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Induction of Defense-Mechanisms in Mungbean Suspension Cultured Cells by an Elicitor from *Macrophomina phaseolina*

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An elicitor molecule has been separated from *Macrophomina phaseolina,* the root rot pathogen of mungbean *(Vigna radiata).* Cell cultures of mungbean cultivars one resistant and another susceptible to the pathogen, which responded to the elicitor have been established. The elicitor treatment induced rapid increases in phenolic synthesis, activation of phenylalanine ammonia-lyase and peroxidase in the cultured cells. Relative increases in phenolic synthesis and peroxidase activity were observed in suspension-cultured cells of the resistant cultivar compared to that of the susceptible one due to elicitor treatment.

Keywords: Cell culture, fungal elicitor, mungbean, peroxidase, phenolics, phenylalanine ammonia-lyase.

Mungbean (*Vigna radiata* L. Wilczek) is an important pulse crop grown in southern parts of Asia, especially in India, Pakistan, Bangladesh, Srilanka, Thailand, Laos, Cambodia, Vietnam, Indonesia, Malaysia and China. It is also grown in eastern and central parts of Africa, the west Indies and USA. It is severely affected by root rot disease caused by *Macrophomina phaseolina* (Tassi) Goid. *M. phaseolina* affects cotton, sunflower, bean, soybean, chickpea, pigeonpea, peanut, sorghum, maize, sesamum, tomato, potato, citrus and many other crops (Rangaswami, 1988).

Plant suspension-cultured cells are in general a useful tool for studying several aspects of plant biology including plant defense mechanisms. Many of the biochemical reactions, such as the production of phytoalexins, activation of cell-wall degrading enzymes, oxidative burst, production of protease inhibitors, pathogenesis related (PR) proteins, cell wall glycoproteins and lignin (Dixon and Harrison, 1990) are caused by the activation of a set of defense-related genes, which can be induced in suspension-cultured plant cells treated with wide range of structurally diverse biotic and abiotic elicitors (Dixon and Lamb, 1990; Vera-Estrella et al., 1993; Honne et al., 1998; Yano et al., 1998).

An elicitor from cell wall of *M. phaseolina* was isolated (Ramanathan and Vidhyasekaran, 1998). Mungbean cell cultures were treated with this elicitor, induction of peroxidase, phenylalanine ammonia-lyase (PAL) and synthesis of phenolics in the cell cultures were studied. Phenylalanine ammonia-lyase (PAL) is a key enzyme in phenyl-

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propanoid metabolism catalyzes the deamination of L-phenylalanine to *trans*-cinnamic acid, which is the first step in the biosynthesis of a large class of plant natural products. The phenylpropanoid derivatives such as lignin-like materials, soluble and wall-bound phenolics, phytoalexins are thought to be parts of the defense related compounds in construction of biochemical and/or physical barriers against the incompatible interaction (Hahlbrock and Scheel, 1989). Peroxidases are a group of glycosylated, H_2O_2 utilizing, monomeric enzymes acting on a large variety of substrates. They participate in a wide spectrum of physiological functions, including oxidation of electron donor molecules, ethylene formation and halogenation. Peroxidase induction in association with plant defense relates to its ability to reinforce existing cell walls by polymerizing phenolic monomers, thus generating an aromatic suberin matrix, and by catalyzing intra- and intermolecular cross-linkages of HRGPs such as extensin (Huystee, 1987). The occurrence of cell wall modifications due to rapidly and transiently increase in peroxidases have been well documented in elicitor treated cells (Kawalleck et al., 1995). Phenolics are one of the most important defense chemicals in plants (Dixon et al., 1994). In this study we have attempted to see the induction of these defense-related activities in suspension cultured cells of two mungbean cultivars, one susceptible and another resistant to the pathogen.

Materials and Methods

Source of host and pathogen

Mungbean cultivars Co 4 (resistant) and Co 5 (susceptible), obtained from the school of Genetics, Tamil Nadu Agricultural University were used in the present studies. Root rot pathogen, *M. phaseolina* was isolated from the infected root bits of mungbean plants and used to study the difference in defense mechanism in suspension-cultured cells of resistant and susceptible cultivars.

Mungbean cell culture

Seeds of both the cultivars were surface sterilized with 0.1% mercuric chloride for 2–3 min and washed with several changes of sterile distilled water. The seeds were placed inside the test tubes containing sterilized Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) and allowed to germinate. Leaf bits of about 5 mm length were collected from 7-day-old aseptically grown seedlings and plated in Petri dishes containing MS medium supplemented with 2,4-D (2 mg/l). The Petri dishes were incubated under continuous light at 25±2 °C for 3 weeks. The calluses that developed were aseptically cut into small pieces and transferred to MS liquid medium, supplemented with 2,4-D (1 mg/l). The cultures were incubated under continuous light conditions (6000 lux) at 25±2 °C. Cultured cells were subcultured once in a week in fresh MS medium supplemented with 2,4-D (1 mg/l). After 4–5 cycle of subculture the suspension-cultured cells were used for all the experiments.

Fungal elicitor

M. phaseolina was grown in Czapek-Dox liquid medium at room temperature for 3 weeks. The mycelial mats were harvested and rinsed with sterile distilled water several times. The mycelia were resuspended in sterile distilled water (10 ml of water per *g* wet weight of mycelia) and then homogenized in a Virtis homogenizer at full speed for 2 minutes. The homogenate was filtered through a coarse sintered-glass funnel, and the residue obtained on the filter was saved. The residue was homogenized three times more in water, once in a mixture of chloroform and methanol (1:1), and finally in acetone. This preparation was air dried and this fraction contained the mycelial walls.

Elicitor was extracted from the mycelial walls by suspending 1 *g* of walls in 100 ml of distilled water and autoclaving for 20 min at 121 °C. The autoclaved suspension was filtered through a coarse sintered-glass funnel. The filtrate was centrifuged at 3000 *g* and concentrated to 10 ml under reduced pressure using Büchi Rotavapor (Büchi Laboratoriums-Technik AG, Flawil, Switzerland). Purification of the elicitor by gel filtration was accomplished according to the procedure described earlier (Ramanathan and Vidhyasekaran, 1998). Purified preparations were reduced through lyophilization using Virtis lyophilizer (USA).

Treatment of mungbean cell cultures with elicitor

Suspension-cultured cells of 4/5 cycle were used to treat with elicitors. Fifty µg of the elicitor preparations in terms of glycoprotein were added to 100 ml of suspension-cultured cells (approximately 5 g) and shaken in Infors AG shaker at 120 rpm at 24 °C. One ml of sterile distilled water was added in control treatments. At different time intervals suspension cultured cells were harvested and various analyses were made.

Peroxidase

Mungbean cell cultures (1.0 g) were homogenized with acetone previously chilled to –15 °C (10 ml g^{-1} fresh weight). The homogenates were filtered through Whatman No.1 filter paper and the residues were rinsed in a container with a second equal volume of chilled acetone. The mats were dried for 1 h under vacuum at room temperature and stored at –15 °C. Peroxidase was extracted by suspending the acetone powder in ice-cold 0.01 M sodium phosphate buffer, pH 6.0, containing 0.1 M sucrose (5 ml buffer for 100 mg acetone powder). The suspensions were stirred occasionally for 1 h in an ice bath, filtered through two layers of cheese-cloth and centrifuged at 7500 *g* for 20 min at 4 °C. The clear supernatants were used for peroxidase assay. The reaction mixture (2.9 ml) was prepared, containing 0.25% (v/v) guaiacol in 0.01 M sodium phosphate buffer, 0.1 M H_2O_2 and enzyme extract (0.1 ml). Enzyme activity was expressed as changes in absorbance at 470 nm min^{-1} g^{-1} fresh tissue (Hammerschmidt et al., 1982).

Phenylalanine ammonia-lyase

Phenylalanine ammonia-lyase (PAL) was extracted from the acetone powder by suspending the powder in cold 0.1 M borate buffer, pH 8.8 (0.1 g powder in 6 ml). The

suspension was incubated at 0 °C for 1 h with occasional stirring, filtered through cheesecloth, and the filtrate centrifuged at 5,000 *g* at 0 °C for 10 min. For the assays, 1.5 ml of the supernatant was added to 1.0 ml of 0.05 M L-phenylalanine and 2.5 ml of 0.01 M borate buffer. Assays were carried out at 40 °C for 1 h and stopped by adding 0.1 ml 5 N HCl. The acidified mixture was extracted with 7.5 ml of ether. The ether was evaporated and the residue dissolved in 4 ml 0.05 N NaOH. Concentration of cinnamic acid in the extract was determined using a known quantity of cinnamic acid as standard and measuring absorbance at 268 nm using Beckman Du spectrophotometer. A mixture in which the reaction was stopped at zero time served as a control (Ross and Sederoff, 1992).

Phenolics

Mungbean cell cultures (0.5 g) were blended with 10 ml of 80% ethanol and boiled for 10 min. The extract was filtered, and the residue further re-extracted four times with 80% ethanol. The filtrates were combined, concentrated *in vacuo* and made up to 2 ml with 80% ethanol (Zieslin and Ben-Zaken, 1993). Water (3 ml), 0.5 ml Folin-Ciocalteau reagent, and 1 ml saturated sodium carbonate solution were added to 100 µl of the extract in a boiling tube. The tube was heated for 1 min in a boiling water bath and cooled in running water. The intensity of blue colour developed was measured at 725 nm using a Beckman DU-spectrophotometer. Catechol was used as the standard.

Three independent experiments with three replications in each experiment were conducted and the data presented are mean of these three replicated experiments. The experimental results were analyzed using microsoft excel version 5.0

Results

When the cell cultures of susceptible and resistant cultivars were treated with the elicitor, phenolic content increased in both the cell cultures. The increase was greater in cell cultures of the resistant variety. Maximum increase was observed within 6 h after elicitor treatment in cell cultures of the resistant cultivar while similar maximum increase was observed at 12 h after elicitor treatment in cell cultures of the susceptible cultivar. Due to elicitor treatment the increase in phenolic content was always higher in cell cultures of the resistant cultivar than that in cell cultures of the susceptible cultivar *(Fig. 1).*

PAL activity increased to the maximum level in cell cultures of susceptible and resistant cultivar within 6 h after elicitor treatment. Almost similar induction of PAL activity was observed in both the cell cultures (resistant and susceptible) at 6 and 12 h after elicitor treatment. Subsequently the activity reached the basal level as that of control cell cultures at 48 h after elicitor treatment *(Fig. 2).*

Peroxidase activity also increased in cell cultures of both susceptible and resistant cultivars due to elicitor treatment. The major difference was its very rapid increase in cell cultures of the resistant cultivar compared to that in cultures of the susceptible cultivar. By 6 h the increase was about 1.5 fold while by 12 h the increase was about 2.0 fold in cell cultures of the resistant cultivar compared to those of the susceptible cultivar. However the

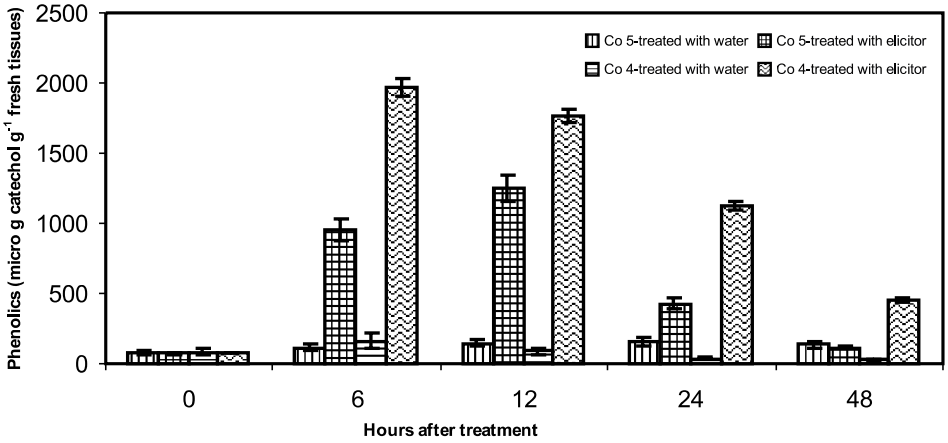

Fig. 1. Study of changes in phenolic content in suspension-cultured cells of resistant (Co 4) and susceptible (Co 5) mungbean cultivars treated with elicitor of *Macrophomina phaseolina*

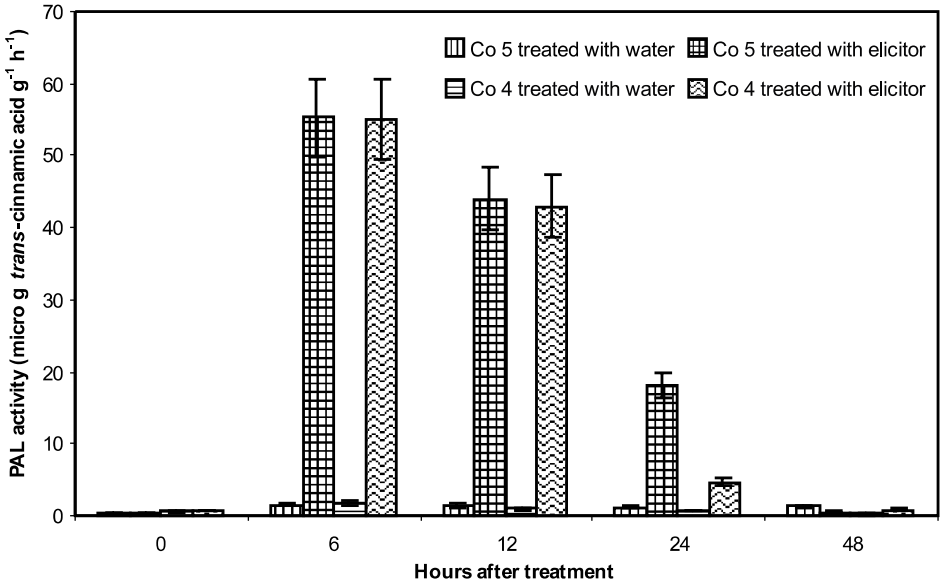

Fig. 2. Study of changes in phenylalanine ammonia-lyase activity in suspension-cultured cells of resistant (Co 4) and susceptible (Co 5) mungbean cultivars treated with elicitor of *Macrophomina phaseolina*

increase in peroxidase activity was observed even at 48 h after elicitor treatment in cell cultures of the susceptible cultivar whereas activity started declining 24 h after elicitor treatment in cell cultures of the resistant cultivar *(Fig. 3).*

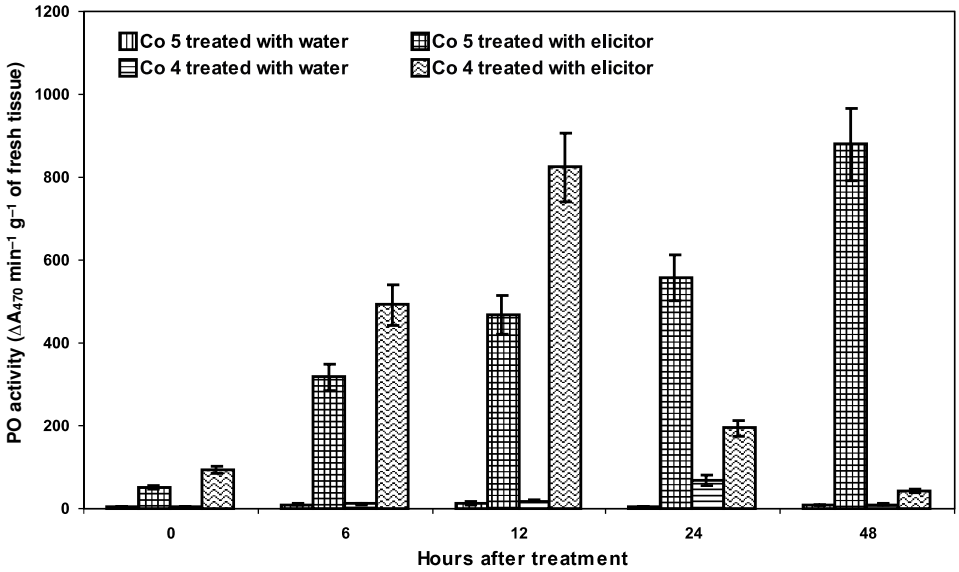

Fig. 3. Study of changes in peroxidase (PO) activity in suspension-cultured cells of resistant (Co 4) and susceptible (Co 5) mungbean cultivars treated with elicitor of *Macrophomina phaseolina*

Discussion

The elicitor activity of a fungal cell wall component is assessed by its ability to induce phenolic synthesis (Dixon et al., 1994) PAL (Hotter, 1997) and peroxidase activity (Mohan and Kolattukudy, 1990; Vera-Estrella et al., 1993). The elicitor active fractions (glycoprotein) isolated from fungal cell wall *(M. phaseolina)* induced phenol synthesis in cultured cells (Ramanathan and Vidhyasekaran, 1998). The similar fungal cell wall component when tested on resistant and susceptible cultured cells rapidly increase phenolic synthesis, peroxidase and PAL activity. Phenolic synthesis, PAL and peroxidase activities in suspension-cultured plant cells were reported by many workers due to elicitor treatment (Dixon and Lamb, 1990; Ryan and Farmer, 1991).

Increased synthesis of phenolics was observed within 6 h after elicitor treatment in suspension cultured cells of both resistant and susceptible mungbean cultivars. Phenolic content has been reported to increase within 1 h in tomato cell cultures after elicitor treatment (Vera-Estrella et al., 1993), within 4 h in elicitor-treated soybean cotyledon tissues (Graham and Graham, 1991) and within 8 h in elicitor-treated tobacco cell cultures (Godiard et al., 1991). PAL activity also showed significant increases in mungbean cell cultures within 6 h of elicitor treatment. PAL activity has been reported to increase within 2 h in alfalfa cell suspension cultures (Gowri et al., 1991) and by about 4

h in tobacco cell suspension-cultures treated with elicitors (Chappell et al., 1991). The rapid increases in PAL activity in suspension-cultured cells due to elicitor treatment have been reported in parsley (Hahlbrock et al., 1995) and pine (Hotter, 1997). Early increase in peroxidase activity in elicitor-treated suspension cultured cells has been reported in tomato (Dixon et al., 1994).

Reaction of suspension-cultured cells of the susceptible and resistant cultivars to *M. phaseolina* elicitor could be distinguished in the magnitude of phenolic synthesis and peroxidase activity. Several studies have indicated that resistant cultivars respond to fungal infection by the rapid and large induction of phenolic synthesis and peroxidase activation compared to susceptible cultivars (Vidhyasekaran, 1988). However similar responses in suspension-cultured cells due to elicitor treatment have been reported only in tomato. Cell suspension cultures from a tomato line resistant to *Verticillium albo-atrum* responded to the fungal elicitor by producing high levels of anionic peroxidase mRNA while the susceptible line showed a very little induction (Mohan and Kolattukudy, 1990). Although several host-specific elicitors have been isolated from fungal pathogens (Schottens-Toma and DeWit, 1988), their reactions on cultured cell have not been reported. Simultaneous early induction of phenolics and peroxidase would have resulted in accumulation of quinones which are much more toxic than soluble phenolics (Vidhyasekaran, 1988), since peroxidase is known to induce phenol oxidation (Huystee, 1987).

Induction of PAL activity by the elicitor was almost similar in cultured cells of both susceptible and resistant cultivars although phenolics accumulated more in the cells of resistant cultivar. The above study revealed that the difference in activation of defense-related genes when the suspension-cultured cells of resistant and susceptible cultivars were used. The difference was due to the non-host specific action of elicitor molecules. Hence the suspension-cultured cells either from resistant or susceptible cultivars treated with cell wall fractions (elicitor) of virulent pathogen in different time duration may be useful system for isolation, characterization and study of expression of defense genes at cellular level. Using this technique the beneficial genes from resistant or susceptible cultivar may be isolated, characterized and transformed into susceptible, high yielding cultivars.

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