

PHYTOTOXICITY OF *Phytolacca americana* LEAF EXTRACTS ON THE GROWTH, AND PHYSIOLOGICAL RESPONSE OF *Cassia mimosoides*

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Abstract—We examined the allelochemical effects of control soil, native soil (treated soil), and leaf extracts of *Phytolacca americana* (pokeweed) on the germination rate and seedling growth of *Cassia mimosoides* var. *nomame*. We also studied the resulting changes in root-tip ultrastructure and peroxidase isozyme biochemistry. *P. americana* leaf extract inhibited seed germination, seedling growth, and biomass when compared to control and treated soil. Root and shoot growth in treated soil was stimulated relative to control soil, but root growth was inhibited by 50% in the leaf extract treatment. Biomass of *C. mimosoides* seedlings grown on leaf extract was reduced sevenfold when compared to the control seedlings. The amounts of total phenolic compounds in the leaf extract, treated soil, and control soil were 0.77, 0.14, and 0.03 mg l⁻¹, respectively. The root tips of *C. mimosoides* treated with leaf extracts of *P. americana* showed amyloplasts and large central vacuoles with electron-dense deposits inside them when compared to control root tips. The activity of guaiacol peroxidase (GuPOX) in whole plant, roots, and shoots of *C. mimosoides* increased as leaf extract increased; maximum activity was observed in extract concentrations of 75% and higher. Root GuPOX activity was three times higher than in shoots. Therefore, we conclude that inhibition of *C. mimosoides* growth is related to the phenolic compounds in the *P. americana* leaf extract and the ultrastructure changes in root-tip cells and increased GuPOX activity is a response to these allelochemicals.

Key Words—Allelochemicals, enzyme activity, phenolic compounds, *Phytolacca americana*, seed germination, seedling growth.

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INTRODUCTION

Allelopathy is an important phenomenon operating in natural and managed ecosystems. Many secondary metabolites, including phenolic, terpenoid, flavonoid, and alkaloid compounds, act as plant allelochemicals. Among these, phenolic compounds are the most abundant under field conditions (Rice, 1984; Williamson, 1990) and are known to affect seed germination, seedling growth, cell division, fungal activity, protein synthesis, and enzyme activity (Thijs et al., 1994; Inderjit, 1996; Seneviratne and Jayasinghearachchi, 2003). The phenolic compound, hydroxamic acid, affected membrane permeability resulting in reduced plant growth; and the interference of hydroxamic acid, 2-benzoxazolinone (BOA), and other hydroxamic acids with auxins inhibited the cell-cycle progression of *L. sativa* (Macias et al., 2003). Rye allelochemicals, BOA and 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA), caused increased cytoplasmic vacuolation, reduced ribosome density and dictyosomes, reduced number of mitochondria, and reduced lipid catabolism (Burgos et al., 2004). Lovett (1982) found that root-tip cells affected by allelochemicals exhibited increased vacuolation, and the alkaloid of thornapple (*Datura stramonium*) caused an increase in amyloplasts as well as the size and number of microbodies in *Helianthus annuus* root tips (Lovett, 1982; Levitt et al., 1984).

The phytotoxic effects of water-soluble phenolic compounds in *Phytolacca americana* have not been studied in the context of morphological and physiological responses associated with seed germination and subsequent seedling growth. In 1979, *P. americana* was introduced into South Korea and became an important ecological problem in 1993 when it spread throughout the country, displacing many native species. It is a perennial weed that occurs worldwide and has been spreading especially in polluted areas of South Korea (Park, 1995). Under field conditions it interferes with the growth and establishment of competing plant species by releasing water-soluble compounds into the soil (Lee et al., 1997). Furthermore, at low concentrations, extracts of *P. americana* inhibited seed germination, seedling growth, and exhibited antimicrobial activity (Kim et al., 2000). Three *Phytolacca* species in South Korea exhibited allelochemical effects that were related to phenolic compounds (Kim et al., 2005).

Recently, the presence of soluble phenolic compounds was used to screen for disease-resistant cultivars of sugarcane and date palm (Daayf et al., 2003; Nutt et al., 2004). And although there is a great need to determine the effect of allelochemicals on enzyme activity, few studies have related physiological effects to peroxidase activity and gene expression in plant differentiation. Consequently, we studied isozymes in *P. americana*.

Impaired enzyme activity, identified by Rice (1984) as a primary target for phytotoxic activity, may explain the inability to metabolize starch. According to

Alscher and Hess (1995), plants produce enzymes such as peroxidase, catalase, and superoxide dismutase (SOD) for self-protection against external stress. In particular, peroxidase exists in the form of isotype enzymes, which have a different molecular structure while activating the same substrate. During organogenesis, the type of isoperoxidase is more important than the total amount of peroxidase (Scandalios, 1990), and is involved in plant growth as well as in many plant defense mechanisms (Lagimini, 1991). Phenolic compounds act as antioxidants by donating electrons to guaiacol peroxidase (GuPOX) for the detoxification of hydrogen peroxide (H_2O_2) produced under stress conditions (Asada, 1992; Sakihama and Yamasaki, 2002). Phenolic compounds also function cooperatively with ascorbate as antioxidants, particularly in vacuoles and apoplasts where they coaccumulate with GuPOX (Yamasaki et al., 1997; Ren et al., 1999; Xiujuan et al., 2002). Phenolic compounds inhibiting plant growth were reported to increase the activities of proteinase, peroxidase, and catalase (Loebenstein and Linsey, 1961; Gaspar et al., 1985), whereas caffeic acid and salicylic acid reduced phosphorylase and GuPOX activity, respectively (Rice, 1984; McCue et al., 2000).

The objectives of the present study were to: (1) determine the effect of *P. americana* leaf extracts, control soil, and soil from natural *P. americana* patches on seed germination and seedling biomass accretion; (2) examine subsequent growth and ultrastructure changes in *Cassia mimosoides* root tips treated with *P. americana* leaf extract; and (3) determine total peroxidase and GuPOX activity in organs of *C. mimosoides* after treatment with leaf extract from *P. americana*.

METHODS AND MATERIALS

Plant and Soil Collection. Seeds from *C. mimosoides* were collected from Chunan, Chungcheong Province, South Korea, and were used to study seed germination, seedling growth, root-tip ultrastructure changes, and guaiacol peroxidase (EC 1.11.1.7) activity. *C. mimosoides* seeds have a high germination rate, are widely distributed throughout South Korea, and are sensitive to water-soluble leaf extracts. These extracts were obtained from leaves of *P. americana* growing on Mt. Pardal located in downtown Suwon, Gyeonggi Province, South Korea. After removing the organic layer, the upper 5 cm of soil was collected under a patch of *P. americana* (treated soil). Control soil was obtained 2 m away in an area where *P. americana* was not present.

Leaf Extract Study. Two hundred g of fresh *P. americana* leaf were extracted with 1 l of distilled water at room temperature for 48 hr and then centrifuged at 15,000 rpm for 30 min (Centrikon T-1045, Kontron Co., Zurich, Switzerland). The supernatant was collected and stored at 4°C until used. The

leaf extract was filtered through Whatman No. 1 filter paper and used undiluted (100%), and diluted to 75%, 50%, 25%, and 10% concentrations, whereas the control consisted of water for bioassay and the GuPOX activity study. After being selected for uniform size, *C. mimosoides* seeds were sterilized for 3 min in a solution of 5% sodium hypochlorite and then rinsed three to five times with distilled water. Thirty seeds were sown in a Petri dish (90 mm diam.) and treated with a 25% leaf-extract solution. Seeds were incubated at 28°C under 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ fluorescent lighting with 16/8 hr L/D periods for 6 d. Seed germination was assessed daily, while root tips were observed after 3 d. Seedling growth, biomass, and GuPOX activity were measured after 6 d. Enzyme samples were stored in the freezer (-50°C). The experiment was replicated three times. The bioassay method used was a modified version of the procedure described by Lodhi (1976).

Transmission Electron Microscopy of Root Tips. Root tips of *C. mimosoides* were cut to 0.1–0.2 mm in length and dehydrated in a Spurr mixed solution (Spurr, 1969) and stained (Reynolds, 1963). Samples were analyzed by transmission electron microscopy (TEM) at 80 kV.

Guaiacol Peroxidase Assay. The enzyme samples were extracted to measure GuPOX activity. One ml of extraction buffer containing 25 mM KPO_4 , 1 mM ascorbate, and 1 mM EDTA was added to 0.3 g of liquid N-frozen root, shoot, and whole plant of *C. mimosoides*, and ground with a mortar and pestle. After being left to stand at 4°C for 20 min, the samples were centrifuged at 13,000 g for 20 min (Centrikon T-126, Kontron). The amount of soluble protein was measured with a protein assay kit (Bio-Rad, Hercules, CA, USA) using the method described by Bradford (1976). GuPOX activity was measured using the method described by Amako et al. (1994) and recalculated to units per microgram protein using enzyme kinetics software.

Statistical Analysis. Data were normally distributed, and significant differences between treatments and controls were calculated using one-way ANOVA and Duncan's mean separation test for the measured parameters. The data shown in figures are the mean \pm SE.

RESULTS

Leaves of *P. americana* showed high concentrations of total phenolic compounds (0.77 mg l^{-1}), whereas the soil under the plants had concentrations about four times higher than the control soil (0.14 vs. 0.03 mg l^{-1}). Seed germination of *C. mimosoides* was significantly inhibited when treated with *P. americana* leaf extract and in the soil collected from under *P. americana* plants. The germination rate was reduced more in the leaf extracts than in the treated soil (Figure 1, $P < 0.001$). Germination was reduced by 40% in the leaf

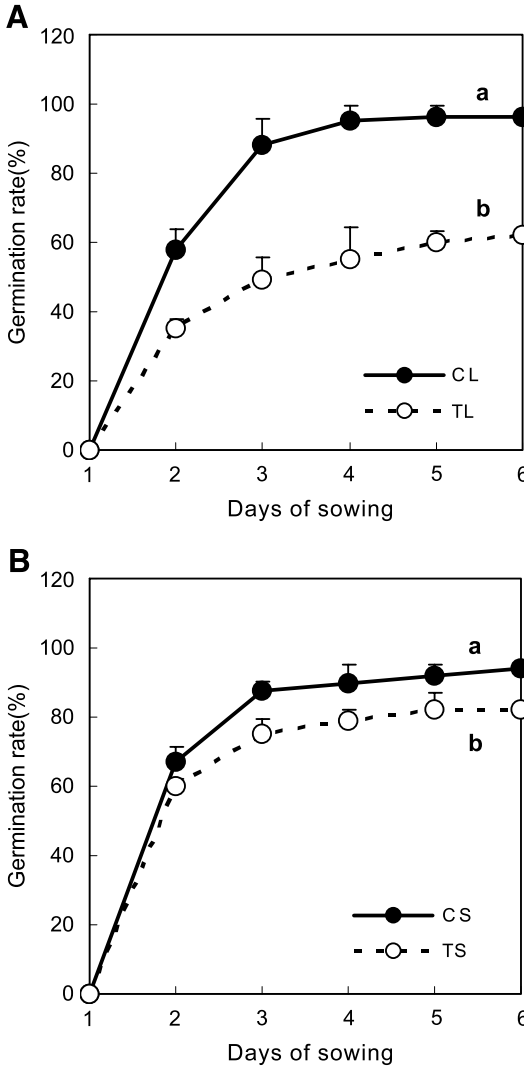


FIG. 1. Comparison of germination rate of *Cassia mimosoides* treated with leaf extract (A) and soil supporting a patch of *Phytolacca americana* (B). CL: control leaf, TL: treated leaf, CS: control soil, TS: treated soil. Means with the same letter are not significantly different (Duncan's multiple range test, $P < 0.001$), \pm SE of $N = 3$ measurements each are given.

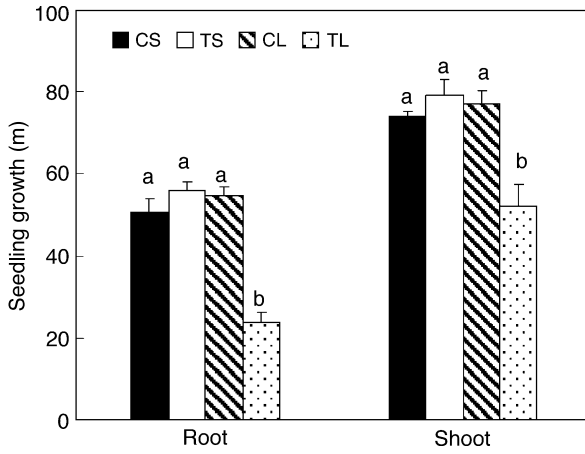


FIG. 2. Comparison of seedling growth of *C. mimosoides* grown with treated leaf extract and soil of *P. americana*. CS: control soil, TS: treated soil, CL: control leaf, TL: treated leaf. Means with the same letter are not significantly different (Duncan’s multiple range test, $P < 0.001$), \pm SE of $N = 3$ measurements each are given.

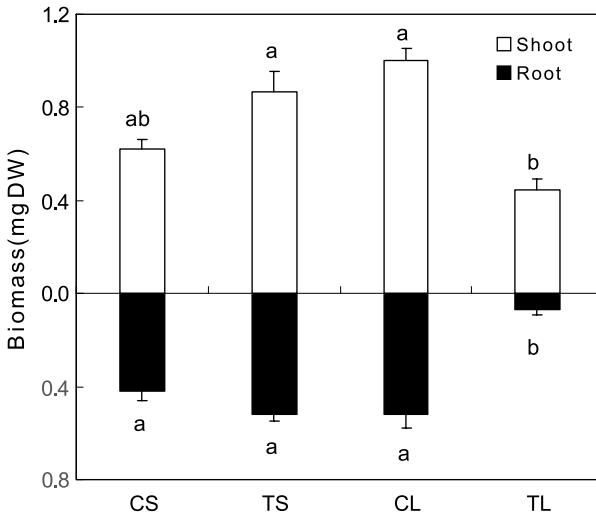


FIG. 3. Comparison of dry weight of *C. mimosoides* seedlings grown with treated leaf extract and soil of *P. americana*. CS: control soil, TS: treated soil, CL: control leaf, TL: treated leaf. Means with the same letter are not significantly different (Duncan’s multiple range test, $P < 0.001$), \pm SE of $N = 3$ measurements each are given.

extract when compared to the control (Figure 1A), whereas germination was reduced by only 10% in the treated soil (Figure 1B). In seedlings treated with leaf extract, shoot and root growth were significantly reduced compared to that of control (Figure 2, $P < 0.001$). Root growth was especially sensitive to the leaf extract, exhibiting more than a 50% growth reduction. Shoot growth was reduced

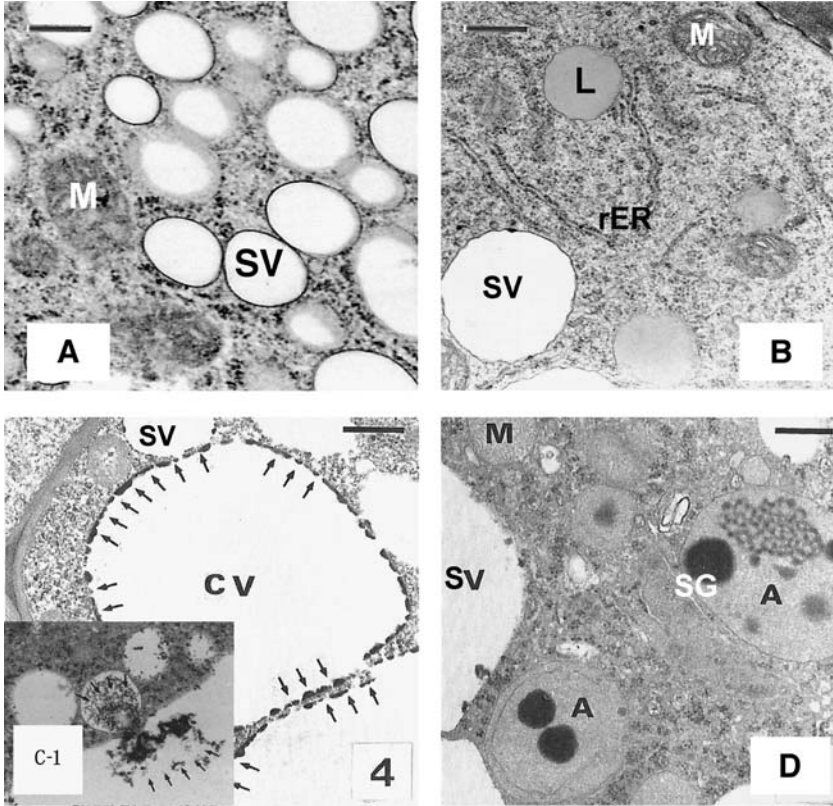


FIG. 4. TEM micrographs of *C. mimosoides* root tips: Control (A and B) and treated (C and D) with leaf extracts of *P. americana*. (A) A large number of small vacuoles and mitochondria are in the parenchyma cell. Bar = 2.4 μ m. (B) Lipid bodies, mitochondria, small vacuoles and rER are in the cytoplasm. Bar = 2.4 μ m. (C) Electron-dense deposits (arrows) are accumulated in the central vacuole (CV) along the tonoplast of a parenchyma cell. Bar = 1.5 μ m, C-1: these arrows point to small vacuoles that are reduced in number, while this amorphous material was discharged into the central vacuole. Bar = 1.5 μ m. (D) An amyloplast (A) near the central vacuole (SV) bounded by a double membrane containing two starch grains. Bar = 2.4 μ m.

by about 33% in seeds treated with leaf extract (Figure 2). Biomass and seedling growth of *C. mimosoides* plants in treated soil was slightly higher than in the control soil, but the biomass differences were not significant (Figures 2 and 3).

The threshold leaf extract concentration was estimated to be 25% because the greatest changes in the bioassay were observed between 10% and 50% (data not shown). In the root tips from control plants, mitochondria and a large number of small vacuoles were observed in parenchyma cells (Figure 4A). Lipid bodies, mitochondria, and rough endoplasmic reticula (rER) were present in the cytoplasm (Figure 4B). In the root tips from seedlings treated with 25% leaf extract, the size of the central vacuole increased, and electron-dense deposits were observed to accumulate along the tonoplast in parenchyma cells of the stele (Figure 4C). Many small vacuoles joined the central vacuole, whereas amorphous materials were discharged from small vacuoles into the central vacuole (Figure 4C-1). Some cellular organelles were reduced in number, and amyloplasts, including starch grains, appeared in parenchyma cells (Figure 4D).

GuPOX activity dramatically increased in the shoots, roots, and the whole plant with increasing extract concentrations. Root GuPOX activity was three

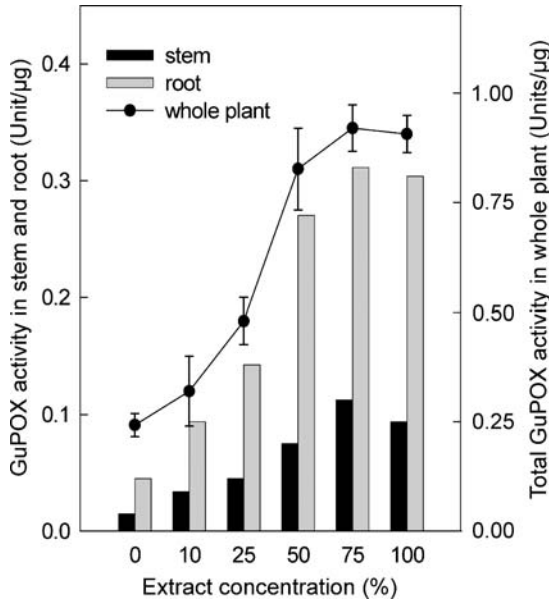


FIG. 5. Comparison of GuPOX activity in root, shoot, and whole plant of *C. mimosoides* treated with *P. americana* leaf extract. Bars represent SE of the mean. Organs treated with extract are indicated by symbols in the legend.

times higher than in the shoots. The highest GuPOX activity was observed in the sample treated with 75% leaf extract, whereas activity in the 100% extract sample was slightly lower. GuPOX activity in the roots exposed to 75% leaf extract increased more than sevenfold over the control (Figure 5).

DISCUSSION

Phenolic compounds found in leaf extracts and treated soil have been reported to reduce seed germination, seedling growth, and biomass (Williams and Hoagland, 1982; Rice, 1984; Einhellig, 1995; Inderjit, 1996) as observed in the present study (Figures 1, 2, and 3). Water-soluble compounds including phenolic compounds released by *Pluchea lanceolata* into the soil were found to significantly decrease seed germination and seedling growth of asparagus bean (*Vigna unguiculata*) (Inderjit and Dakshini, 1992). In the present study, seed germination in treated soil was inhibited (Figure 1B), whereas seedling growth and biomass were only slightly stimulated (Figures 2 and 3). Lodhi (1976) also found increased seedling growth in treated soil containing phenolic compounds. Under field conditions, Rice (1984) suggested that high concentrations of allelochemicals might not be maintained as a result of leaching and microbial degradation. In our study, the observed increase in seedling biomass in response to the presence of soil phenolic compounds has been reported previously (Lodhi, 1976; Rice, 1984). Concentrations of phenolics were found to be higher in leaf extracts (0.77 mg l^{-1}) than in treated soil (0.14 mg l^{-1}). Upon entry into the soil, chemical compounds may be degraded, transformed, and retained depending on the number and types of soil microbes present (Blum, 1995). Thus, bioassays using allelopathic compounds in treated soils may better reflect plant responses such as seedling growth and biomass accumulation, as observed in the present study.

TEM, combined with biochemical assays, suggested possible mechanisms of plant responses to phenolic compounds from *P. americana* leaf extract (Figure 4). In control root tips, a large number of small vacuoles, mitochondria, rER, and lipid bodies were observed in parenchyma cells (Figure 4A and B). In root tips treated with leaf extract, the arrows point to small vacuoles that were reduced in number, whereas an amorphous material was discharged from the small vacuoles into the central vacuole (Figure 4C-1). It also showed the accumulation of electron-dense deposits along the inside of the tonoplast (Figure 4C). Elakovich and Wooten (1991) reported that plant cells were not affected by osmotic potential below 143 mOsm kg^{-1} . In this research, the osmotic potential of diluted extract 75%, 50%, 25%, and 10% is 72, 46, 18, and 8 mOsm kg^{-1} , respectively. We treated *C. mimosoides* with a 25% concen-

tration of *P. americana* leaf extract. Therefore, the result in Figure 4 suggested that the observed changes in the root tip ultrastructure was not a result of high osmotic potential as reported by Lee and Kim (2000), but may be attributable to phenolic compounds in the leaf extract of *P. americana* that caused the development of starch grains in amyloplasts and electron-dense deposits in the central vacuole. Lovett (1982) and Rizvi and Rizvi (1992) reported a similar response in vacuole size of root tips affected by allelochemicals. Kutchan et al. (1986) also suggested that vacuoles are important in segregating phytotoxic secondary metabolites and play a compartmentalizing role in detoxification (Lovett, 1982; Burgos et al., 2004). The accumulation of electron-dense deposits along the tonoplast has not been previously observed (Figure 4A and B), but is consistent with a detoxification role. The amyloplasts (Figure 4D) observed in the present study may function to accumulate toxic substances for self-protection against *P. americana* leaf extract. The amyloplasts were also found in sunflower (*H. annuus*) root-tip cells following treatment with alkaloids from thornapple (*D. stramonium*) (Levitt et al., 1984; Lovett et al., 1987).

Enzymes play a major role in protecting the plant from oxygen free radicals (Asada, 1992; Sakihama and Yamasaki, 2002; Xiujuan et al., 2002). The isoenzyme form of peroxidase was reported to be the most important one in the differentiation process of plants and many plant defense mechanisms (Scandalios, 1990). Enzyme activity associated with root differentiation was found to increase with stress from toxins, drought, and freezing damage (Alscher and Hess, 1995). Among peroxidase isozymes, GuPOX and AsPOX are representative enzymes that are induced in plants that are mechanically damaged, infected by pathogens, or externally stressed. Lee and Kim (2000) reported that AsPOX was not responsible for the increase in peroxidase activity on *C. mimosoides* treated with *P. americana* leaf extract. Therefore, we investigated GuPOX activity and isozyme forms of peroxidase to understand the physiological response to phenolic allelochemicals. GuPOX activity increased as *P. americana* leaf extract concentration increased. Root activity was three times higher than shoot activity in *C. mimosoides* treated with different concentrations of *P. americana* extract. In the present study, there was a positive relation between GuPOX activity and *P. americana* leaf extract concentration (Figure 5). In response to elevated GuPOX activity, root differentiation was inhibited (Figures 2 and 3). Yoo and Kim (1988) reported that root activity was highly sensitive to external factors. GuPOX activity is known to be sensitive to external stress and plays a major role in protection mechanisms (Gaspar et al., 1985; Ren et al., 1999; Xiujuan et al., 2002). As GuPOX activity increased, inhibition of root growth increased as well. *C. mimosoides* may have been protected by increased GuPOX activity in response to the stress of *P. americana* leaf extract. We suggest that up-regulation of GuPOX activity may be a protection mechanism.

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