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Effects of ferulic acid on growth and lignification of conventional and glyphosate-resistant soybean

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ABSTRACT

Ferulic acid (allelochemical commonly found in soil extracts) affects the root growth and lignification of soybean (*Glycine max* L. Merr.) seedlings. Glyphosate-resistance is conferred in soybean by incorporating a gene encoding a glyphosate-insensitive enzyme (CP4-EPSP synthase), which acts in the shikimate pathway to synthesize aromatic amino acids and lignin. To evaluate the possible tolerance of glyphosate-resistant soybean to ferulic acid, comparative studies were done for its effects on the root growth and lignification of two soybean cultivars (conventional and glyphosate-resistant). Three-day-old seedlings of CD 201 (conventional soybean) and CD 214RR (glyphosate-resistant soybean) were cultivated in half-strength Hoagland nutrient solution (pH 6.0), with or without 1.0 mM ferulic acid, 0.5 mM glyphosate or a mixture of 1.0 mM ferulic acid plus 0.5 mM glyphosate in a growth chamber (25°C, 12 h/12 h light/dark photoperiod, irradiance of 280 µmol m^{-2} s^{-1}, 24 to 96 h). The ferulic acid decreased the length, fresh weight and dry weight of roots of both cultivars. Root growth inhibition has been associated with an increase in lignin contents. The mixture of ferulic acid and glyphosate showed similar effects in both cultivars. These results confirm the susceptibility of both conventional and glyphosate-resistant seedlings to ferulic acid and suggest that the genetic modification in the glyphosate-resistant soybean does not develop tolerance to ferulic acid.

Key words: Allelochemical, ferulic acid, *Glycine max*, glyphosate-resistant soybean, lignin, roots.

INTRODUCTION

Ferulic acid (4-hydroxy-3-methoxycinnamic acid) is a central metabolite of the phenylpropanoid pathway, which synthesizes the phenolic compounds and many secondary plant products, including lignin. Lignin is the main structural component to thicken the plant cell walls and imparts mechanical support and efficient flow of water and solutes over long distances within the vascular systems (5). Besides ferulic acid's role in the phenylpropanoid pathway, it is frequently found in soil extracts (13). It affects plant growth at concentrations up to 10 mM (21). Exposure of plant roots to ferulic acid reduces water use (17), inhibits foliar expansion (4) and root elongation (1,22) and decreases the nutrients uptake (3,7,20). Further, this allelochemical rapidly depolarizes the root cell

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membrane, causing a generalized increase in membrane permeability, inducing lipid peroxidation and affecting certain enzymatic activities (2,11,25,26).

Glyphosate [*N*-(phosphonomethyl)glycine, Roundup®, Monsanto] is a broad-spectrum, nonselective herbicide that kills plants by specifically inhibiting 5-enolpyruvylshikimic acid-3-phosphate synthase (EPSPS, EC 2.5.1.19), an enzyme in the shikimate pathway, which leads to the synthesis of aromatic amino acids (tryptophan, tyrosine and phenylalanine) and other ring-containing metabolites, including lignin, by the phenylpropanoid pathway (5). Consequently, the inhibition of EPSPS reduces the biosynthesis of aromatic amino acids, which leads to the arrest of protein production, resulting in the plant's death (15). Glyphosate resistance is conferred in soybean by incorporating a gene encoding a glyphosate-insensitive EPSPS enzyme (CP4-EPSP synthase). Glyphosate-resistant soybean remains unaffected when treated with the herbicide (23).

Reduction in the growth of roots is one of the first effects of allelochemicals and is associated with premature lignification of the cell walls (6,26). Based on this, we postulate that if ferulic acid interferes with the phenylpropanoid pathway (26), and the glyphosate-resistant soybean has a genetic modification related to this pathway (15), this soybean line might exhibit tolerance to the allelochemical. Thus, the main question of the present work was whether exogenously supplied ferulic acid affects the root growth (length and weight) and lignin contents of CD 201 (conventional) and CD 214RR (glyphosate-resistant) soybean seedlings.

MATERIALS AND METHODS

Soybean [*Glycine max* L. Merr. *cv.* CD 201 – conventional – and CD 214RR – glyphosate-resistant – (Coodetec, Brazil)] seeds, surface-sterilized with 2% sodium hypochlorite for 5 min and rinsed extensively with deionized water, were dark-germinated (at 25 C) on three sheets of moistened filter paper. Twenty-five 3-day-old seedlings of uniform size were grown 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 (prepared in water). Subsequent experiments were performed with 0.5 mM glyphosate (prepared in water) or with a mixture of 1.0 mM ferulic acid plus 0.5 mM glyphosate. The container was kept in a growth chamber (25°C, 12 h/12 h light/dark photoperiod, irradiance of 280 µmol m^{-2} s^{-1}). Roots were measured at the beginning and at the end of experiments. Fresh root weight was determined immediately after incubation and dry weight estimated after oven drying at 80°C, for 24, 48, 72 and 96 h. Ferulic acid and *N*-(phosphonomethyl)glycine (glyphosate) were purchased from Sigma Chemical Co. (St. Louis, USA) and all other reagents used were of the purest grade available.

Lignin

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 (14). The pellet was centrifuged (1400 *g*, 4 min) and washed by successive stirring and centrifugation as follows: twice with phosphate buffer, pH 7.0 (7 ml); three times with 1% (v/v) Triton® X-100 in phosphate buffer, pH 7.0 (7 ml); twice with 1 M NaCl in phosphate buffer, pH 7.0 (7 ml); twice with distilled water (7 ml), and twice with 100% acetone (5 ml). The pellet was dried in an oven (60°C, 24 h) and cooled down in a vacuum desiccator. The dry matter obtained 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 (95ºC, 4 h). After cooling at room temperature, the sample was centrifuged (1400 *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 (30ºC, 18 h, 115 oscillations min^{-1}) in 0.5 M NaOH (6 ml). After centrifugation (1400 *g*, 5 min), the supernatant was stored. The pellet was washed again with 0.5 M NaOH (3 ml) and after centrifugation the supernatant was mixed with the supernatant obtained earlier. The combined alkali extracts were acidified with concentrated HCl (1.8 ml). After precipitation (0°C, 4 h), LTGA was recovered by centrifugation (1400 *g*, 5 min) and washed twice 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 content was expressed as mg LTGA g^{-1} dry weight.

Statistical analysis

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 four independent experiments ± S.E. The one-way variance analysis to test the significance of the observed differences was performed with the Sisvar package (Version 4.6, UFLA, Brazil). The difference among parameters was evaluated by the Scott–Knott test, and *P* values 0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

Root lengths were decreased in conventional (CD 201) soybean seedlings grown for 96 h in nutrient solution containing 1.0 mM ferulic acid (Fig. 1A). Root lengths were reduced by 48.1, 65.6, 69.2 and 54.2% after 24, 48, 72 and 96 h of ferulic acid treatment, respectively, compared to control. Root lengths were also decreased in glyphosate-resistant (CD 214RR) soybean seedlings grown in nutrient solution containing the allelochemical (Fig. 1B). In this variety, root lengths were reduced by 52.3, 69.8, 71.6 and 58.9% after ferulic acid treatment than control. There were non-significant differences in root lengths of ferulic acid treated conventional and glyphosate-resistant cultivars. Fresh weights of roots incubated in ferulic acid were significantly different from the control (Fig. 1C). Fresh weights of conventional soybean seedlings were decreased by 29.4, 53.2 and 25% after 48, 72 and 96 h exposure to ferulic acid, over the control. The allelochemical also reduced the root fresh weights of glyphosate-resistant soybean seedlings (Fig. 1D). Root fresh weights were 31.2, 39.1 and 27.1% lower than control for 48, 72 and 96 h of ferulic acid, respectively. There were no significant differences between the conventional and glyphosate-resistant cultivars after allelochemical treatments. Ferulic acid affected the root

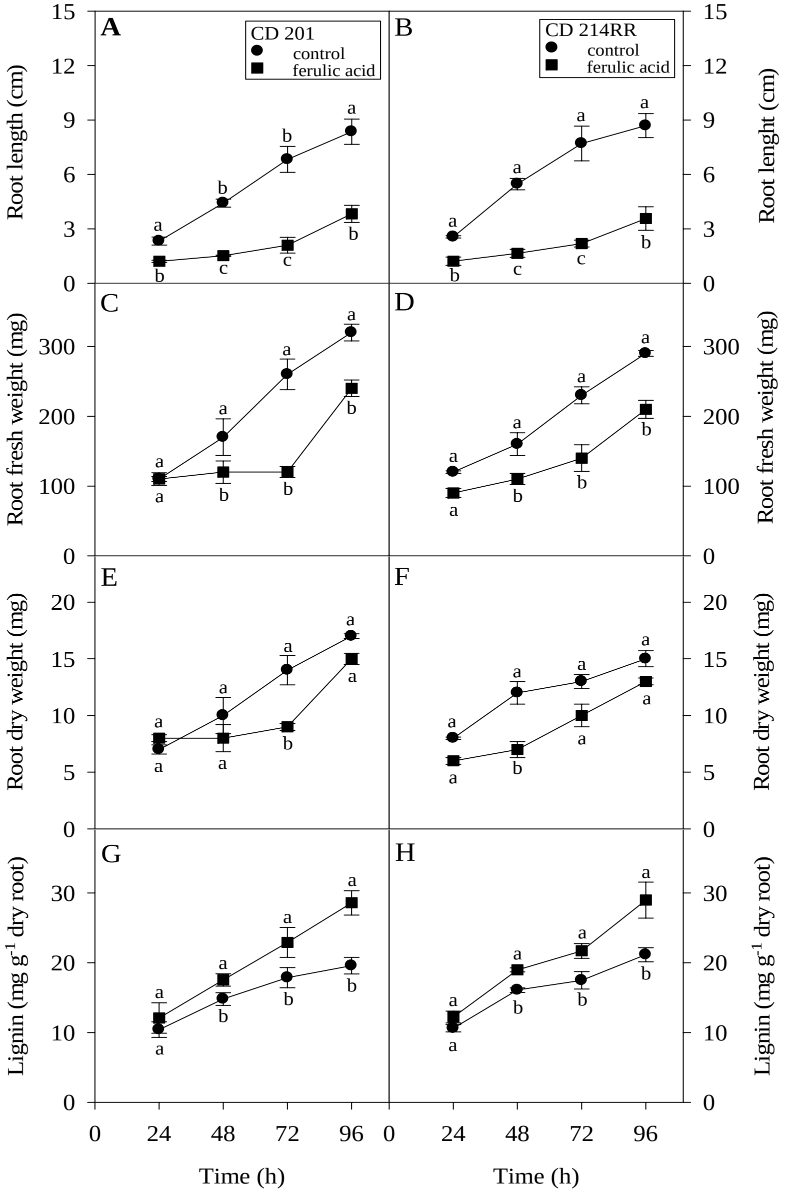

Figure 1. Effects of ferulic acid on root length (A and B), root fresh weight (C and D), root dry weight (E and F), and lignin content (G and H) of CD 201 (conventional) and CD 214RR (glyphosate-resistant) soybean seedlings. Values (N = 4 ± S.E.) differing statistically (Scott-Knott´s test) from the control (*P* 0.05) and between cultivars are shown.

dry weights. The dry weight of conventional soybean roots was reduced by 35.7% at 72 h after incubation with the allelochemical than untreated roots (Fig. 1E). The dry weight of glyphosate-resistant roots after 48 h exposure to ferulic acid was 41.7% less than control (Fig. 1F). No significant differences were observed between the conventional and glyphosate-resistant cultivars after 24 h and 96 h.

Following ferulic acid treatment, the lignin content in conventional soybean roots increased significantly by 18.4, 28.3 and 45.9% at 48, 72 and 96 h, respectively (Fig. 1G). Similarly, the allelochemical increased the lignin content of glyphosate-resistant soybean seedlings (Fig. 1H). Lignin contents were 18, 24.2 and 37% higher than control for 48, 72 and 96 h ferulic acid treatment, respectively. Once more, there were no significant differences between the conventional and glyphosate-resistant cultivars after ferulic acid treatments.

Root growth (root length and fresh and dry weights) of conventional soybean decreased with exposure to 1.0 mM ferulic acid (Fig. 1A,C,E). Studies using 1.0 mM ferulic acid applied in hydroponic cultures have shown root growth inhibition of different plant species. The hypocotyl length of mung bean (*Phaseolus aureus* L.) decreases by 40% after 1.0 mM ferulic acid treatment (10). The root length and fresh weight of pea (*Pisum sativum* L.) were significantly reduced (>70%) by 1.0 mM ferulic acid (27). Application of 1.0 mM ferulic acid decreases the growth of maize (*Zea mays* L.) seedlings, affecting both shoots (>60%) and roots (>40%) (11). Additionally, application of 1.0 mM FA drastically decreases the root length (>21%) and root dry weight (>30%) of sorghum (*Sorghum bicolor* Moench.) seedlings (12). Patterson (24) showed that, in conventional soybean, 1.0 mM ferulic acid significantly reduced the total dry weight (>45%). The length and the fresh and dry weights of conventional soybean roots decreased after 1.0 mM allelochemical treatment (26). Consistent with these cited reports, 1.0 mM ferulic acid also reduced the root length and the fresh and dry weights of glyphosate-resistant soybean seedlings (Fig. 1B,D,F).

Our main finding is that ferulic acid not only reduced the root growth of conventional and glyphosate-resistant soybean seedlings, but also significantly increased the lignin content (Fig. 1G,H). Lignin is complex polymer of hydroxylated and methoxylated phenylpropane units. The phenylpropanoid pathway synthesizes phenolic compounds, which are derived by enzymatic deamination of phenylalanine and tyrosine generated in shikimate pathway via nitrogen-free skeletons of cinnamate or *p*-coumarate. Once formed, the latter compound is converted in phenylpropanoid metabolism, into phenolic acids which are polymerized in lignin (5). Cell walls are known to become lignified when cell expansion decreases, when the cell differentiates into xylem and, notably, when the cell is under biotic or abiotic stresses (8). Premature lignification of the cell walls, associated with reduced growth of the plant roots, is one of stress effects of ferulic acid on soybean (26). This hypothesis has been strengthened by the fact that ferulic acid may be esterified with cell wall polysaccharides or incorporated into lignin structures or may form bridges which connect lignin with the wall polysaccharides. This forms a complex network that solidifies the plant cell wall by increasing its rigidity and subsequently restricting the root growth (18,25,26).

Subsequent experiments were done to compare the actions of 1.0 mM ferulic acid and 0.5 mM glyphosate herbicide, 48 h after treatment (Fig. 2). Root lengths were

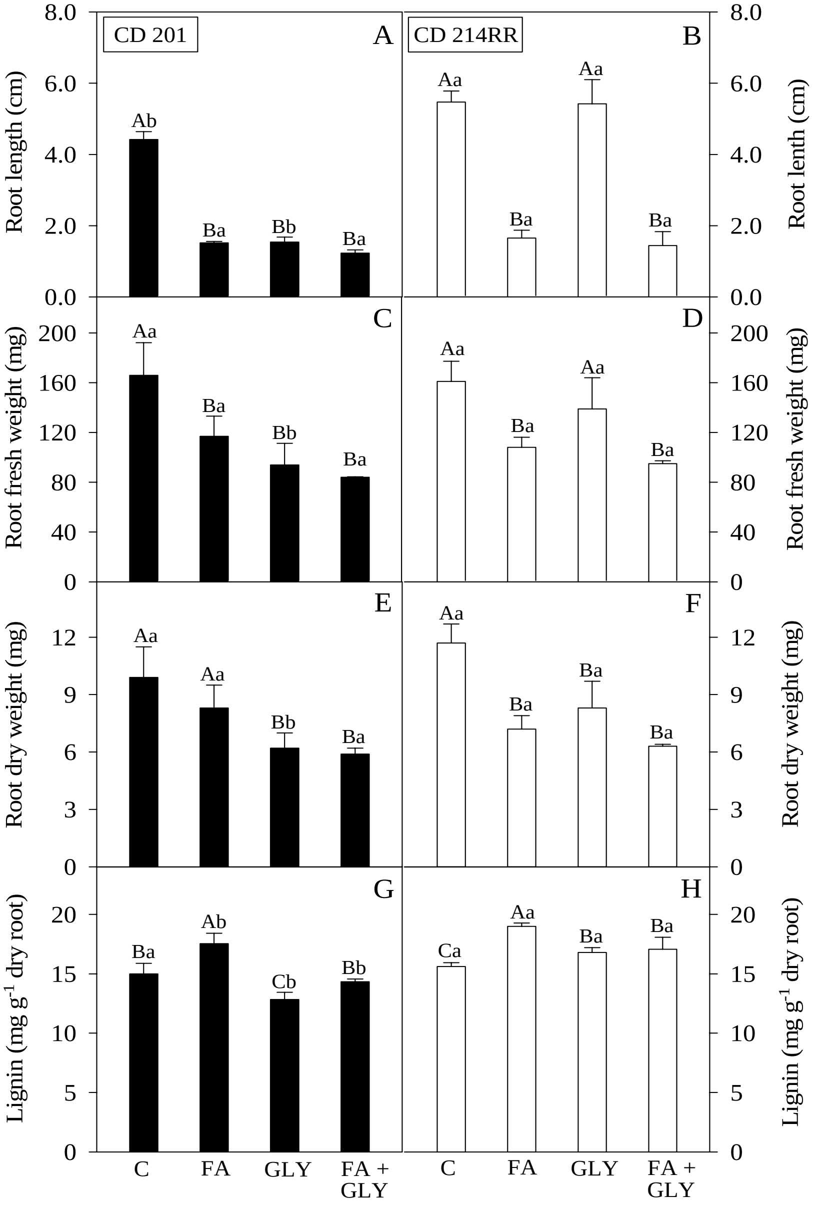

Figure 2. Effects of ferulic acid and glyphosate on root length (A and B), root fresh weight (C and D), root dry weight (E and F) and lignin content (G and H) of CD 201 (conventional) and CD 214RR (glyphosate-resistant) soybean seedlings grown during 48 h. C, control; FA, 1.0 mM ferulic acid; GLY, 0.5 mM glyphosate, and FY+GLY, 1.0 mM ferulic acid plus 0.5 mM glyphosate. Values (N = 4 ± S.E.) differing statistically (Scott-Knott´s test) from the control (*P* 0.05) are marked in capital letters, while values differing between cultivars are marked in lower case letters.

decreased in glyphosate-resistant soybean seedlings grown in nutrient solution containing FA or FA+GLY (Fig. 2B). No significant differences were observed between the conventional and glyphosate-resistant cultivars after these treatments. As expected, GLY did not affect the root growth of CD 214RR seedlings. Root fresh weights were significantly different from the control after treatment of conventional seedlings with FA, GLY or FA+GLY (Fig. 2C). Root fresh weights of the glyphosate-resistant seedlings were reduced by incubation in FA or FA+GLY, while no significant change was observed after GLY treatment, compared to control roots (Fig. 2D). Similar to the root lengths, a significant difference between cultivars was noted only in the GLY treatment. Except the treatment of conventional soybean with FA, the root dry weights were decreased in all experimental conditions than controls (Fig. 2E,F). No significant differences were verified between the conventional and glyphosate-resistant cultivars after FA and FA+GLY exposures.

The lignin content in conventional soybean roots increased significantly after FA treatment, decreased in response to GLY and was not affected by the FA+GLY treatment (Fig. 2G). On the other hand, the lignin content in glyphosate-resistant roots increased in all experimental conditions, compared to control roots (Fig. 2H). Significant differences in lignin content were observed between the conventional and glyphosate-resistant cultivars after all treatments.

To compare the actions of ferulic acid and glyphosate on root growth and lignification, soybean seedlings were grown for 48 h in nutrient solution containing ferulic acid, glyphosate or the mixture of these compounds. Acting alone or jointly, both compounds decreased the root growth (length and weight) of conventional soybean (Fig. 2A,C,E). Similar behavior was observed for glyphosate-resistant soybean when ferulic acid or the mixture FA+GLY were applied (Fig. 2B,D,F). By contrast, glyphosate did not affect the root growth (length and fresh weight) of genetically modified soybean – an expected result since this cultivar tolerates the herbicide (15,23). However, the mixture of the compounds (FA+GLY), reduced the root growth (length and weights) of glyphosate-resistant soybean. Thus, current results clearly indicate that ferulic acid inhibits the root growth of this cultivar, independently of the genetic modification related to the shikimate pathway.

Root lignification was also changed by the action of ferulic acid or glyphosate (Fig. 2G,H). Ferulic acid increased the lignin contents of conventional and glyphosate-resistant soybeans, although no significant difference was observed between cultivars. A dual behavior was noted for the lignin content of seedlings exposed to glyphosate: a reduction for CD 201 and an increase for CD 214RR than controls. There is no simple explanation for this, although there are some supporting results. Application of glyphosate significantly reduced the deposition of lignin in bean (*Phaseolus vulgaris* L.) roots (19). By contrast, unexpected effects on the lignin metabolism due to lignin overproduction in glyphosate-resistant soybean have been reported after application of the herbicide (9,16). Although there were differences among the cultivars for all experimental conditions, FA+GLY significantly reduced the lignin contents of both cultivars than ferulic acid (FA) treatments. This is evidence that the increase in root lignification may be due to the action of ferulic acid.

In conclusion, current results confirm the susceptibility of both conventional and glyphosate-resistant soybeans to ferulic acid by showing the role of this phenolic acid as an allelochemical. In addition, data suggest that the genetic modification in glyphosate-resistant soybean does not generate resistance to the allelopathic action of ferulic acid.

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