SANTOS, W. D., FERRARESE, M. L. L., and FERRARESE-FILHO, O. 2006. High performance liquid chromatography method for the determination of cinnamyl alcohol dehydrogenase in soybean roots. *Plant Physiol. Biochem.* 44:511–515.
- EINHELLIG, F. A. 1995. Characterization of the mechanisms of allelopathy. Modeling and experimental approaches, pppp. 132–141, in H. H. Cheng, Inderjit, and K. M. M. Dakshini (eds.). *Allelopathy, Organisms, Processes and Applications* American Chemical Society, Washington, DC.
- FERRARESE, M. L. L., SOUZA, N. E., RODRIGUES, J. D., and FERRARESE-FILHO, O. 2001. Carbohydrate and lipid status in soybean roots influenced by ferulic acid uptake. *Acta Physiol. Plant.* 23:421–427.
- FERRARESE, M. L. L., ZOTTIS, A., and FERRARESE-FILHO, O. 2002. Protein-free lignin quantification in soybean (*Glycine max*) roots. *Biologia.* 57:541–543.
- GERRITS, P. O. 1991. The Application of Glycol Methacrylate in Histotechnology: Some Fundamental Principles. Department of Anatomy and Embryology State University Groningen, Netherlands.
- HAMADA, K., YSUTSUMI, Y., and NISHIDA, T. 2003. Treatment of poplar callus with ferulic and sinapic acids II. Effects on related monolignol biosynthetic enzyme activities. *J. Wood Sci.* 49:366–370.

- HO, L. C. M. 1988. Metabolism and compartmentation of imported sugars in sink organs in relation to sink strength. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 39:355–378.
- HSU, Y. T., and KAO, C. H. 2007. Heat shock-mediated H₂O₂ accumulation and protection against CD toxicity in rice seedlings. *Plant Soil.* 300:137–147.
- IYAMA, K., LAM, T. B. T., and STONE, B. A. 1990. Phenolic acid bridges between polysaccharides and lignin in wheat internodes. *Phytochemistry.* 29:733–737.
- INDERJIT, and DUKE, S. O. 2003. Ecophysiological aspects of allelopathy. *Planta.* 217:529–539.
- JENSEN, W. A. 1962. Botanical histochemistry, principles and practice. W. H. Freeman, San Francisco.
- JOHANSEN, D. A. 1940. Plant microtechnique. McGraw-Hill Book Co., New York.
- KAUR, H., INDERJIT, and KAUSHIK, S. 2005. Cellular evidence of allelopathic interference of benzoic acid to mustard (*Brassica juncea* L.) seedling growth. *Plant Physiol. Biochem.* 43:77–81.
- KIM, H., RALPH, J., YAHIAOUI, N., PEAN, M., and BOUDET, A. M. 2000. Cross-coupling of hydroxycinnamyl aldehydes into lignins. *Organic. Lett.* 2:2197–2200.
- LAM, T. B. T., KADOYA, K., and IYAMA, K. 2001. Bonding of hydroxycinnamic acids to lignin: ferulic and *p*-coumaric acids are predominantly linked at the benzyl position of lignin, not the β -position, in grass cell walls. *Phytochemistry.* 57:987–992.
- LI, L., CHENG, X. F., LESHKEVICH, J., UMEZAWA, T., and HARDING, S. A. 2001. The last step of syringyl monolignols biosynthesis in angiosperms is regulated by a novel gene encoding sinapyl alcohol dehydrogenase. *Plant Cell.* 13:1567–1585.
- LIU, D. L., and LOVETT, J. V. 1993. Biologically active secondary metabolites of barley. II. Phytotoxicity of barley allelochemicals. *J. Chem. Ecol.* 19:2231–2244.
- MANGOLIN, C. A., PRIOLI, A. J., and MACHADO, M. F. P. S. 1994. Isozyme patterns in callus cultures and in plants regenerated from calli of *Cereus peruvianus* (cactaceae). *Biochem. Gen.* 32:237–247.
- O'BRIEN, T. P., FEDER, N., and MCCULLY, M. E. 1964. Polychromatic staining of plant cell walls by toluidine blue O. *Protoplasma.* 59:368–373.
- PASSARDI, F., COSIO, C., PENEL, C., and DUNAND, C. 2005. Peroxidases have more functions than a Swiss army knife. *Plant Cell. Rep.* 24:255–265.
- PEREIRA, A. J., VIDIGAL-FILHO, P. S., LAPENTA, A. S., and MACHADO, M. F. P. S. 2001. Differential esterase expression in leaves of *Manihot esculenta* Crantz infected with *Xanthomonas axonopodis* pv. manihotis. *Biochem. Genet.* 39:289–296.
- POLITYCKA, B. 1996. Peroxidase activity and peroxidation lipidic in roots of cucumber seedlings influenced by derivatives of cinnamic and benzoic acids. *Acta Physiol. Plant.* 18:365–370.
- ROS BARCELÓ, A., GÓMEZ ROS, L. V., GABALDÓ, N. C., LÓPEZ-SERRANO, M., POMAR, F., CARRIÓN, J. S., and PEDRENO, M. A. 2004. Basic peroxidases: the gateway from lignin evolution? *Phytochem. Rev.* 3:61–78.
- SÁNCHEZ, M., PEÑA, M. J., REVILLA, G., and ZARRA, I. 1996. Changes in dehydrodiferulic acids and peroxidase activity against ferulic acid associated with cell walls during growth of *Pinus pinaster* hypocotyl. *Plant Physiol.* 111:941–946.
- SCHOCH, G. A., NIKOV, G. N., ALWORTH, W. L., and WERCK-REICHHART, D. 2002. Chemical inactivation of the cinnamate 4-hydroxylase allows for the accumulation of salicylic acid in elicited cells. *Plant Physiol.* 130:1022–1031.
- SHANN, J. R., and BLUM, U. 1987a. The uptake of ferulic acid and *p*-hydroxybenzoic acids by *Cucumis sativus*. *Phytochemistry.* 26:2959–2964.
- SHANN, J. R., and BLUM, U. 1987b. The utilization of exogenously applied ferulic acid in lignin biosynthesis. *Phytochemistry.* 26:2977–2982.
- WALLACE, G., and FRY, S. C. 1999. Action of diverse peroxidases and laccases on six cell wall-related phenolic compounds. *Phytochemistry.* 52:769–773.
- WEIR, T. L., PARK, S. W., and VIVANCO, J. M. 2004. Biochemical and physiological mechanisms mediated by allelochemicals. *Curr. Opin. Plant Biol.* 7:472–479.
- WOJTASZEK, P. 1997. Oxidative burst: an early plant response to pathogen infection. *Biochem. J.* 322:681–692.
- YAMAUCHI, K., and FUKUSHIMA, K. 2004. The regulation from guaiacyl to syringyl lignin in the differentiating xylem of *Robinia pseudacacia*. *C. R. Biologies.* 327:791–797.