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BRIEF COMMUNICATION

Peroxidase activity and lignification in soybean root growth-inhibition by juglone

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Abstract

The changes in activities of soluble and cell wall-bound peroxidases and lignin contents in juglone-stressed soybean (*Glycine max*) seedlings and their relationships with root growth were investigated. Soybean seedlings (3-d-old) were cultivated in nutrient solution supplemented with 0.5 to 25 μM juglone for 24 h. Length and dry mass of roots decreased after 5 to 25 μM juglone treatments. Low juglone concentrations (≤ 1 μM) increased soluble peroxidase activity, while high concentrations (≥ 10 μM) inhibited activities of soluble and cell wall-bound peroxidases. Juglone (≤ 1 μM) did not affect lignin content but highly increased lignification after 5 to 25 μM treatments. Results indicate that lignification may be an important step in root growth reduction of juglone-stressed soybean.

Additional key words: allelochemical, allelopathy, *Glycine max*, lignin, naphthoquinone.

Although juglone (5-hydroxy-1,4-naphthoquinone) produced by black walnut affected growth of various vegetables, field crops, ornamental plants, and several woody species (Cook 1921, Rietveld 1983, Chary and Rao 1998, Kobayashi and Ito 1998, Willis 2000), its mechanism has not been fully explained. In general, juglone concentrations in soil vary from 8 to 22 μM (Ponder and Tadros 1985, Jose and Gillespie 1998a). Its toxicity includes effects on growth, chlorophyll content, photosynthesis, transpiration, stomatal conductance, relative growth rate, and oxygen uptake in soybean, pea and *Lemna minor* (Köeppe 1972, Hejl *et al.* 1993, Jose and Gillespie 1998b). Furthermore, disruption of root oxygen uptake was positively correlated with concentrations of juglone, suggesting that this compound may reach mitochondria in root cells (Hejl and Koster 2004). Recently, Terzi *et al*. (2003/4) related the effects of juglone on anatomical parameters including xylem vessels of muskmelon. Reduction of seedling root has been attributed to the enhancement of cell wall-bound peroxidase activity, which leads to premature lignification of the cell walls (Ros-Barceló *et al.* 2002, Santos *et al*. 2004). To date, no reports on the effects of exogenous juglone on lignification are available. Therefore, the influence of juglone on peroxidase activity and lignin content and their relationships with root growth of soybean were analyzed in current research.

Soybean [*Glycine max* (L.) Merr. cv. BRS-133] seeds, surface-sterilized with 2 % sodium hypochlorite for 2 min and rinsed extensively with deionized water, were dark-germinated (at 25 °C) on two sheets of moistened filter paper. Twenty-five 3-d-old seedlings of uniform size were supported on an adjustable acrylic plate and transferred into a glass container (10 × 16 cm) filled with 200 cm^3 of half-strength Hoagland's solution (pH 6.0) without or with 0.5 to 25 μM juglone. The container was kept in a growth chamber (25 °C, 12-h photoperiod, irradiance of 280 μmol m^{-2} s^{-1}). Roots were exposed to juglone for 24 h. All roots were measured at the start and at the end of experiments. Dry mass of all roots was estimated after oven drying at 80 °C for 24 h. Juglone was purchased from *Sigma Chemical Co* (St Louis, USA) and all other reagents used were of the purest grade available.

Peroxidase was extracted from fresh roots (0.5 g) with 67 mM phosphate buffer (5 cm^3, pH 7.0). The extract was centrifuged (10 000 *g*, 15 min, 4 °C) and the supernatant determined the activity of soluble peroxidase.

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Abbreviations: d.m. - dry mass; ROS - reactive oxygen species.
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To isolate the cell wall-bound peroxidase, the pellet was washed with deionized water until no soluble peroxidase activity was detected in the supernatant. Pellet was then incubated in 1 M NaCl (4 cm^3, 4 °C, 60 min) and the homogenate was centrifuged (10 000 *g*, 15 min). The supernatant contained the cell wall-(ionically)-bound peroxidase. Guaiacol-dependent activities of the soluble and cell wall-bound peroxidase were determined according to Santos *et al.* (2004). The reaction mixture contained 25 mM sodium phosphate buffer (3 cm^3, pH 6.8), 2.58 mM guaiacol and 10 mM H_2O_2. The reaction started by adding the enzyme extract in phosphate buffer. Guaiacol oxidation was followed for 5 min at 470 nm (*SP75 spectrophotometer, Sanyo Gallemback*, Leicestershire, UK), and enzyme activity was calculated from the coefficient of absorbance (25.5 mM^{-1} cm^{-1}) for tetraguaiacol. Blank consisted of a reaction mixture without enzyme extract whose absorbance was subtracted from the mixture with enzyme extract.

For lignin determination, dry roots (0.3 g) were homogenized in 50 mM potassium phosphate buffer (7 cm^3, pH 7.0) with mortar and pestle, and transferred to a centrifuge tube (Ferrarese *et al.* 2002). The pellet was centrifuged (1 400 *g*, 4 min) and washed by successive stirring and centrifugation, as follows: twice with phosphate buffer pH 7.0 (7 cm^3); three times with 1 % (v/v) *Triton X-100* in pH 7.0 buffer (7 cm^3); twice with 1 M NaCl in pH 7.0 buffer (7 cm^3); twice with distilled water (7 cm^3) and twice with acetone (5 cm^3). Pellet was left overnight in a desiccator for drying. Pellet was finally dried in an oven (24 h, 60 °C), cooled down in a vacuum desiccator, and the dry matter obtained was defined as the protein-free cell wall fraction. Further, dry protein-free tissue (0.1 g) was placed into a screw-cap centrifuge tube containing the reaction mixture (1.2 cm^3 of thioglycolic acid plus 6 cm^3 of 2 M HCl) and heated (95 ºC, 4 h). After cooling at room temperature, the sample was centrifuged (1 400 *g*, 5 min) and the supernatant decanted. The pellet was washed three times with distilled water (7 cm^3), and the product extracted by shaking at 30 ºC for 18 h and 115 oscillations min^{-1} (*Marconi MA095*, São Paulo, Brazil) in 0.5 M NaOH (6 cm^3). After centrifugation (1 400 *g*, 5 min), the supernatant was stored and mixed with supernatant obtained from a second pellet washed with 0.5 M NaOH (3 cm^3). The combined alkali extracts were acidified with concentrated HCl (1.8 cm^3). The lignothioglycolic acid formed after 4 h at 0 ºC was recovered by centrifugation (1 400 *g*, 5 min) and washed twice in distilled water (7 cm^3). 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.

Data from 6 separate experiments were combined. Statistical tests were performed with *InStat*® package (Version 1.12a, *GraphPAD Software*, San Diego, USA). Statistical significance of the difference between parameters was evaluated by Student's *t*-test ($P < 0.05$).

In soybean seedlings grown in nutrient solution supplemented with juglone root lengths and dry mass decreased with increasing concentrations (Fig. 1*A*,*B*). Mean total root lengths were 59, 98, and 100 % less than controls for 5, 10, and 25 μM treatments, respectively. Low concentrations (0.5 and 1 μM) did not impair root growth. A similar behavior was also evident in dry masses that were 8, 25, and 38 % less than those of controls with 5 to 25 μM juglone (Fig. 1*B*). No appreciable change in dry mass of roots exposed to juglone at low concentrations (≤ 1 μM) was recorded. Studies using concentrations of juglone ranging from 1 μM to 1 mM applied in hydroponic culture have shown growth inhibition of different plant species. Rietveld (1983) verified that a concentration of 1 μM juglone did not affect the root growth of black alder (*Alnus glutinosa*), albeit 10 μM juglone did cause inhibition. Neave and Dawson (1989) confirmed Rietveld's findings

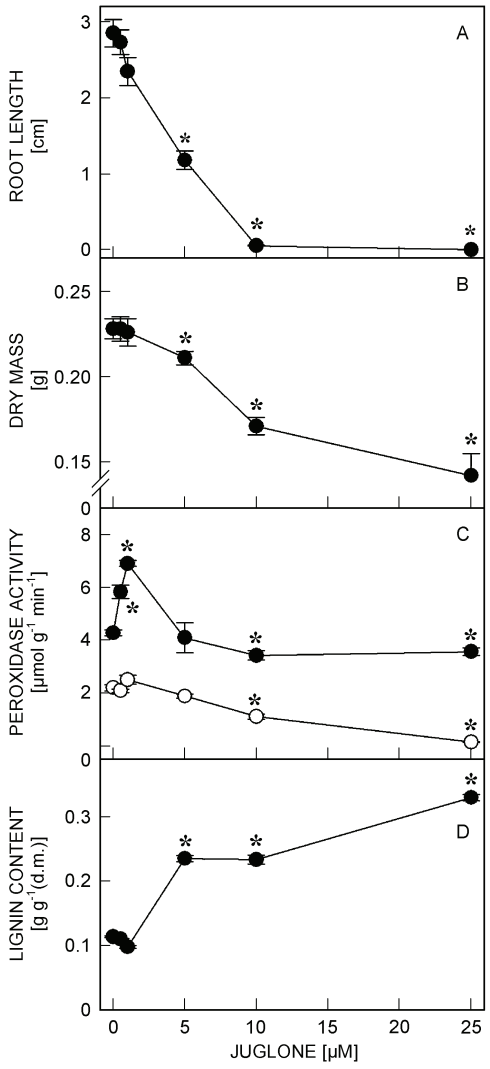

Fig. 1. Growth parameters, peroxidase activity and lignin content in soybean seedlings untreated or treated with juglone after 24 h: *A* - root length; *B* - root dry mass; *C* - soluble (*closed circles*) and cell wall-bound (*open circles*) peroxidase activities; *D* - lignin content. * - Mean values ± SE ($n = 6$) differed significantly from those of control ($P < 0.05$).

when they used concentrations of 2 and 20 μM. Application of 10 and 100 μM juglone resulted in a significant decrease in shoot and root relative growth rates in maize and soybean (Jose and Gillespie 1998b, Hejl and Koster 2004). At 1 mM, juglone inhibited lettuce radicle elongation (Kobayashi and Ito 1998). Results reported here showed juglone effects starting from 5 μM.

Soluble peroxidase activity increased after juglone treatment (Fig. 1*C*), with significant elevation over controls occurring with 0.5 μM (37 %) and 1 μM (62 %). In contrast, high concentrations (10 and 25 μM) inhibited soluble peroxidase activity by about 20 and 17 %, respectively. Similarly, juglone decreased the cell wall-bound peroxidase activities by about 50 and 94 % for 10 and 25 μM treatments, respectively. The inhibitions were associated to drastic decreases in root length and dry mass (Fig. 1*A*,*B*) and elevated increases in lignin content (Fig. 1*D*). Soluble peroxidase is one of the antioxidant enzymes that protect cells from the destructive influence of reactive oxygen species (ROS). Since soluble peroxidase activity has been enhanced and root growth does not decrease, it is possible that, at low concentrations (≤ 1 μM), this scavenging system is not impaired by juglone. However, if the capacity of the cells to scavenge ROS is exceeded, the consequence may be a state of oxidative stress leading to the depolarization of the root cell membrane enhancing its permeability with electrolyte leakage, blocking the plant nutrient uptake and hindering the root growth. It seems that these facts may, at least partially, explain results obtained with high juglone concentrations (Fig. 1*C*). Similar to the soluble form, cell wall-bound peroxidase activity decreased by high juglone concentrations. As mentioned earlier, there are even evidences that the cell wall-bound form is involved in lignification but this hypothesis cannot be inferred from the available data.

Results showed that juglone (at 1 μM or below) did not affect lignin content (Fig. 1*D*). However, roots increased (over 200 %) lignin content after 5 to 25 μM juglone treatments. Cell walls are know to become lignified when cell expansion ceases, when the cell is under biotic stress, and when it differentiates to particular specialization, notably the xylem (Christensen *et al*. 1998). In fact, Terzi *et al*. (2003/4) reported that xylem vessel radius of stem decreases with decreasing growth of muskmelon after juglone treatment. In these authors' opinion, the narrowing of the xylem vessels may be a defense mechanism of the seedlings to limit juglone translocation, hindering the access of water and nutrients from roots to leaves. In addition, others have shown that water uptake and acid efflux decreased in soybean seedlings treated with juglone (Hejl and Koster 2004).

From the foregoing discussion it seems quite plausible to assume that juglone-induced inhibition in root growth of the soybean seedlings may be due to cell wall stiffening process related to lignin production. If the cell wall is a target site of juglone, findings corroborate as to the way this compound affects the growth of plant species.

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