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Peroxidase and Lipid Peroxidation of Soybean Roots in Response to *p*-Coumaric and *p*-Hydroxybenzoic Acids

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ABSTRACT

The scope of the present study was to investigate how the p-coumaric (p-CA) and p-hydroxybenzoic (p-HD) acids affect the peroxidase (POD, EC 1.11.1.7) activity, the lipid peroxidation (LP) and the root growth of soybean (Glycine max (L.) Merr.). Three-day-old seedlings were cultivated in nutrient solution containing p-CA or p-HD (0.1 to 1 mM) for 48 h. After uptake, both compounds (at 0.5 and 1 mM) decreased root length (RL), fresh weight (FW) and dry weight (DW) while increased soluble POD activity, cell wall (CW)-bound POD activity (with 1 mM p-CA and 0.5 mM p-HD) and LP.

Key words: *Glycine max* (L.) Merr., roots, phenolic acid

INTRODUCTION

Higher plants regularly release organic compounds in the environment; their decay products are often added to the soil matrix and some of these have been reported as agents of plant-plant interactions, a characteristic of the well-known phenomenon called allelopathy. These compounds are released by a number of mechanisms, including rainwater drag, excretion or exudation from roots, and natural decay of above-ground or below-ground plant parts. All these possibilities involve the contact of the secondary chemicals with the rhizosphere or the bulk soil, where plants can absorb them (Rice, 1984).

Phenolic acids are ubiquitous in plants and they are important in plant-soil systems. They are a component of structures of the plant and flower pigments, act as protectants against invading organisms and also as allelochemicals. These derivatives interfere to some degree with many vital plant processes, including water use, transpiration, shoot and root growth, inhibition of nutrient uptake by roots, photosynthesis reduction, and leaf expansion (Einhellig, 1995). Despite these observations, little interest has been given for these effects on soybean (Baziramakenga *et a*l., 1995; McClure et al., 1987; Patterson, 1981). Due to this fact, the goal of the present work was to investigate how the allelochemicals p-CA and p-HD affect RL, FW, DW, POD activity and LP of soybean roots grown in nutrient solution.

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MATERIALS AND METHODS

General procedures. Soybean (Glycine max (L.) Merr., cv BR-16) seeds were soaked in a solution of 2 % (v/v) sodium hypochlorite for two minutes and washed thoroughly with deionized water. The seeds were grown between paper toweling placed in polystyrene containers (10 x 16 cm) with a small amount of deionized water at the bottom. The containers were incubated in a germination chamber in darkness at 25 °C (± 0.2), and 80 % relative humidity. Three-day-old seedlings of uniform size were transferred to containers (10 x 16 cm), filled with 200 ml of full-strength Hoagland's solution (Hoagland and Arnon, 1950) with or without *p*-CA or *p*-HD (0.1 to 1 mM). Nutrient solution was buffered with 17 mM potassium phosphate buffer and adjusted to pH 6.0 (Ferrarese et al., 2000). Each container held 25 uniform seedlings suspended in the solution by floating styrofoam boats. The containers were kept in a growth chamber at 25 °C under fluorescent light (280 μmol m^{-2} s^{-1}) on a 12-h photoperiod. The nutrient solution was aerated continuously by air bubbling. The roots were exposed to allelochemicals for 48 h with the nutrient solution completely renewed after the first 24 h. Roots were measured before and at the end of experiments. Roots FW were determined gravimetrically immediately after 48 h and DW was determined after oven drying at 80 °C for 24 h. Phenolic compounds used in this investigation were purchased from Sigma Chemical Co (USA). All other reagents used were of the purest grade available or chromatographic grade.

Depletion of *p*-CA and *p*-HD. For depletion experiments, *p*-CA or *p*-HD concentrations in nutrient culture were determined from the initial 200 ml solutions (Shann and Blum, 1987). The phenolic acid remaining after 6, 12, 15 and 24 h of treatment was determined by chromatography. Samples of nutrient solution were filtered through a 0.45 μm disposable syringe filter, and sample injection (20 μl) and analysis were accomplished by HPLC. A reversed-phase ODS column (150 x 4.6 mm, 5 μm) was used at room temperature in conjunction with the same type of guard-column (10 x 4.6 mm). The HPLC solvent used was methanol/4 % aqueous acetic acid (70/30, v/v) at a flow rate of 0.8 ml min^{-1} for an isocratic run of 20 min. *p*-CA or *p*-HD detections were performed from 254 nm with a UV-detector. The compounds were identified by comparing of retention times with those of the standards.

Enzymatic activities. After 48 h, seedlings were removed and the roots were detached for enzyme extraction. For POD, fresh roots (0.25 g) were extracted with 2.5 ml of 67 mM phosphate buffer (pH 7.0) as described by Shann and Blum (1987). The extract was centrifuged at 10,000 *x*g for 15 min at 4 °C and the supernatant was used to determine the activity of soluble POD. To isolate CW-bound POD, the pellets were washed with deionized water until no activity of soluble POD was detected in the supernatants. The pellets were washed twice with 1 ml of 1 M NaCl. The washes were pooled and used to assay CW-(ionically) bound POD. Guaiacol-dependent activities of the soluble and CW-bound POD were determined according Cakmak and Horst (1991) with some 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 initiated by the addition of diluted enzyme extract in phosphate buffer. Guaiacol oxidation was followed for 5 min at 470 nm, and the enzyme activity was calculated using the extinction coefficient of 25.5 mM^{-1} cm^{-1} for tetraguaiacol. POD activities were expressed as μmol min^{-1} g^{-1} FW.

Determination of LP. After 48 h, seedlings were removed and roots were detached for determination of LP (Baziramakenga et al., 1995). Fresh roots (0.25 g) were extracted with 2.5 ml of 67 mM phosphate buffer (pH 7.0). The extract was centrifuged at 10,000 *x*g for 15 min at 4 °C, and the supernatant was used to determine the LP. An aliquot of 0.5 ml of the supernatant was added to 4 ml of 0.5 % of thiobarbituric acid (prepared in 20 % of trichloroacetic acid). The mixture was placed in a water bath at 90 °C (10 min) and then quickly cooled on an ice-bath for 15 min. Samples were centrifuged at 3,500 *x*g (5 min). The absorbance of the supernatant was measured at 532 nm, and this value was subtracted from the nonspecific absorbance (at 600 nm) reading value. For the calculation of malondialdehyde quantity, the extinction coefficient value of 156 mM^{-1} cm^{-1} was used and LP expressed as μmol g^{-1} FW.

Statistical Analysis. The experimental design was completely randomized with four replications for the evaluation of all variables. The treatments were disposed in a factorial scheme 2x3+1. The first factor had two levels (*p*-CA and *p*-HD) and the second factor had three levels (concentrations of 0.1, 0.5 and 1 mM) plus control. Dunnett's test at 0.05 level of probability was used for the comparison of the factorial treatments compared with the control. The data analysis was carried out with the assistance of Statistical and Genetic Analysis System program (SAEG) of the Federal University of Viçosa, Brazil.

RESULTS AND DISCUSSION

Uptake of the both compounds by the roots was carried out by depletion technique (Shann and Blum, 1987). As can be seen in Figure 1, depletion of 1 mM *p*-CA or *p*-HD in the nutrient solution by the soybean roots was very rapid. Starting at 6 h, the depleted amounts reached their maximum with 15 h (for *p*-HD) and 24 h (for *p*-CA) when ceased.

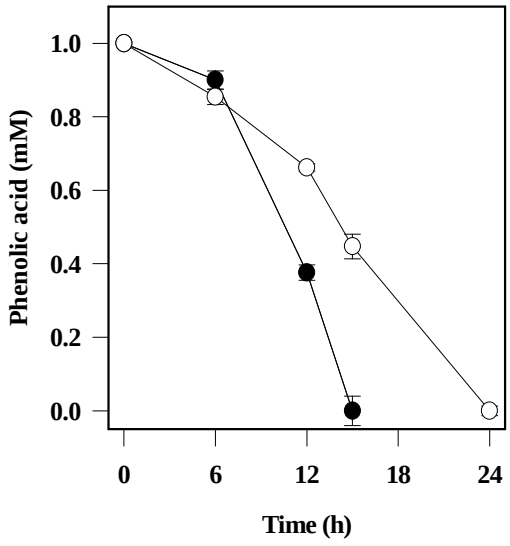

Figure 1 - Depletion of 1 mM *p*-coumaric (**O**) or *p*-hydroxybenzoic (●) acids by soybean roots.

In agreement with results of the present investigation, Shann and Blum (1987) reported that ferulic acid, another cinnamic acid derivative, and *p*-HD depletion by cucumber (*Cucumis sativus* L.) roots were also rapid. Besides, they did not detect any exudation of the compound by the root into the nutrient solution. Since in those experiments the uptake measured as depletion from solution was closely related to isotope net uptake, data obtained here (Fig. 1) shown uptake of these compounds by soybean roots. This has led researchers (Ferrarese et al., 2000, Lehman and Blum, 1999; Silva et al., 2000) to assume that the depletion technique may be used in uptake studies although some limitations as chemistry or microbial degradation of the compounds may occur. In this aspect, Shann and Blum (1987) demonstrated that there was minimal microbial utilization of ferulic acid or *p*-HD during nutrient culture experiments with cucumber roots.

The effects of *p*-CA and *p*-HD (0.1 to 1 mM) on RL, FW and DW were investigated 48 h after the root treatment (Table 1). It was clear that *p*-CA or *p*-HD, at the highest concentration, prompted a significant decrease in the RL (71 % and 42 %, respectively) compared with the control. On the other hand, at the lowest concentration, no significant ($p \geq 0.05$) alteration on RL occurred after *p*-CA or *p*-HD treatments. The two compounds also affected FW and DW. Comparison between the highest phenolic acid concentrations showed that the root FW diminished 28 % (with *p*-CA) and 20 % (with *p*-HD). Under these same conditions, both compounds reduced the root DW by 22 % and 15 %. Some researchers have reported alterations in roots treated with phenolic compounds in different plant species. Application of 1 mM ferulic acid caused considerable decrease in the growth of maize (*Zea mays* L.) seedlings, both of shoots and roots, as reported by Devi and Prasad (1996). Vaughan and Ord (1990) verified that root growth and FW in the pea (*Pisum sativum* L.) were inhibited by 1 mM *p*-CA or *p*-HD. At 1 mM *p*-CA, primary RL and FW of canola (*Brassica napus* L.) were drastically reduced (Baleroni et al., 2000). In soybean, Patterson (1981) has demonstrated that *p*-CA, at 1 mM, significantly reduced total DW. This same compound, at 0.4 mM, reduced RL and root DW (Einhellig and Eckrich, 1984). Schuab et al. (2001) demonstrated significant reduction of

the RL and root FW under the action of 1 mM *p*-CA. Data in the present work agreed with results obtained by the authors quoted above.

In order to verify the effects on POD, seedlings were cultivated with of *p*-CA or *p*-HD exogenous treatment (0.1 to 1 mM) for 48 h (Table 1). It was found that the soluble POD activity gradually increased against concentration since significant results over the control occurred at 0.5 mM (42 % with *p*-CA and 45 % with *p*-HD) and at 1 mM (70 % with *p*-CA and 53 % with *p*-HD). As revealed by the same table, *p*-CA was capable to increase the CW-bound POD activity by about 54 %, only at 1 mM. However, contrasting with the results obtained with *p*-CA, 1 mM *p*-HD did not alter CW-bound POD activity but increased the enzymatic activity by 55 %, at 0.5 mM.

Effects of the phenolic compounds on LP are summarized in Table 1, which showed that both *p*-CA and *p*-HD, at 0.5 mM and 1 mM, affected LP significantly ($p \leq 0.05$). The results were more expressive at 0.5 mM (81 % with *p*-CA and 62 % with *p*-HD) than at 1.0 mM (62 % with *p*-CA and 36 % with *p*-HD). With respect to POD, various lines of evidence indicated that the soluble form, associated with cytosol, has been involved in the catalysis of most of the peroxidative reactions in the cell while the bound form responsible for the oxidative polymerization of monolignols to produce lignin (Sánchez et al., 1996).

Table 1 - Effects of *p*-coumaric (*p*-CA) and *p*-hydroxybenzoic (*p*-HD) acids on root length (RL), fresh weight (FW), dry weight (DW), soluble and bound peroxidases (POD) activities and lipid peroxidation (LP).

mM	RL (cm)	FW (g)	DW (g)	Soluble POD $\mu mol\ min^{-1}\ g^{-1}$	Bound POD $\mu mol\ min^{-1}\ g^{-1}$	LP $\mu mol\ g^{-1}$
none	7.08	4.23	0.255	6.970	2.490	0.042
			p-coumaric acid			
0.1	6.10 (ns)	3.40 (-)	0.190 (-)	7.320 (ns)	2.050 (ns)	0.054 (ns)
0.5	5.66 (-)	2.70 (-)	0.213 (-)	9.920 (+)	2.060 (ns)	0.076 (+)
1.0	2.06 (-)	3.03 (-)	0.200 (-)	11.87 (+)	3.840 (+)	0.068 (+)
			p-hydroxybenzoic acid			
0.1	7.12 (ns)	3.51 (-)	0.238 (ns)	8.710 (ns)	2.690 (ns)	0.048 (ns)
0.5	3.95 (-)	3.15 (-)	0.190 (-)	10.08 (+)	3.860 (+)	0.068 (+)
1.0	4.08 (-)	3.38 (-)	0.217 (-)	10.65 (+)	3.190 (ns)	0.057 (+)

Means followed by (-) are different and inferior to controls at 0.05 level of probability by Dunnett's test. Means followed by (+) are different and superior to controls at 0.05 level of probability by Dunnett's test. ns, not significant.

In this view, some researchers have reported alterations in POD activity under action of allelochemicals. For example, in cucumber root treated with ferulic acid (0.5 or 1 mM), the soluble and bound forms of POD increased significantly while vanillic acid did not affect them (Politycka, 1996; Shann and Blum, 1987). Application of 1 mM ferulic acid caused a significant increase in both soluble and bound POD in maize roots and correlated with a pronounced decrease in root length (Devi and Prasad, 1996). Baziramakenga et al. (1995) exploited the effects of benzoic and cinnamic acids (but no their derivatives as such as *p*-CA or *p*-HD) on soluble POD of soybean roots grown hidroponically. The results showed dual behavior: increase in the activity with 0.05 mM cinnamic acid (no alteration with benzoic acid) and a similar decrease under action of the compounds, at 0.2 mM. Based on these results, the researchers attributed that the effects of phenolic compounds were due to the production of free radicals. At the same time, a key role of CW-bound POD in the stiffening of the cell wall through the formation of biphenyl bridges between wall polymers and, consequently, in the decrease of the cell wall extensibility has been proposed (Fry, 1986). In fact, the dimerization of ferulic acid in pine (*Pinus pinaster* Aiton) hypocotyl, due the oxidative capacity of CW-bound POD, was inversely related to the growth capacity (Sánchez et al., 1996). Thus, it seems feasible that these facts may explain the increases of POD activities observed here in relation to root growth capacity decreased (Table 1).

With respect to LP, Baziramakenga et al. (1995) also reported that benzoic and cinnamic acids

induced LP, which resulted from free radical formation in plasma membranes. To the authors, the phenolic acid-induced decrease in soybean nutrient absorption may be a consequence of damage to cell membrane integrity caused by lipid peroxidation. Similarly, treatment of cucumber roots with *p*-CA caused an increase of LP in association with the deterioration of membrane integrity (Politycka, 1996). Taking into account these reports, the results observed here clearly demonstrate that both allelochemicals affect root growth in association with pronounced increase in the POD activities and LP. It could be reasonable to suppose that after uptake, *p*-CA and *p*-HD acids cause stress followed by oxidative reactions with effective participation of POD. The increase of POD activities accompanied by the reduction of root growth strengthened the hypothesis of phenolic acid synthesis, incorporation of these compounds in lignin, increase of the cell wall rigidity, and growth reduction (Sánchez et al., 1996). At the level of cellular membrane, the phenolic acid oxidation leads to the production of quinones, which are toxic compounds responsible for the generation of reactive oxygen species (Appel, 1993). These free radicals are extremely dangerous to cells because they provoke enzyme inactivation, membrane LP and decrease of the nutrient absorption by the roots. In summary, these are some of the essential factors, which may be associated with the reduction of the root growth of soybean.

In addition, it is important to note that a possible discussion about the relationship between *p*-CA and *p*-HD structures and their effects cannot be inferred from the available results. Since there is no report on this aspect to date, at least for soybean, further experiments with different allelochemicals are necessary to give a clear-cut answer to this question.

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RESUMO

A proposta do presente trabalho foi investigar como os ácidos *p*-cumárico (*p*-CA) e *p*-hidroxibenzóico (*p*-HD) afetam a atividade da peroxidase (POD, EC 1.11.1.7), a peroxidação lipídica (LP) e o crescimento de raízes de soja (*Glycine max* (L.) Merr.). Plântulas de três dias foram cultivadas em solução nutritiva com *p*-CA ou *p*-HD (0,1 a 1 mM) por 48 horas. Após absorção, ambos os compostos (a 0,5 e 1 mM) reduziram o comprimento das raízes (RL), a biomassa fresca (FW) e a biomassa seca (DW) enquanto aumentaram a atividade da POD solúvel, a atividade da POD ligada à parede celular (com *p*-CA 1 mM e *p*-HD 0,5 mM), e a LP.

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