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Table: 1, Figs : 3

Mimosine-inhibited soybean (*Glycine max*) root growth, lignification and related enzymes

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ABSTRACT

The phytotoxic mechanism of mimosine and its effects on phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) and peroxidase (POD, EC 1.11.1.7) activities as well as lignin content in soybean (*Glycine max* (L.) Merr.) roots were investigated. Seedlings (3-d-old) were cultivated in half-strength Hoagland nutrient solution (pH 6.0) with or without 0.05 to 1.0 mM mimosine in a growth chamber (25°C, 12/12 h light/dark photoperiod, irradiance of 280 µmol m^{-2} s^{-1}) for 24 h. In general, length and fresh and dry weights of roots decreased in response to mimosine. Root growth inhibition has been associated with reduction in PAL and POD activities and lignin content. These findings suggest that PAL and POD enzymes are responsible for the mimosine allelochemical action.

Key words: Allelopathy, *Leucaena leucocephala*, lignin, peroxidase, phenylalanine ammonia-lyase, root, soybean.

INTRODUCTION

Plants are known to release organic compounds into the environment by volatilization through their aerial parts; leaching of the aerial parts by rain; exudation through roots; leaching from plant litter; and decomposition of organic matter. These secondary plant metabolites may accumulate in the soil environment and influence the growth and development of neighboring plants (allelopathic effect) (6,24). The above-mentioned allelopathy process is proper to *Leucaena leucocephala* (Lam.) de Wit (9,22).

Leucaena is the most productive and versatile multi-purpose legume tree in tropical agriculture. It is widely recommended for agroforestry due to its fast growth rate, fodder, fuel and wood value, ability to fix nitrogen and improve overall land productivity (1,11). This plant species has been used as a cover crop to control weeds by its production of potential allelochemicals such as gallic, protocatechuic, *p*-hydroxybenzoic, *p*-hydroxyphenylacetic, vanillic, ferulic, caffeic and *p*-coumaric acids and quercetin (3). However, the production and release of a non-protein amino acid named mimosine (-(3-hydroxy-4-pyridon-1-yl)-L-alanine) from its leaves and seeds (2 to 10% of dry weight) has been considered the main cause of allelopathy (9,11). Mimosine affects the growth of various plants such as lettuce (*Lactuca sativa*), rice (*Oryza sativa*), radish (*Raphanus sativus*), turnip (*Brassica rapa*), wheat (*Triticum aestivum*) and maize (*Zea mays*). In

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general, mimosine inhibits germination, seedling vigor, root growth, food mobilization, starch solubilization, protein breakdown and enzyme activities (1,14,15,16). Despite these reports, the mechanism of action of mimosine is not yet fully understood.

The phenylpropanoid pathway is one of the most important metabolic routes due to its responsibility for the synthesis of phenolic compounds and a wide range of secondary plant products, including lignin (2). Phenylalanine ammonia-lyase (PAL) is considered to be the primary enzyme of the phenylpropanoid biosynthetic pathway, and peroxidase (POD) within the cell wall, in either the free or bound state, has been associated with monolignol polymerization and, therefore, with lignin synthesis (2,13). To date, no reports concerning the effects of exogenous mimosine on the lignification of soybean roots are available. Consequently, the aim of the present work has been to investigate whether the lignification process is a target of mimosine action. PAL and POD activities and lignin content were determined after treatment of soybean roots with mimosine.

MATERIALS AND METHODS

General Procedures

Soybean (*Glycine max* (L.) Merr. *cv.* BRS-184) 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 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 with or without 0.05 to 1.0 mM mimosine. Each experiment was represented by one glass container. Nutrient solution was buffered with 17 mM potassium buffer, adjusted to pH 6.0 and monitored over time. The container was kept in a growth chamber (25°C, 12/12 h light/dark photoperiod, irradiance of 280 µmol m^{-2} s^{-1}). Root lengths were measured at the beginning of the experiment and after 24 h. At the end of the experiment, the roots were carefully blotted with an absorbent paper and the fresh weight was determined. Dry root weight was estimated after oven-drying at 80°C until it reached a constant weight. Mimosine was purchased from Sigma Chemical Co (St Louis, USA), and all other reagents used were of the purest grade available or of chromatographic grade.

Enzymatic Assays

After exposure to increasing mimosine concentrations, all roots were detached and enzymes were extracted. Phenylalanine ammonia-lyase (PAL) was extracted as described by Ferrarese *et al.* (7). Fresh roots (2 g) were ground at 4ºC in 0.1 M sodium borate buffer (pH 8.8). Homogenates were centrifuged (2200 *g*, 15 min) and the supernatant was used as the enzyme preparation. The reaction mixture (100 moles sodium borate buffer pH 8.7 and a suitable amount of enzyme extract in a final volume of 1.5 ml) was incubated at 40°C for 5 min for the PAL activity assay. Fifteen µmoles of L-phenylalanine were added to start the reaction, which was stopped after 1 h of incubation by the addition of 50 µl of 5 N HCl. Samples were filtered through a 0.45 µm disposable syringe filter and analyzed (20 µl) with a Shimadzu® Liquid Chromatograph (Tokyo, Japan) equipped with a LC-10AD pump, a Rheodine® injector, a SPD-10A UV detector, a

CBM-101 Communications Bus Module and a Class-CR10 workstation system. A reversed-phase Shimpack® GLC-ODS (M) column (150 4.6 mm, 5 µm) was used at room temperature, with an equivalent pre-column (10 4.6 mm). The mobile phase was methanol:water (70%:30%) with a flow rate of 0.5 ml min^{-1}. Absorption was measured 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 standard values. Parallel controls without L-phenylalanine or with *t*-cinnamate (added as an internal standard in the reaction mixture) were performed as described elsewhere (7). PAL activity was expressed as µmol *t*-cinnamate h^{-1} g^{-1} of fresh weight.

Peroxidase (POD) was extracted from fresh roots (0.5 g) with 67 mM phosphate buffer (5 ml, pH 7.0). The extract was centrifuged (2200 *g*, 5 min, 4°C), and the supernatant was used to determine the activity of soluble POD. For cell wall-bound POD isolation, the pellet was washed with deionized water until no soluble POD activity was detected in the supernatant. The pellet was then incubated in 1 M NaCl (2 ml, 1 h, 4°C), and the homogenate was centrifuged (2200 *g*, 5 min). The supernatant contained the cell wall-(ionically)-bound POD. Guaiacol-dependent activities of soluble and cell wall-bound POD were determined according to Cakmak and Horst (4), with slight modifications. The reaction mixture (3 ml) contained 25 mM sodium phosphate buffer, pH 6.8, 2.58 mM guaiacol and 10 mM H_2O_2. The reaction was started by adding the enzyme extract to the phosphate buffer. Guaiacol oxidation was followed for 5 min at 470 nm, and the enzyme activity was calculated from the extinction coefficient (25.5 mM^{-1} cm^{-1}) of tetraguaiacol. The blank consisted of a reaction mixture without enzyme extract; this absorbance was subtracted from the mixture with enzyme extract. POD activities were expressed as µmol tetraguaiacol min^{-1} g^{-1} of fresh weight.

Lignin Quantification

After exposure to increasing mimosine concentrations, 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 (8). 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); 3 times with 1% (v/v) Triton® X-100 in pH 7.0 buffer (7 ml); 2 times with 1 M NaCl in pH 7.0 buffer (7 ml); 2 times with distilled water (7 ml); and 2 times with acetone (5 ml). The pellet was dried in an oven (60°C, 24 h) and cooled 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 3 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 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 2 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 mg LTGA g^{-1} of dry weight.

Statistical analysis

The experimental design was completely randomized, and each experiment was represented by one glass container with twenty-five seedlings. Data are expressed as the mean of three to five independent experiments ± S.E. Significant differences were verified by one-way analysis of variance with the *Sisvar* package (Version 4.6, UFLA, Brazil). Differences among parameters were evaluated by the Scott-Knott test, and P 0.05 was considered as statistically significant. Relationship between root growth and lignin content was tested by correlation analysis.

RESULTS AND DISCUSSION

Root length and fresh and dry weights decreased in soybean seedlings grown during short-term exposure (24 h) in nutrient solution containing increasing concentrations mimosine (Table 1). This allelochemical inhibited root length from 18.6 to 86.6% after 0.05 to 1.0 mM treatments when compared to control. Fresh root weights were 14.8, 19.5 and 20.3% less than control for 0.25, 0.5 and 1.0 mM treatments, respectively. No significant changes in the fresh weight of roots exposed to mimosine, at low concentrations (0.1 mM), were recorded. Dry root weights were 16.7 to 45.0% less than control for all treatments.

Table 1. Changes in the root length, fresh weight and dry weight of soybean seedlings treated for 24 h with mimosine

Mimosine (mM)	Root length (cm)	Inhibition (%)	Fresh weight (g)	Inhibition (%)	Dry weight (g)	Inhibition (%)
0	2.53 0.067^{a}		2.56 0.053^{a}		0.180 0.017^{a}	
0.05	2.06 0.182^{b}	18.6	2.39 0.034^{a}	6.6	0.139 0.002^{c}	22.8
0.1	1.48 0.188^{c}	41.5	2.55 0.067^{a}	0.4	0.150 0.003^{b}	16.7
0.25	0.43 0.043^{d}	83.0	2.18 0.074^{b}	14.8	0.131 0.004^{c}	27.2
0.5	0.35 0.009^{d}	86.2	2.06 0.024^{b}	19.5	0.099 0.001^{d}	45.0
1.0	0.34 0.031^{d}	86.6	2.04 0.057^{b}	20.3	0.120 0.003^{c}	33.3

Mean ± S.E. values (N = 5) followed by the same letter are not significantly different according to Scott-Knott test (P 0.05). The symbol % indicates inhibition of statistically significant means in comparison to control (0 mM).

Previous studies have shown inhibitory actions of mimosine on the growth of many plant species. For example, application of 0.05 and 0.1 mM mimosine resulted in significant inhibitory effects on the radicle growth of lettuce, radish, rice and turnip (11,20). At 1.0 mM, mimosine caused a significant reduction in seed germination, radicle and plumule length of rape (*Brassica campestris*), radish, wheat (*Triticum aestivum*), bean (*Phaseolus vulgaris*), and mung bean (*Vigna mungo*). Significant inhibitions in seed germination, root and shoot lengths and fresh weights of rice seedlings were verified using 0.5 mM mimosine treatment (14). As reported by Pires *et al*. (15), inhibition of maize root growth by mimosine was proportional to the aqueous extract concentration (0.1 to 0.95 mM allelochemical). More recently, Xuan *et al*. (22) demonstrated that mimosine (0.25 to 5.0 mM) strongly suppressed the length of radicles and hypocotyls of bean, turnip, ryegrass (*Lolium muliflorum*), *Bidens pilosa*, *Leucaena leucocephala* and *Mimosa pudica*. Consistent with the above-quoted reports, mimosine (up to 0.05 - 0.25 mM) also reduced soybean root growth and fresh and dry weights (Table 1), indicating susceptibility of this plant to the compound.

Mimosine-affected PAL activities were significantly different from those of control (Fig. 1). Enzymatic activities decreased 49.8 and 65.4% at 0.5 and 1.0 mM treatments, respectively. Mimosine at 0.25 mM was non-inhibitory to PAL activity. Similar behavior was also evident in soluble POD (Fig. 2A). Enzyme activities decreased 26.4 and 36.3% at 0.5 and 1.0 mM mimosine, respectively, when compared to untreated roots. The cell wall-bound POD activities (Fig. 2B) decreased from 24.9 to 61.2% from 0.05 to 1.0 mM mimosine compared to control. The allelochemical significantly decreased the lignin content of soybean roots (Fig. 3). Inhibition as much as 24.1 to 37.8% was observed with 0.25 and 1.0 mM mimosine. No significant changes in the lignin content of roots exposed to mimosine at 0.1 mM were recorded.

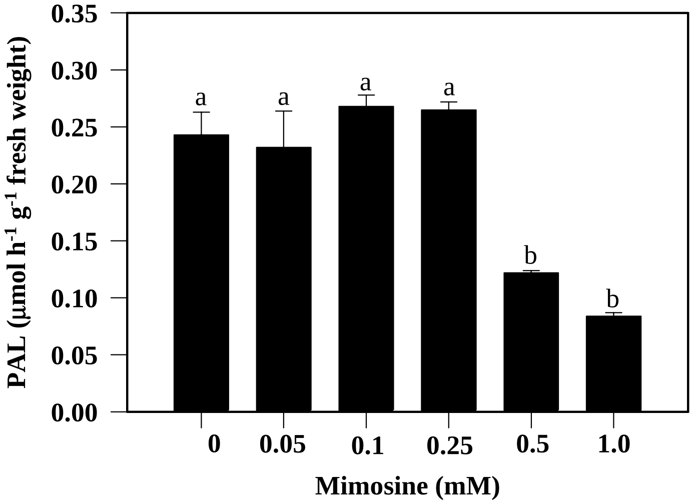

Figure 1. Effects of mimosine on phenylalanine ammonia-lyase (PAL). Mean ± S.E. values (N = 3) followed by the same letter are not significantly different according to Scott-Knott test (*P* 0.05).

The discovery that mimosine decreased lignin production (Fig. 3) coupled to a reduction in soybean root growth (Table 1) is of particular interest. Lignin biosynthesis involves the polymerization of monolignols primarily derived from the phenylpropanoid pathway. It is well established that PAL, the primary enzyme of phenylpropanoid metabolism, regulates the production of phenolic compounds (2). PAL activity, which is believed to be critical for controlling lignin cell wall deposition (12), is lowered by treating

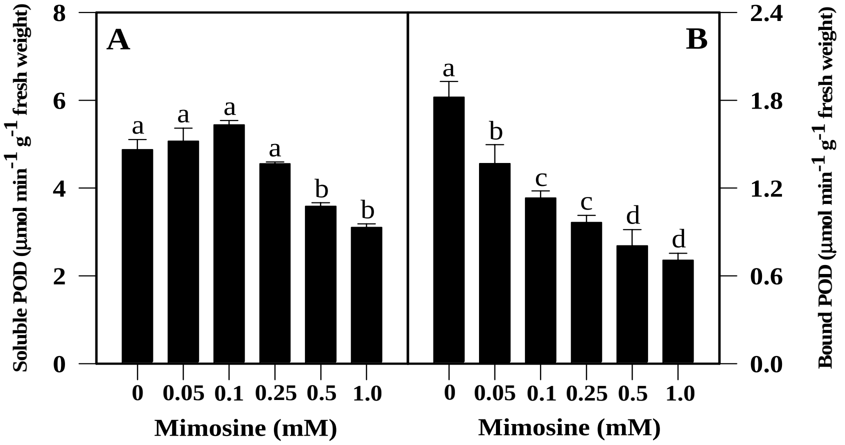

Figure 2. Effects of mimosine on soluble (A) and cell wall-bound (B) peroxidases (POD). Mean ± S.E. values (N = 3) followed by the same letter are not significantly different according to Scott-Knott test (*P* 0.05).

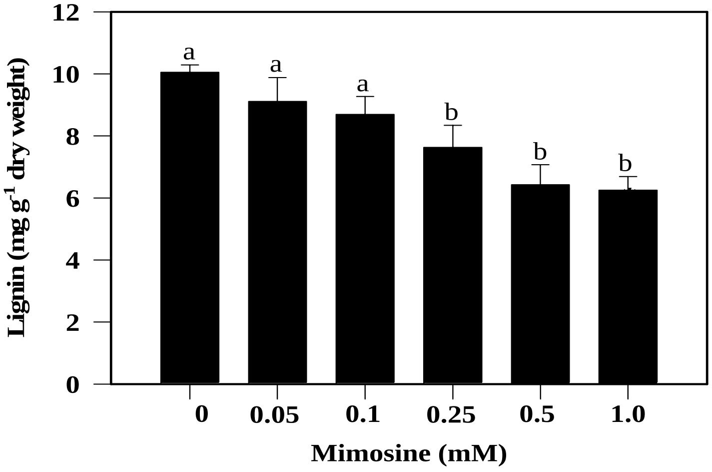

Figure 3. Effects of mimosine on lignin content. Mean ± S.E. values (N = 4) followed by the same letter are not significantly different according to Scott-Knott test (*P* 0.05).

soybean roots with high mimosine concentrations (Fig. 1). A possible explanation for the inhibitory action of mimosine on PAL is that the allelochemical is a structural analog of L-tyrosine. It is possible that mimosine acts as L-tyrosine by inhibiting protein biosynthesis due to its incorporation into polypeptide chains of the new protein (14) or to

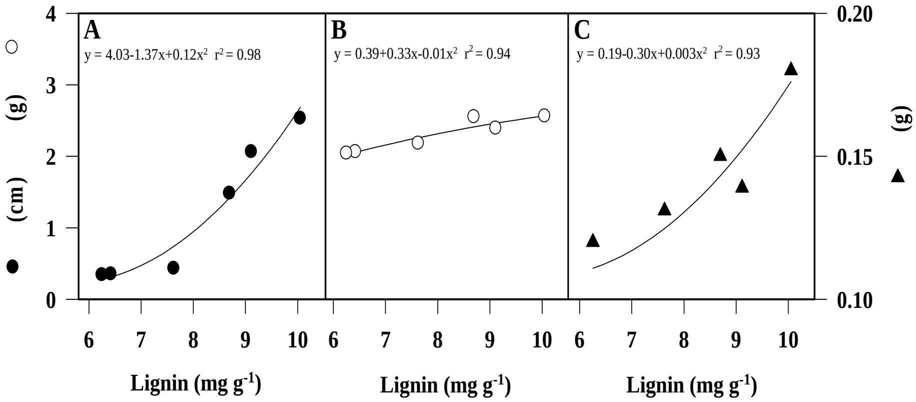

Figure 4. Relationships between root length (A), fresh weight (B) or dry weight (C) values and lignin content of roots. The regression lines were calculated by the equations described for each variable.

competition with L-phenylalanine, the highly specific substrate of PAL (10). Some evidence, in fact, supports this hypothesis. First, mimosine inhibits DNA replication and protein synthesis (5) and blocks mitosis (15). Second, exogenous L-tyrosine inhibits pure PAL activity (10). As previously cited, PAL regulates the production of phenolics, such as ferulic, *p*-coumaric, caffeic and sinapic acids. Subsequent enzymatic steps produce monolignols, which are later polymerized by POD action to complete the lignification process. One of the noteworthy features of POD is its association with cell elongation processes and growth-restricting reactions. POD may induce cell wall loosening and growth by elongation as well as cross-linking of cell wall components (13). As has been observed, mimosine decreased the free and cell wall-bound POD activities of soybean roots (Fig. 2A,B). The simplest explanation for the inhibitory mechanism of mimosine on POD may be the fact that this allelochemical is a strong iron chelator (22). Structurally, POD contains an iron (III) protoporphyrin IX (protohemin) prosthetic group located at the active site. This metal center is essential for the catalytic activity of the enzyme, involving exchanges of electrons and protons (21). The metal-chelating ability of the 3-hydroxy-4-oxo function of the pyridone ring in mimosine (23) may disturb the action of POD by reducing its activity. Evidence exists to support this hypothesis. At 0.25 and 0.5 mM, mimosine significantly inhibited POD activity and suppressed the synthesis of its isozymes in rice (14). Chelation of iron may be the possible mechanism of mimosine, since other iron-containing enzymes such as nitrogenase, nitrate reductase, and catalase, have been inhibited (1,14).

Lignification plays important roles in plant growth. Lignin biosynthesis occurs during secondary plant cell wall development, and its deposition hardens the stem walls. This process creates structural support, enabling increased growth in height (17). To evaluate the relationships between root growth and lignification, regression analyses were performed between the values of root length (Fig. 4A), fresh (Fig. 4B) and dry (Fig. 4C) weights and lignin content, after mimosine treatments. Results revealed significant positive non-linear correlations between the root length (r^2 = 0.98), fresh weight (r^2 =

0.94), dry weight (r^2 = 0.93) and lignin content. There is some evidence that the reduction of root growth is associated with premature lignification of seedlings treated with ferulic acid, a cinnamic acid derivative (18,19). However, after short-term (24 h) exposure to the amino acid mimosine this hypothesis cannot be inferred from the available data. Thus, based on the results of current research, it seems reasonable to suppose that in the presence of mimosine, decreases in PAL activity may reduce the production of phenolic acids. This is plausible since a decrease in PAL activity is suggested as a regulatory mechanism for the production of phenolic acids in the phenylpropanoid pathway (2). Since these compounds are not available for POD in the polymerization steps, a further reduction in the lignin synthesis of cell walls has been associated with restricted root growth. In short, the results obtained indicate that, at least for soybean, PAL and POD activities are sensitive to mimosine, which suggests that these enzymes might be responsible for the allelochemical action.

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