# Individual and interactive effects of phenolic acids on peroxidase and lipid peroxidation in soybean, *Glycine max* (L.) Merr. roots (Leguminosae-Faboidae)

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**ABSTRACT.** The purpose of the present work was to evaluate the individual and simultaneous effects of *p*-coumaric acid (*p*-CA) and *p*-hydroxybenzoic acid (*p*-HB) on soybean, *Glycine max* (L.) Merr. (Leguminosae-Faboidae) peroxidase (POD, EC 1.11.1.7), lipid peroxidation (LP) and root growth. Three-day-old seedlings were cultivated in nutrient solution containing *p*-CA or *p*-HB (0.5 and 1.0 mM) or equimolar mixtures for 24 hours. Individually, the two allelochemicals decreased root length (RL), root fresh weight (FW), root dry weight (DW), increased soluble POD and cell wall (CW)-bound POD but did not affect LP. The joint effects of these compounds were lower than the sum of the effects of each one tested separately. The results indicate that the mixtures of these allelochemicals can affect growth of soybean roots in an antagonistic manner.

**Key words:** allelochemicals, antagonism, lipid peroxidation, peroxidase, roots, soybean.

**RESUMO. Efeitos individuais e interativos de ácidos fenólicos sobre a peroxidase e peroxidação lipídica em raízes de soja, *Glycine max* (L.) Merr. (Leguminosae-Faboidae).** O propósito do presente trabalho foi avaliar os efeitos individuais e simultâneos dos ácidos *p*-cumárico (*p*-CA) e *p*-hidroxibenzóico (*p*-HB) sobre a peroxidase (POD, EC 1.11.1.7) de soja *Glycine max* (L.) Merr. (Leguminosae-Faboidae), peroxidação lipídica (LP) e crescimento das raízes. Plântulas de três dias de desenvolvimento foram cultivadas, durante 24 horas, em solução nutritiva contendo *p*-CA ou *p*-HB (0,5 e 1,0 mM) ou misturas equimolares. Individualmente, os dois aleloquímicos diminuíram o comprimento das raízes (RL), a biomassa fresca das raízes (FW), a biomassa seca das raízes (DW), aumentaram a POD solúvel e POD ligada à parede celular mas não afetaram a LP. Os efeitos simultâneos desses compostos foram menores que a soma dos efeitos de cada um, testado separadamente. Os resultados indicam que as misturas desses aleloquímicos podem afetar o crescimento das raízes de soja de maneira antagônica.

**Palavras-chave:** ácidos fenólicos, aleloquímicos, antagonismo, raízes, soja.

## Introduction

The cinnamic acid derivatives, such as *p*-coumaric acid (*p*-CA), and the benzoic acid derivatives, such as *p*-hydroxybenzoic acid (*p*-HB), are among the most common phenolic acids liberated by plants through leaching of leaf exudates and rhizodeposition, and affect the growth of various plant species (Macias, 1995). In order to be effective, concentrations of these allelochemicals must be enough in soil to reach inhibitory levels, and this depends on the type of soil, microbial degradation, thermal effects or drainage (Wink and Latz-Bruning, 1995).

Mixtures of non-inhibitory concentrations of individual phenolic acids have been reported that may inhibit plant growth in an additive (equal to the sum of the effects of each allelochemical tested separately), antagonistic (lower than the sum of the effects of each allelochemical) or synergistic (greater than the sum of the effects of each allelochemical) manner (Blum *et al*., 1985; Lehman *et al*., 1994). These possibilities have been considered for mixtures of some phenolic compounds on cucumber (*Cucumis sativus* L.) radicle growth and leaf expansion (Blum *et al*., 1984; Blum *et al*., 1985; Lehman *et al*., 1994).

No attention has been paid to the joint actions of phenolic acids on soybean (*Glycine max* (L.) Merr.) roots, which are susceptible to various phenolic acids tested separately (Patterson, 1981; Einhellig and Eckrich, 1984; McClure *et al*., 1987; Baziramakenga *et al*., 1995; Schuab *et al*., 2001). The present investigation was undertaken in order to evaluate the individual and interactive effects of *p*-CA and *p*-HB on peroxidase (POD), which protects the plants against biotic and abiotic stresses, and on lipid peroxidation (LP), which indicates the membrane integrity, and on root growth of soybean seedlings cultivated in nutrient solution.

## Material and methods

*Glycine max* (L.) Merr. (cv BRS-133) seeds, sterilized with 2% NaClO (three minutes) and rinsed extensively with deionized water, were dark-germinated (at 25°C) on two sheets of moist filter paper. 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 (Ferrarese *et al*., 2000), with or without *p*-CA or *p*-HB (0.5 or 1.0 mM). Nutrient solution was buffered with 17 mM potassium phosphate buffer, adjusted to pH 6.0 and monitored over time. 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-hours photoperiod. The nutrient solution was aerated continuously by air bubbling, and the roots were exposed to allelochemicals for 24 hours. All roots were measured before and at the end of experiments. Root FW was determined gravimetrically after 24 hours, and root DW was determined after oven drying at 80°C. In order to test possible interactive effects, equimolar mixtures containing *p*-CA and *p*-HB were prepared at final concentrations of 0.5 and 1.0 mM. Phenolic compounds used in this study were purchased from Sigma Chemical Co. (St. Louis, USA). All other reagents used were of the purest grade available.

After 24 hours, all the roots were detached from treated or untreated seedlings and used for enzyme extraction. For POD, fresh roots (0.5 g) were extracted with 5 mL of 67 mM phosphate buffer (pH 7.0) and 0.1g of polyvinylpyrrolidone as described by Shann and Blum (1987) with some modifications. The extract was centrifuged at 10,000x*g* for 15 minutes at 4°C and the supernatant was used to determine the activity of soluble POD. To isolate CW-bound POD, the pellet was washed with 5 mL of deionized water until no activity of soluble POD was detected in the supernatant. The pellet was extracted with 4ml of 1.0 M NaCl (one hour at 4°C). After centrifugation, the supernatant was obtained and considered as the CW-(ionically) bound POD. Guaiacol-dependent activities of the soluble and CW-bound POD were determined according to Cakmak and Horst (1991) with some modifications. The reaction mixture (3ml) 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 minutes, at 470 nm, and the enzyme activity was calculated using the extinction coefficient (25.5 $mM^{-1}$ $cm^{-1}$) for tetraguaiacol. The reaction mixture without enzyme extract was used as a blank, and this value was subtracted from those with enzyme extract. POD activities were expressed as μmol $min^{-1}$ $g^{-1}$ FW.

To determine LP, fresh roots (0.5 g) were extracted with 5ml of 67 mM phosphate buffer (pH 7.0) and 0.1g of polyvinylpyrrolidone (Baziramakenga *et al*., 1995). The extract was centrifuged at 10,000x*g* for 15 minutes, at 4°C, and the supernatant was used to determine the LP. An aliquot of 0.75 mL of the supernatant was added to 3ml of 0.5% of thiobarbituric acid (prepared in 20% of trichloroacetic acid). The mixture was placed in a water bath at 90°C (10 minutes) and then quickly cooled on an ice-bath for 15 minutes. Samples were centrifuged at 3,500xg (5 minutes). The absorbance of the supernatant was measured at 532 nm, and this value was subtracted from the nonspecific absorption (at 600 nm) reading value. For the calculation of malondialdehyde quantity, the extinction coefficient value of 155 $mM^{-1}$ $cm^{-1}$ was used and LP expressed as μmol $g^{-1}$ FW.

Statistical tests were performed using the InStat® package (version 1.12, GraphPAD Software, San Diego, USA). The statistical significance of the difference between parameters was evaluated by means of Student's *t*-test ($p \leq 0.05$). Data in the tables are expressed as means of four separate experiments ± SE.

## Results and discussion

The individual actions of *p*-CA and *p*-HB on RL, root FW and root DW were investigated 24 hours after root treatment (Table 1). As can be seen, *p*-CA and *p*-HB, at 0.5 or 1.0 mM, prompted a significant decrease in the RL in comparison to the control. Similarly, both compounds reduced root FW and root DW. The same Table also shows that soluble and CW-bound POD activities increased under

action of 0.5 or 1.0 mM *p*-CA and *p*-HB while no significant alteration occurred on LP, at any concentration. Comparisons between the compounds show that the effects of 0.5 mM *p*-HB on RL, root DW, soluble POD and CW-bound POD activities were more pronounced while the effects of 1.0 mM *p*-HB on RL and root DW were less pronounced than *p*-CA. On the other hand, no significant difference on POD occurred between compounds, at 1.0 mM, or on root FW and LP for any concentration.

**Table 1.** Changes in the root length (RL), root fresh weight (FW), root dry weight (DW), soluble and bound peroxidase (POD) and lipid peroxidation (LP) in roots of soybean seedlings treated with *p*-CA and *p*-HB

| mM | RL (cm) | FW (g) | DW (g) | Soluble POD µmol $min^{-1}$ $g^{-1}$ | Bound POD µmol $min^{-1}$ $g^{-1}$ | LP µmol $g^{-1}$ |
|---|---|---|---|---|---|---|
| none | 3.63 | 2.45 | 0.19 | 3.73 | 2.22 | 0.095 |
| | | | *p*-coumaric acid | | | |
| 0.5 | 2.46 (*) | 1.98 | 0.17 (*) | 6.82 (*) | 2.91(*) | 0.096 |
| | (-32.2) | (-19.2) | (-10.5) | (+82.8) | (+131.1) | (1.0) |
| 1.0 | 0.53 (*) | 1.83 | 0.15 (*) | 7.63 | 3.30 | 0.096 |
| | (-85.4) | (-25.3) | (-21.1) | (+204.6) | (+148.6) | (1.0) |
| | | | *p*-hydroxybenzoic acid | | | |
| 0.5 | 1,69 (*) | 1.97 | 0.16 (*) | 7.98 (*) | 3.75 (*) | 0.097 |
| | (-53.4) | (-19.6) | (-15.8) | (+213.9) | (+168.9) | (2.1) |
| 1.0 | 1,75 (*) | 1.99 | 0.16 (*) | 8.10 | 3.76 | 0.096 |
| | (-51.2) | (-18.8) | (-15.8) | (+217.2) | (+169.4) | (1.0) |

Means followed by (-, inferior or +, superior) are significantly different from control (Student *t*-test, $p \leq 0.05$, n=4). Means followed by (*) are significantly different between the two compounds at the same concentration. Data in parenthesis are % of inhibition or activation in comparison to control

Likewise some researchers have reported changes in root growth of different plant species treated individually with phenolic acids. For example, mung bean (*Phaseolus aureus* L.) hypocotyl length was decreased by 1.0 mM *p*-CA and *p*-HB (Demos *et al*., 1975). Application of 0.4 mM ferulic acid reduced RL and root DW in sorghum (*Sorghum bicolor* Moench.) seedling (Einhellig and Eckrich, 1984). This same allelochemical, at 1.0 mM, decreased the growth of maize (*Zea mays* L.) seedlings as reported by Devi and Prasad (1996). At 1.0 mM *p*-CA or *p*-HB, RL and root FW of pea (*Pisum sativum* L.) were drastically reduced (Vaughan and Ord, 1990). Lettuce (*Lactuca sativa* L.) root growth was severely reduced by 0.4 mM *p*-HB (Pramanik *et al*., 2000). Baleroni *et al*. (2000) reported that primary RL and root FW of canola (*Brassica napus* L.) were inhibited by 1.0 mM *p*-CA. In soybean, Patterson (1981) has demonstrated that *p*-CA, at 1.0 mM, significantly reduced root DW. In addition, Schuab *et al.* (2001) demonstrated significant reduction of the RL and root FW under the action of 1.0 mM *p*-CA.

The increase of soluble POD activity has demonstrated to be accompanied by a decrease of root growth. In cucumber (Politycka, 1996; Shann and Blum, 1987) and maize (Devi and Prasad, 1996) roots treated with 1.0 mM ferulic acid, soluble POD increased significantly and correlated with a pronounced decrease in root length. For these authors, one possible explanation is that the effects are attributed to the production of free radicals. Indeed, at cellular membrane level, the phenolic acid oxidation by peroxidases leads to the production of quinones, which are toxic compounds responsible for the generation of reactive oxygen species (Bolwell and Wojtaszek, 1997). These free radicals are extremely dangerous to cells because they cause enzyme inactivation, membrane lipid peroxidation and decrease in nutrient absorption by the roots. Since soluble POD catalyzes the oxidation of structurally diverse phenolic substrates it is often regarded as an antioxidant enzyme protecting cells from the destructive influence of derived oxygen species (Bolwell and Wojtaszek, 1997; Chen and Schopfer, 1999). However, it is interesting to note that LP was unaffected by the two allelochemicals, at least up to 24 h of treatment (Table 1). In line with this, Baziramakenga *et al*. (1995) reported that cinnamic and benzoic acids affected the LP after 48-hours treatment, alone. On the other hand, some reports indicate that the bound form POD is responsible for the oxidative polymerization of monolignols to produce lignin. In fact, after treatment of cucumber roots with ferulic acid, the soluble and bound forms of POD increased significantly while the treatment with vanillic acid, a benzoic acid derivative, did not alter these forms (Shann and Blum, 1987; Politycka, 1996).

Taking into account these reports, the results observed here demonstrate that the individual treatments with *p*-CA and *p*-HB increased both soluble and CW-bound POD in association with a pronounced decrease in root length. It is likely that *p*-CA and *p*-HB cause stress followed by oxidative reactions with effective participation of soluble POD. In parallel, the increase of CW-bound POD activity accompanied by the reduction of root growth strengthens the hypothesis of phenolic acid oxidation, incorporation of these compounds in lignin, increase of the cell wall rigidity, and consequently, growth reduction (Fry, 1986; Sánchez *et al*., 1996).

In order to scrutinize the interactive effects of these allelochemicals, seedlings were cultivated with equimolar mixtures of *p*-CA or *p*-HB for 24 hours (Table 2). It was found that acting jointly, the combination tested (0.5 or 1.0 mM) produced less negative effects on all variables than the sum of the effects of each allelochemical tested separately. Under such conditions, very few investigations

about the effects of phenolic acids mixture have been carried out. In cucumber, Blum *et al*. (1985) demonstrated that single and multiple treatments of ferulic acid, vanillic acid and *p*-CA reduced the leaf expansion and dry weight. The effects of the mixture of allelochemicals were additive (to 0.5 mM ferulic acid plus 0.5 mM *p*-CA mixture) and antagonistic (to 0.5 mM ferulic acid plus 0.5 mM vanillic acid mixture). Actions of ferulic acid plus vanillic acid, ferulic acid plus *p*-HB, *p*-CA plus *p*-HB mixtures on leaf area expansion were antagonistic while other combinations among these same compounds were additive (Blum *et al*., 1989; Gerig and Blum, 1991). Finally, Lehman *et al*. (1994) reported that the interactive effects of ferulic acid and *p*-CA on leaf expansion were additive. The inhibition of leaf expansion was directly related to the product of the concentrations of the acid(s) and the proportion of roots treated with the acid(s).

**Table 2.** Changes in the root length (RL), root fresh weight (FW), root dry weight (DW), soluble peroxidase (POD), (CW)-bound POD and lipid peroxidation (LP) in roots of soybean seedlings treated with *p*-CA and *p*-HB. Sum = data of *p*-CA plus *p*-HB tested separately. Mixture = data of equimolar mixtures

| Condition | RL (cm) | FW (g) | DW (g) | Soluble POD µmol $min^{-1}$ $g^{-1}$ | Bound POD µmol $min^{-1}$ $g^{-1}$ | LP µmol $g^{-1}$ |
|---|---|---|---|---|---|---|
| 0.5 mM | | | | | | |
| Sum | 4.15 | 3.95 | 0.33 | 14.80 | 6.66 | 0.193 |
| Mixture | 1.18 | 2.00 | 0.16 | 7.35 | 3.50 | 0.097 |
| | (71.5) | (49.4) | (51.5) | (50.3) | (47.4) | (49.7) |
| 1.0 mM | | | | | | |
| Sum | 2.28 | 3.82 | 0.31 | 15.73 | 7.06 | 0.192 |
| Mixture | 0.93 | 2.13 | 0.17 | 6.43 | 3.15 | 0.092 |
| | (59.2) | (44.2) | (45.2) | (59.1) | (55.4) | (52.0) |

Data in parenthesis are % of inhibition in comparison to the sum (Student *t*-test, $p \leq 0.05$, n=4)

In conclusion, the results presented in this work suggest that, at least for soybean, the interactive effects of the *p*-CA plus *p*-HB mixture are antagonistic. Although the mechanisms involved are still unknown, the enzymatic alterations may be important in explaining the reduction in the root growth. It is necessary to comment that the experiments here were conducted under controlled conditions. Of particular interest is the fact that the toxicity of allelochemicals, either individually or in mixtures, may vary with soil type, pH, mineral nutrition, and the susceptibility of the species (Blum, 1998). However, if the plants are continually exposed to compounds, the turnover rates of allelochemicals pools in soils will probably be substantial enough to cause antagonistic effects. Furthermore, lower root-allelochemical interactions of *p*-CA or *p*-HB in mixtures cannot be discarded. It is another possibility since the uptake of one allelochemical by the roots may be affected by the presence of other phenolic acids or alternative carbon sources (Blum, 1998). In consequence, analyses of uptake by the soybean roots of *p*-CA and *p*-HB, alone and in mixtures, could supply conclusive additional data. This is the challenge of a new study in progress.

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