



Effects of cinnamic and ferulic acids on growth and lignification of maize roots

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ABSTRACT

Cinnamic acid and its hydroxylated derivative, ferulic acid, are known allelochemicals that affect the root growth of several plant species. Some studies have indicated that the impairment of root growth by allelochemicals is associated with lignification of the cell walls. However, this hypothesis has not been tested in monocot plants. Here, we evaluated the effects of these two allelochemicals on growth, activities of phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL), lignin content and the composition of *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) monomers in maize roots. Cinnamic acid reduced root growth, TAL activity and lignin (H+G+S) content. When applied in conjunction with piperonylic acid, cinnamic acid reduced total lignin and H, G and S content. Ferulic acid reduced root growth and PAL/TAL activities, but it increased G monomer and lignin (H+G+S). When applied in conjunction with 3,4-(methylenedioxy)cinnamic acid, ferulic acid reduced total lignin and G and S content. The results indicated that decreased maize root growth by the cinnamic acid was not related to the lignin production while exogenous ferulic acid could be channeled into the phenylpropanoid pathway and resulted in an excessive production of lignin, thus solidifying the cell wall and restricting the plant growth.

Keywords: Allelochemical, lignin, monolignol, phenylpropanoid pathway, root growth, *Zea mays* L.

Introduction

Allelochemicals are secondary metabolites regularly released by higher plants into the surrounding soil which can influence the growth and development of neighboring plants, both positively and negatively. This phenomenon is known as allelopathy, defined as the ability of plants to protect themselves as well as chemical communication between microbes, plant/microbe, plant/insect or plant/herbivore (Weir *et al.*, 2004). A large number of allelochemicals are commonly found in soils, affecting plant growth at concentrations of up to 10 mM (Siqueira *et al.*, 1991; Macias, 1995) and due to their high metabolic activity, plant roots are quite susceptible to the stress induced by allelochemicals. After uptake by the roots, allelochemicals alter physiological and biochemical processes such as water utilization, mineral uptake, photosynthesis, amino acid metabolism, protein synthesis, glycolysis, mitochondrial respiration and ATP synthesis, among countless others (Weir *et al.*, 2004). However, conclusive modes of allelochemical action on plants are far from being understood.

One of the most striking events in the development of higher plants is the lignification of cell walls, a dynamic and flexible process important for many specialized functions such as regulation of water flow, hydrophobicity, rigidity and protection against biotic and abiotic stresses (Harrington *et al.*, 2012). Lignification, which refers to the anchorage of lignin in the plant cell wall, is also a crucial step for the root growth. Furthermore, the wall of the root cell is the first tissue that is affected by stress signals (Komatsu *et al.*, 2010).

Lignin is a heterogeneous polymeric end-product of the phenylpropanoid pathway generated by combinatorial radical coupling of *p*-hydroxycinnamyl alcohols (called monolignols) (Vanholme *et al.*, 2012). In the first two steps of this metabolic route, the non-oxidative deamination of L-phenylalanine by phenylalanine ammonia-lyase (PAL) yields *t*-cinnamate, followed by hydroxylation of the aromatic ring catalyzed by cinnamate 4-hydroxylase (C4H) to generate *p*-coumarate. The coupling of these enzymes establishes a rate-limiting step in the pathway. PAL is found in dicots and monocots, but a

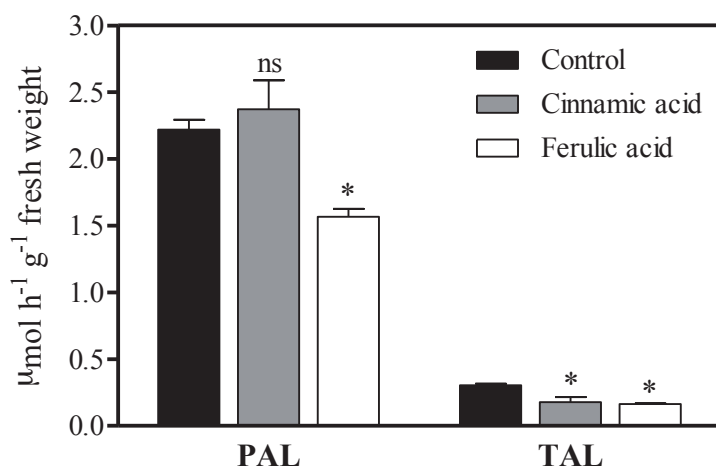


Figure 1. Effects of 1.0 mM cinnamic and ferulic acids on phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) activities of maize roots. *Means ($n = 6 \pm \text{SE}$) significantly smaller than the control (Dunnett's multiple comparison test, $P \leq 0.05$). ns, not significant.

secondary tyrosine ammonia-lyase (TAL) activity is associated with the enzyme from monocots, including maize (Rosler *et al.*, 1997). TAL is highly selective for L-tyrosine, which is directly converted to *p*-coumarate, reducing the number of enzymes necessary for the production of this metabolite from two to one (Ferrer *et al.*, 2008). The next step is the activation of the acid to a thioester by action of 4-coumarate: CoA ligase (4CL) which yields *p*-coumaroyl-CoA. The *p*-coumaroyl-CoA is transesterified and hydroxylated by action of 4-hydroxycinnamoyl-CoA: shikimate/quinic 4-hydroxycinnamoyltransferase and *p*-coumarate 3-hydroxylase to generate caffeoyl-CoA. A methoxylation reaction catalyzed by caffeoyl-CoA *O*-methyltransferase produces feruloyl-CoA from caffeoyl-CoA. By the sequential action of cinnamoyl-CoA reductase, ferulate 5-hydroxylase, caffeic acid 3-*O*-methyltransferase and cinnamyl alcohol dehydrogenase (CAD) the CoA thioesters are converted in monolignols (*p*-coumaryl, coniferyl and sinapyl alcohols). Finally, oxidative polymerization of these three monolignols, by action of cell wall-bound peroxidase (POD), generates the *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units of the lignin.

Cinnamic and ferulic acids are known allelochemicals that affect the growth of several plant species (Cheng, 1995; Panteli and Voutsas, 2010). Some studies have suggested that a correlation between a reduction in plant growth and increased root lignin content is the consequence of the plant defenses acting against allelochemical stress (Devi and Prasad, 1996; Politycka, 1999; dos Santos *et al.*, 2004; Bohm *et al.*, 2006; Soares *et al.*, 2011). Stress-induced lignification can limit cell expansion, the capacity for nutrient uptake and the ability to sustain

plant growth. In previous reports, ferulic (dos Santos *et al.*, 2008), *p*-coumaric (Zanardo *et al.*, 2009), caffeic (Bubna *et al.*, 2011) and cinnamic (Salvador *et al.*, 2013) acid are shown to impair soybean root growth which was associated with changes in the key enzymes of the phenylpropanoid pathway, resulting in premature cell wall lignification. These data suggest that allelochemicals could be directly channeled into the phenylpropanoid pathway, increasing the lignin content, solidifying the cell wall and reducing soybean root growth.

All of the previously cited studies have evaluated the effects of allelochemicals in dicot plants. To extend these studies, the effects of cinnamic and ferulic acids in maize as a monocot plant model were evaluated on root growth, PAL and TAL activities, total lignin content and the composition of H, G and S monomers. To evaluate the possible entry of the exogenous allelochemicals into the phenylpropanoid metabolic route, experiments were performed using two selective enzymatic inhibitors of this pathway: piperonylic acid (PIP, a *quasi*-irreversible inhibitor of the enzyme C4H) and 3,4-(methylenedioxy)cinnamic acid (MDCA, a competitive inhibitor of the 4CL enzyme).

Material and Methods

General procedures

Maize (*Zea mays* L. cv. IPR-114) seeds were surface-sanitized with 2 % sodium hypochlorite for 5 min, rinsed extensively with deionized water and dark-germinated at 25 °C on two sheets of moistened filter paper. Twenty-five 3-d-old seedlings of uniform size

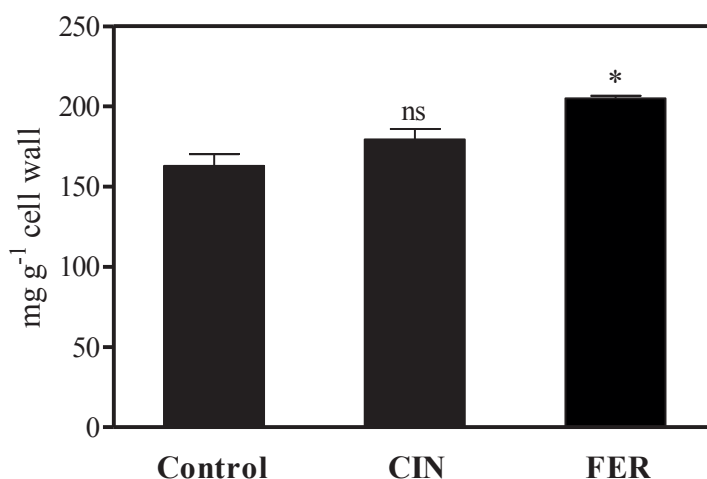


Figure 2. Effects of 1.0 mM cinnamic (CIN) and ferulic (FER) acids on lignin content of maize roots. *Mean ($n = 5 \pm \text{SE}$) significantly higher than the control (Dunnett's multiple comparison test, $P \leq 0.05$). ns, not significant.

were supported by an adjustable acrylic plate and dipped into a 10×16 cm glass container filled with 200 mL of half-strength Hoagland's solution (pH 6.0), with or without 1.0 mM cinnamic or ferulic acids. All nutrient solutions were buffered with 67 mM potassium phosphate buffer to eliminate the effects of very low pH. The concentrations of the allelochemicals were based on a survey of the literature (Siqueira *et al.*, 1991; Macias, 1995; Ng *et al.*, 2003; Batish *et al.*, 2008; Singh *et al.*, 2009; Zanardo *et al.*, 2009; Bubna *et al.*, 2011; Salvador *et al.*, 2013; Lima *et al.*, 2013). Additional experiments with 0.1 mM PIP or 1.0 mM MDCA were conducted, as indicated in the figure legends. The containers were kept in a growth chamber at 25 °C, with a light/dark photoperiod of 12/12 h and a photon flux density of $280 \mu\text{mol m}^{-2} \text{s}^{-1}$ for 24 h. The roots were measured before incubation and at the end of experiments, and the difference in the lengths were calculated for all of the samples. Where indicated, the fresh root weight was determined immediately after incubation and the dry weight was estimated after oven-drying at 80 °C to a constant weight. Cinnamic and ferulic acids, PIP and MDCA were purchased from Sigma-Aldrich (St Louis, MO, USA) and all other reagents used were of the purest grade available or chromatographic grade.

Enzymatic assays

After incubation, all treated and untreated seedling roots were detached and enzymes were extracted. Phenylalanine ammonia-lyase (PAL) was extracted as described by Ferrarese *et al.* (2000). Fresh roots (1.0 g) were ground at 4 °C in 0.1 M sodium borate buffer (pH 8.8), homogenates were centrifuged (2200 g, 15 min)

and the supernatant used as the enzyme preparation. The reaction mixture (100 μmoles sodium borate buffer, pH 8.7, and a suitable amount of enzyme extract in a final volume of 1.55 mL) was incubated (40 °C, 5 min) for the PAL activity assay. L-Phenylalanine (15 μmoles) was added to start the reaction, which was stopped after 1 h of incubation by the addition of 50 μL of 5 M HCl. Samples were filtered through a 0.45- μm disposable syringe filter (Hamilton® Co., Nevada, USA) and 20 μL aliquots were analyzed in a high performance liquid chromatography (HPLC) system (Shimadzu® 10AVP, Tokyo, Japan) equipped with a 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 column (150 mm $\times$ 4.6 mm, 5 μm) protected with an equivalent pre-column, was used at 30 °C. The mobile phase was 70 % methanol (v/v) with a flow rate of 0.8 mL min^{-1} for an isocratic run of 10 min and UV detection was carried out at 275 nm. Data collection and integration were performed with Class-CR10 software (Shimadzu®, Tokyo, Japan). *t*-Cinnamate, the product of PAL, was identified by comparing its retention time with a standard compound. Parallel controls without L-phenylalanine or with *t*-cinnamate (added as an internal standard in the reaction mixture) were performed as described elsewhere (Ferrarese *et al.*, 2000). PAL activity was expressed as $\mu\text{mol t-cinnamate h}^{-1} \text{g}^{-1}$ of fresh weight.

Tyrosine ammonia-lyase (TAL) was extracted as described by Khan *et al.*, (2003) with modifications. Fresh roots (1 g) were ground at 4 °C in 50 mM Tris-HCl buffer (pH 8.5), homogenates were centrifuged (2200 g, 10 min) and the supernatant used as the enzyme

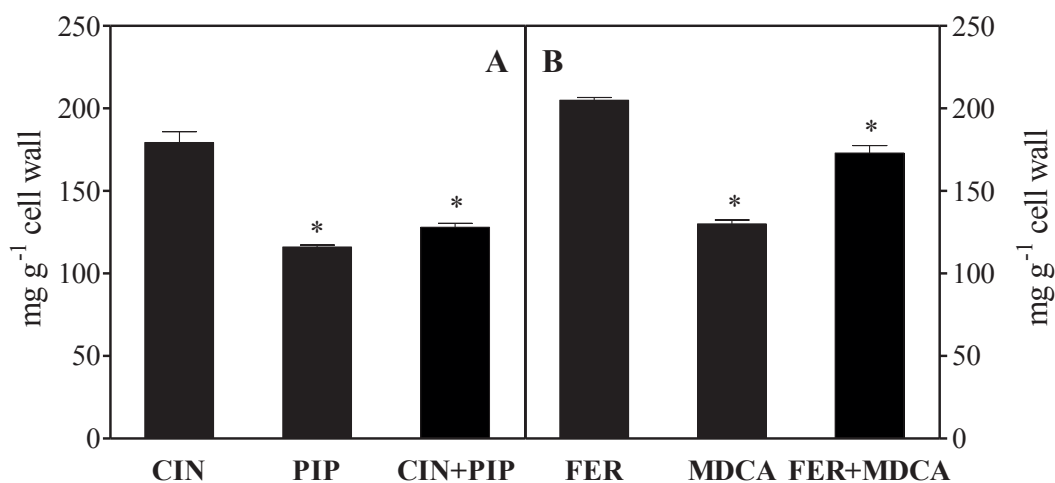


Figure 3. Lignin content in maize roots treated with 1.0 mM cinnamic acid (CIN), 0.1 mM piperonylic acid (PIP), 1.0 mM cinnamic acid plus 0.1 mM piperonylic acid (CIN+PIP), 1.0 mM ferulic acid (FER), 1.0 mM 3,4 (methylenedioxy)cinnamic acid (MDCA) and 1.0 mM ferulic acid plus 1.0 mM 3,4 (methylenedioxy)cinnamic acid (FER+MDCA). *Means ($n = 5 \pm SE$) significantly smaller than CIN (A) or FER (B) treatments (Dunnett's multiple comparison test, $P \leq 0.05$).

preparation. The reaction mixture (100 μ moles Tris-HCl buffer, pH 8.0, and a suitable amount of enzyme extract in a final volume of 0.95 mL) was incubated (40 °C, 5 min) for the TAL activity assay. L-Tyrosine (5.5 μ moles) was added to start the reaction, which was stopped after 1 h of incubation by the addition of 50 μ L of 5 M HCl. Samples were filtered through a 0.45- μ m disposable syringe filter (Hamilton® Co., Nevada, USA) and 20 μ L aliquots were analyzed by HPLC as described earlier. The mobile phase was methanol:4 % acetic acid (30 %:70 %) with a flow rate of 1.0 mL min⁻¹ for an isocratic run of 15 min and UV detection was carried out at 320 nm. *p*-Coumarate, the product of the TAL reaction, was identified by comparing its retention time with a standard compound. Parallel controls without L-tyrosine or with *p*-coumarate (added as an internal standard in the reaction mixture) were performed. Since cinnamic acid is converted into *p*-coumarate by the action of C4H, controls with and without L-tyrosine were made to eliminate any endogenous *p*-coumarate. TAL activity was expressed as μ mol *p*-coumarate h⁻¹ g⁻¹ of fresh weight.

Quantification of lignin and monomer 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 homogenate was centrifuged (1400 *g*, 6 min) and the pellet washed by successive washing 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 (80 °C, 24 h) and cooled in a vacuum desiccator. The dry matter obtained was defined as a protein-free cell wall fraction. Lignin was assayed by the acetyl bromide method (Morrison, 1972). Samples (20 mg) of the protein-free cell wall fraction were placed in a screw-cap centrifuge tube containing 0.5 mL of 25 % acetyl bromide (v/v in glacial acetic acid) and incubated at 70 °C for 30 min. After complete digestion, the samples were quickly cooled on ice, mixed with 0.9 mL 2 M NaOH, 0.1 mL 7.5 M hydroxylamine-HCl and 2 mL glacial acetic acid. After centrifugation (1000 *g*, 5 min), the absorbance of the supernatant was measured at 280 nm and lignin content expressed as mg g⁻¹ cell wall.

The alkaline nitrobenzene technique was used to oxidize lignin and determine its monomeric composition (Zanardo *et al.*, 2009). The protein-free cell wall fraction (50 mg) was sealed in a Pyrex® ampule containing 1 mL 2 M NaOH and 0.1 mL nitrobenzene and heated to 170 °C for 150 min with occasional shaking. The sample was then cooled at room temperature, washed twice with chloroform, acidified to pH 2 with 5 M HCl and extracted twice with chloroform. The organic extracts were combined, dried, resuspended in 1 mL methanol and diluted in methanol/4 % acetic acid in water (20:80, v/v). All the samples were filtered through a 0.45- μ m disposable syringe filter (Hamilton® Co., Nevada, USA) and 20 μ L aliquots analyzed by HPLC as previously

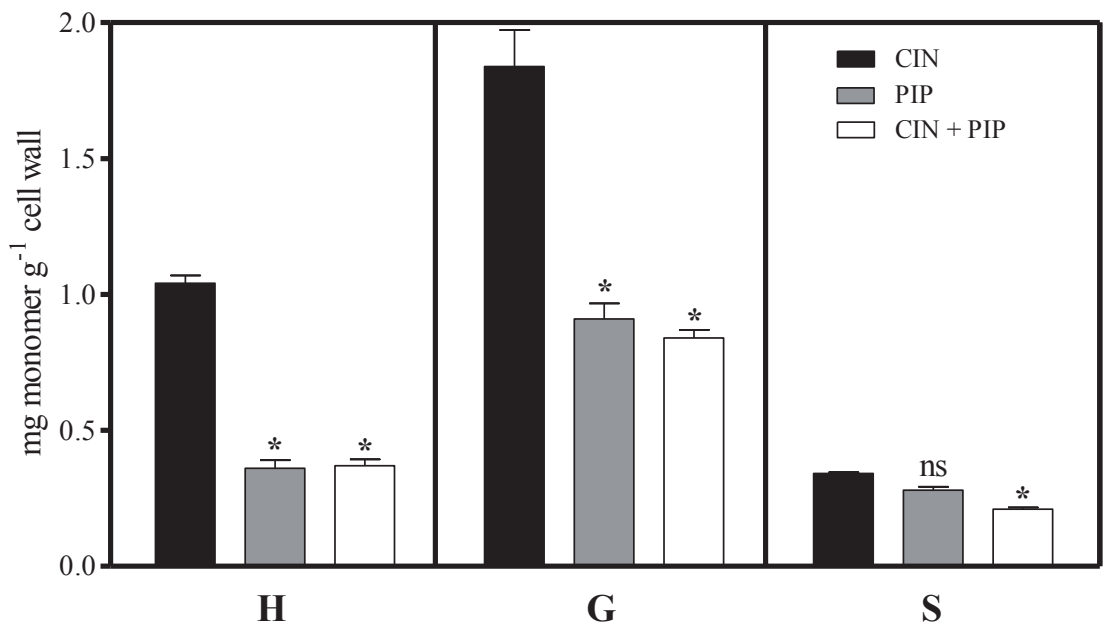


Figure 4. Lignin monomer composition in maize roots treated with 1.0 mM cinnamic acid (CIN), 0.1 mM piperonylic acid (PIP) and 1.0 mM cinnamic acid plus 0.1 mM piperonylic acid (CIN+PIP). H, *p*-hydroxyphenyl; G, guaiacyl; and S, syringyl monomers. *Means ($n = 3 \pm \text{SE}$) significantly smaller than CIN treatment (Dunnett's multiple comparison test, $P \leq 0.05$) ns, not significant.

described. The mobile phase consisted of a mixture of methanol and 4 % acetic acid in water (20:80, v/v) with a flow rate of 1.2 mL min⁻¹ for an isocratic run of 20 min. Quantification of the monomer aldehyde products (*p*-hydroxybenzaldehyde, vanillin and syringaldehyde) released by nitrobenzene oxidation was performed at 290 nm using the corresponding standards and results are expressed as mg monomer g⁻¹ cell wall.

Statistical analyses

The experimental design was randomized and each plot represented one glass container with 25 seedlings. The data were expressed as the mean of three to six independent experiments $\pm$ SE. An analysis of variance was used to test the significance of differences (Prism 5, GraphPad Software Inc., San Diego, CA). The differences between the parameters were evaluated using Dunnett's multiple comparison test. P values ≤ 0.05 were considered to be statistically significant.

Results

• Effects on root growth

Table 1 summarizes the effects of allelochemicals on maize root growth (lengths and weights). Cinnamic

acid was the most effective inhibitor of root length, *i.e.*, 93.7 % reduction compared with the control, while ferulic acid reduced root growth by 77.9 %. The effects of both compounds were also evident for root fresh weights, which significantly decreased after cinnamic (27.6 %) and ferulic (22.5 %) acid treatments when compared with the controls. Similarly, root dry weights were reduced by cinnamic (25.2 %) and ferulic (19.6 %) acids.

• Effects on PAL and TAL activities

In agreement with the effects observed on root growth, phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) enzyme activities in seedlings treated with cinnamic and ferulic acids were different from those of controls (Fig. 1). Roots exposed to ferulic acid had significantly decreased PAL activity by 29 % in comparison to the control (2.21 $\mu\text{mol h}^{-1} \text{g}^{-1}$ fresh weight). Compared with the control (0.31 $\mu\text{mol h}^{-1} \text{g}^{-1}$ fresh weight), cinnamic and ferulic acids treatments decreased TAL activities by 42 % and 46 %, respectively.

• Effects on lignin content and its monomeric composition

Cinnamic acid (CIN) did not affect the lignin content in comparison with the control (163 mg g⁻¹ cell

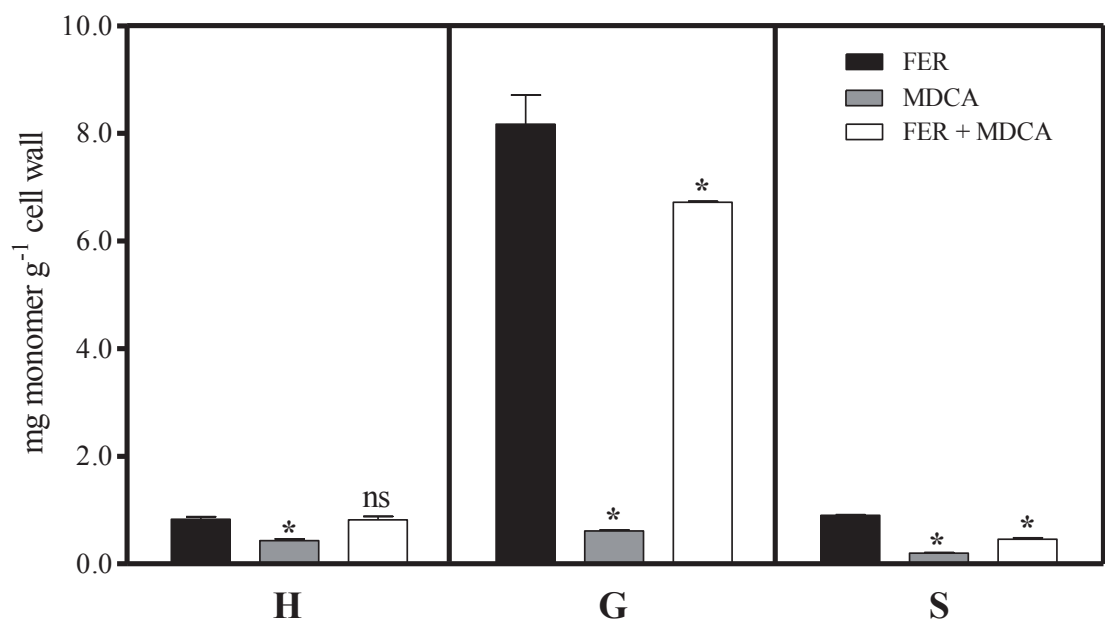


Figure 5. Lignin monomer composition in maize roots treated with 1.0 mM ferulic acid (FER), 1.0 mM 3,4 (methylenedioxy) cinnamic acid (MDCA) and 1.0 mM ferulic acid plus 1.0 mM 3,4 (methylenedioxy)cinnamic acid (FER+MDCA). H, *p*-hydroxyphenyl; G, guaiacyl; and S, syringyl monomers. *Means ($n = 3 \pm SE$) significantly smaller than CIN treatment (Dunnett's multiple comparison test, $P \leq 0.05$) ns, not significant.

wall) (Fig. 2). By contrary, the exposure of maize roots to ferulic acid (FER) increased lignin content by 25.8 %.

Table 2 shows the effects of allelochemicals on lignin monomer composition. Compared with the corresponding controls, cinnamic acid decreased the contents of G (50.9 %) and S (67.9 %) monomers. The analysis of monomers revealed an increase (117.9 %) in the content of G and a slight decrease (15 %) in the S monomer after exposure to ferulic acid. Table 2 also summarizes the results of lignin (referred to as the sum of H+G+S) measurements. While cinnamic acid decreased the H+G+S contents by 43.8 %, ferulic acid increased it by 72.8 % in comparison to the controls. The H:G:S ratio was altered by the action of allelochemicals as follows: cinnamic acid increased H and ferulic acid increased G, compared with the control ratio.

- Effects of cinnamic and ferulic acids and enzyme inhibitors on lignin content and its monomeric composition

Subsequent analyses were conducted to evaluate the changes in lignin content and monomeric composition in response to these allelochemicals when selective inhibitors of phenylpropanoid pathway were used. PIP (a *quasi*-irreversible inhibitor of the enzyme C4H) reduced lignin content by 35 % in comparison to the cinnamic acid (CIN) alone (Fig. 3A). Similarly, a reduction (29 %) in the lignin content was noted in roots exposed to cinnamic acid plus inhibitor (CIN+PIP) compared with the cinnamic acid (CIN) treatment alone. Treatment of roots with MDCA

Table 1.

Changes in the root length and fresh and dry weights of maize treated for 24 h with 1.0 mM cinnamic and ferulic acids.

Condition	Root length (cm)	%	Fresh weight (g)	%	Dry weight (g)	%
Control	3.03 ± 0.02		2.75 ± 0.13		0.194 ± 0.002	
Cinnamic acid	0.19 ± 0.02*	-93.7	1.99 ± 0.04*	-27.6	0.145 ± 0.001*	-25.2
Ferulic acid	0.67 ± 0.04*	-77.9	2.13 ± 0.07*	-22.5	0.156 ± 0.004*	-19.6

Means ($n = 4 \pm SE$) significantly smaller than the control (Dunnett's multiple comparison test) are marked with an asterisk (*). The column % represents inhibition of statistically significant means in comparison to control (0 mM).

Table 2. Changes in the lignin monomer composition of maize treated for 24 h with 1.0 mM cinnamic and ferulic acids. The results are expressed as mg monomer g⁻¹ cell wall.

Monomer	Control	Cinnamic acid	Ferulic acid
H	0.92 ± 0.03	1.04 ± 0.03 ^{ns}	0.83 ± 0.05 ^{ns}
G	3.75 ± 0.05	1.84 ± 0.13*	8.17 ± 0.54*
S	1.06 ± 0.01	0.34 ± 0.05*	0.90 ± 0.02*
H + G + S	5.73 ± 0.08	3.22 ± 0.18*	9.90 ± 0.60*
H : G : S	16 : 65 : 19	32 : 57 : 11	8 : 83 : 9

H, *p*-hydroxyphenyl; G, guaiacyl and S, syringyl. Means ($n = 3 \pm \text{SE}$) significantly smaller than the control (Dunnett's multiple comparison test) are marked with an asterisk (*). ns, not significant.

(a competitive inhibitor of the enzyme 4CL) and ferulic acid plus MDCA (FER+MDCA) reduced lignin content by 37 % and 16 %, respectively, in comparison to the ferulic acid (FER) alone (Fig. 3B).

Lignin monomer composition was evaluated by alkaline nitrobenzene oxidation (Fig. 4). Following treatment with PIP, the H and G monomer contents decreased by 65 % and 51 %, respectively, compared with the cinnamic acid (CIN) alone. The exposure of roots to the allelochemical plus inhibitor (CIN+PIP) significantly reduced H (64 %), G (54 %) and S (38 %) contents compared with cinnamic acid (CIN) treatment. The analysis of monomer content revealed significant decreases in H (48 %), G (93 %) and S (78 %) after exposure to MDCA compared with the allelochemical (FER) treatment (Fig. 5). In conjunction with ferulic acid, MDCA reduced the content of G (18 %) and S (19 %) monomers compared with the allelochemical treatment.

Discussion

Plant roots are susceptible to allelochemical stress during early seedling growth (Inderjit and Duke, 2003; Weir *et al.*, 2004) due to high metabolic activity and the start of lignification at this stage (Donaldson, 2001). Studies with cinnamic and ferulic acids have shown inhibitory effects on root growth (length and weight) in different plants species (Baleroni *et al.*, 2000a; Ng *et al.*, 2003; Ding *et al.*, 2007; Macias *et al.*, 2007; Politycka and Mielcarz, 2007; Reigosa and Pazos-Malvido, 2007; Salvador *et al.*, 2013) including maize (Janovicek *et al.*, 1997; Prasad and Devi, 2002; Gelsomino *et al.*, 2015). This study further confirmed the susceptibility of maize for both compounds, which reduced the length as well as the fresh and dry weights of roots.

Different modes of action have been proposed for the inhibition of plant growth by allelochemicals

(Dayan *et al.*, 2000). Disruption and depolarization of the cell membrane, increases in lipid peroxidation and reactive oxygen species (ROS), decreased H-ATPase activity and loss of viability (Onnerud, 2002; Yu *et al.*, 2003; Ding *et al.*, 2007). As a cascade, many other metabolic processes such as photosynthesis and cellular respiration (Weir *et al.*, 2004), protein synthesis (Baziramakenga *et al.*, 1997), lipid mobilization (Baleroni *et al.*, 2000b), glucose metabolism (Ferrarese *et al.*, 2001) and enzyme activities (Doblinski *et al.*, 2003; Salvador *et al.*, 2013) can be affected. Despite the relevance of the cell membrane as a possible site of allelochemical action, the cell wall can be considered one of the first tissues affected by stress signals, which are then transmitted to the cell interior and influence additional processes (Komatsu *et al.*, 2010). It is also known that changes in plant growth can be attributed to changes in the conductive tissues, such as phloem and xylem (Chen *et al.*, 2006). Previous work suggests that the impairment of root growth by allelochemicals is associated with premature lignification of the cell walls (Komatsu *et al.*, 2010; Bubna *et al.*, 2011; Salvador *et al.*, 2013) as well as structural changes in root cells consistent with enhanced lignin production (dos Santos *et al.*, 2008). However, all these reports have addressed the effect of allelochemicals on soybean, a dicotyledonous plant. In the current work, experiments were conducted with maize, a monocotyledonous plant, to test whether phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) activities and the production and composition of lignin were affected by allelochemicals.

As described earlier, PAL and TAL reactions form cinnamate derivatives which form monolignols that are polymerized within lignin (Vanholme *et al.*, 2012). After ferulic acid treatment, PAL and TAL activities decreased in spite of lignin production and there was enhanced formation of G monomer and a high

H+G+S value. Effects of phenolic allelochemicals on PAL activity are contradictory and can be stimulatory (Politycka, 1999; dos Santos *et al.*, 2004), have no effect (Shann and Blum, 1987) or be inhibitory (Sato *et al.*, 1982; Zanardo *et al.*, 2009; Bubna *et al.*, 2011). While the action of allelochemicals on PAL activity have been reported, their effects on TAL have not been described to date. It was hypothesized that the high influx of exogenous ferulic acid into the phenylpropanoid pathway, via the 4CL reaction, inhibited PAL/TAL, presumably by a feedback process (Blount *et al.*, 2000). As a consequence, the low levels of phenylpropanoid derivatives could be supplied by exogenous ferulic acid with subsequent lignin production. In soybean roots, other hydroxylated cinnamic acid derivatives such as *p*-coumaric and caffeic acids decreased PAL activity but increased lignin production (Zanardo *et al.*, 2009; Bubna *et al.*, 2011). This study showed that cinnamic acid decreased TAL activity, the levels of G and S monomers as well as lignin (sum of H+G+S). Upon initial observation, there were no clear explanations for these findings. However, as the phenylpropanoid pathway was subject to complex control, it was possible that cinnamic acid inhibited other enzymes such as CAD which catalyzed the final step in a branch of phenylpropanoid synthesis specific for the production of lignin monomers. Another possibility was that cinnamic acid itself could induce potential side effects, affecting maize root lignification.

Since exogenous allelochemicals can be incorporated into the structure of lignin by means of the phenylpropanoid pathway (dos Santos *et al.*, 2008; Zanardo *et al.*, 2009; Bubna *et al.*, 2011; Salvador *et al.*, 2013), changes in lignin monomer composition caused by specific inhibitors could be enlightening. Because cinnamic acid forms *p*-coumaric acid through the action of the C4H enzyme, maize roots were stressed by the allelochemical, alone or jointly with PIP, a *quasi*-irreversible inhibitor of the enzyme (Schoch, 2002). As expected, the exposure of maize roots to the PIP reduced the total lignin content and the amount of H and G monomers in comparison to the ferulic acid treatment alone. Jointly with cinnamic acid, PIP reduced total lignin content and the content of all monomers. These findings indicated that the C4H inhibitor prevented either the entry of the allelochemical into the metabolic pathway or the formation of lignin and lignin monomers. These observations were supported by reductions in the H+G+S sum and alterations of the H:G:S ratio (32:57:11) which differed from controls (16:65:19). These findings clearly indicated that exogenously applied cinnamic acid entered into the phenylpropanoid pathway via the C4H reaction and reduced the production of lignin

and its monomers. Based on these data, decreased maize root growth caused by cinnamic acid was not related to enhanced lignification, as hypothesized for soybean (Salvador *et al.*, 2013).

To examine whether exogenous ferulic acid can be channeled into the phenylpropanoid metabolic route, experiments were performed using MDCA, a competitive inhibitor of the 4CL enzyme which converts hydroxycinnamic acids to hydroxycinnamoyl-CoA thioesters (Chakraborty *et al.*, 2009). Results showed that MDCA reduced the total lignin and lignin monomers compared to the ferulic acid treatment while treatment with ferulic acid and MDCA reduced the content of total lignin and G/S monomers. As MDCA inhibited 4CL, the access of exogenous ferulic acid to the phenylpropanoid pathway was blocked. In contrast with the results for cinnamic acid, ferulic acid increased the sum of H+G+S and changed the H:G:S ratio (8:83:9) primarily due to the enhanced formation of the G monomer. These findings strengthened the hypothesis that ferulic acid could be channeled into the phenylpropanoid pathway via the 4CL reaction. Furthermore, increased lignin production was associated with decreased maize root growth, which was similar to effects reported for soybeans (dos Santos *et al.*, 2004; 2008). Supporting evidence for this hypothesis comes from studies in which the incorporation of exogenous ferulic acid leads to the formation of feruloyl-CoA and further to coniferyl and sinapyl alcohols in poplar (Hamada *et al.*, 2003) and that labeled ferulic acid is incorporated into G and S monomers followed by an increase in the cell-wall lignification in robinia (Yamauchi and Fukushima, 2004).

Conclusions

The results obtained in the present research suggested that 1) cinnamic acid reduced maize root growth, TAL activity and lignin (H+G+S) content. The use of PIP as a selective inhibitor of the C4H reaction indicated the specific entry point of this allelochemical into the phenylpropanoid pathway. However, for this study there was no direct link between the reduction of root growth and the lignification process. 2) Ferulic acid reduced maize root growth and PAL/TAL activities but increased G monomer production and lignin (H+G+S) levels. The use of MDCA as a specific inhibitor of the 4CL reaction indicated that ferulic acid could be channeled into the phenylpropanoid pathway. In summary, inhibition of maize root growth may be attributed to the excessive production of lignin due to exogenously applied ferulic acid, as enhanced lignin polymerization solidified the cell wall and restricted plant growth.

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