Journal of Plant Biology, March 2008, 51(2) : 145-149

Effect of Chitin Hexamer and Thuricin 17 on Lignification-related and Antioxidative Enzymes in Soybean Plants

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Inducers of disease resistance in crop plants have a role in sustainable agriculture. We describe a set of bacteriocins that can potentially improve plant growth by controlling specific pathogens and inducing generalized resistance. Solutions of the bacteriocin thuricin 17 and/or a chitin hexamer (a known inducer and positive control) were applied to leaves of two-week-old soybean plants, and levels of lignification-related and antioxidative enzymes were monitored. Phenyl ammonia lyase (PAL) activity in thuricin 17-treated leaves was highest at 60 h after treatment, being 61.8% greater than the control. PAL activity also was increased 18.1% at 72 h after treatment with the chitin hexamer. Tyrosine ammonia lyase (TAL) activity in leaves was 57.0% higher than the control at 48 h after treatment with thuricin 17, while such activity in chitin hexamer-treated leaves was increased by 23.8% at 72 h. At 36 h after treatment with the chitin hexamer or chitin hexamer + thuricin 17, the total concentration of phenolic compounds was 15.3 or 19.3%, respectively, greater than the control. At 72 h, total phenolic concentrations increased by 23.2 and 19%, respectively, in response to thuricin 17 and chitin hexamer + thuricin 17. POD activity in thuricin 17-treated leaves increased by 74.6 and 81.2% at 48 and 72 h, respectively, whereas SOD activity increased by 24.9 and 79.9%, respectively, in chitin hexamer- and thuricin 17-treated leaves at 48 h. A peroxidase isozyme (31 kDa isomer) was induced in thuricin 17-treated leaves at 60 h, while catalase (59 kDa isomer) was induced in chitin hexamer-treated leaves. PAGE showed that two major SOD bands (Fe-SODs) were produced by both types of treatment. Collectively, these results indicate that the bacteriocin thuricin 17 can act as an inducer of plant disease defenses (i.e., activated lignification-related enzymes, antioxidative enzymes, and related isozymes) and that this induction is similar, but not identical, to that of the chitin hexamer elicitor. Although treatment with thuricin 17 + chitin hexamer also induced those responses, it did not present a clear pattern of additivity or synergy.

Keywords: chitin hexamer, peroxidase (POD), phenylalanine ammonia lyase (PAL), soybean, superoxide dismutase (SOD), thuricin 17, tyrosine ammonia lyase (TAL)

Plants synthesize a broad range of secondary metabolites to improve their resistance to pathogen attack. In many cases elicitors, such as oligosaccharides, are produced only upon exposure to compounds that indicate the presence of the pathogen (Somssich et al., 1986). Understanding these responses and how to manipulate them would be an important tool for future sustainable crop production.

The major molecular events of plant–pathogen interactions involve three steps: i) generation and recognition of signal compounds, ii) inter-and intracellular signal conversion and transduction, and iii) activation of signal-specific responses in target cells (Ebel and Cosio, 1994). Various elements of a multi-component plant defense mechanism that are induced by elicitors include the hypersensitive reaction (HR) (Arlat et al., 1994; Park and Paek, 2007), the production of activated oxygen species (oxidative burst) (Apostol et al., 1989), the modification of plant cell walls through callose deposition (Conrath et al., 1989), and the synthesis and accumulation of antimicrobial phytoalexins (Dixon et al., 1983). In addition to these localized defenses, systemic acquired resistance (SAR), which increases the plant's resistance to subsequent pathogen attack, is activated in many species; it can also be induced by specific elicitor compounds (Somssich et al., 1986).

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Elicitor molecules produced by microorganisms are extremely diverse in nature. Four major classes of elicitor-active oligosaccharides have been identified -- oligoglucan, oligochitin and oligochitosan from fungi, and oligogalacturonide from plants (Côté and Hahn, 1994). Chitin, an elicitor molecule, is a component of fungal cell walls, and is a polysaccharide composed of β-1-4-linked N-acetylglucosamine (GlcNAc) units. Chitin oligomers act as potent elicitor signals for several plant response systems, such as acidification of the cytoplasm (Kuchitsu et al., 1997), induction of phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase (TAL) (Khan et al., 2003), biosynthesis of phytoalexins (Yamada et al., 1993), the biosynthesis of JA (Nojiri et al., 1996), induction of lignification in wheat (Barber et al., 1989), and the generation of reactive oxygen species (Kuchitsu et al., 1995).

Bacillus thuringiensis NEB17 (BtNEB17), originally isolated from soybean root nodules (Bai et al., 2002), has been shown to produce a bacteriocin, now named thuricin 17. This bacteriocin is a small peptide (molecular weight 3162 Da) that shows antibacterial activity against closely related bacteria (Gray et al., 2006). However, its effect on the induction of defense-related responses in plants is not known.

In this study we examined the influence of chitin hexamer (a hexamer of GlcNAc) and thuricin 17 (a small bacterial peptide) on lignification-related enzymes (PAL and TAL) and

antioxidative enzymes (peroxidase, catalase, and superoxide dismutase) in soybean plants.

MATERIALS AND METHODS

Plant Material

Seeds of soybean (*Glycine max* L. Merr. cv. OAC Bayfield) were surface-sterilized in 10% bleach (final concentration 0.5% sodium hypochlorite), rinsed several times with distilled water, then germinated in vermiculite (Holiday®, Montréal). Their seedlings were then reared in the same medium in a growth chamber under a 16-h photoperiod (natural light supplemented with high-pressure sodium lamps to approximate daylight), at 25 ± 1°C, until they reached the vegetative cotyledon (VC) stage (Fehr and Caviness, 1977).

Known Elicitor and Bacteriocin Treatments

We used chitin (hexamer) and thuricin 17 as elicitor molecules. The former was purchased from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan) whereas the latter was isolated in our laboratory following the methods of Gray et al. (2006). The concentration used for the chitin hexamer was based on previously published work (Khan et al. 2003), while unpublished data from our laboratory had shown that 10^8 mol L^{-1} is the most active concentration for thuricin 17. Both elicitors were added when seedlings reached the first trifoliate stage (~2 weeks old). Chitin hexamer and thuricin 17 treatments were applied through cut stems, as described by Orozco-Cardenas and Ryan (1999). Briefly, plants were excised at the base of their stems with a sharp scalpel and promptly placed in 2-mL Eppendorff tubes containing 0.5 mL of 100 μmol L^{-1} chitin hexamer [$(GlcNAc)_6$], 0.5 mL of 1 x 10^8 mol L^{-1} thuricin 17, or a mixture of 0.25 mL chitin hexamer + 0.25 mL thuricin 17; all solutions were made up in 15 mM sodium phosphate buffer (pH 6.5). Control plants were treated with phosphate buffer solution alone. Once all the solution was taken up by the plants (4 to 6 h), they were immediately transferred to glass test tubes containing 20 mL of distilled water. These plants were then kept under constant white light (85 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Leaves were collected at 12, 24, 36, 48, 60, or 72 h after elicitor treatment, weighed, placed in plastic bags, and stored immediately at –80°C.

Determination of Phenyl Ammonia Lyase (PAL) and Tyrosine Ammonia Lyase (TAL) Activities

Leaf samples (300 mg fresh weight, FW) were extracted in 4 mL of buffer [50 mM Tris (pH 8.5), 14.4 mmol L^{-1} 2-mercaptoethanol, and 1% w/v insoluble polyvinylpolypyrrolidone], then centrifuged at 6,000 g for 10 min at 4°C. The total protein concentration in these soluble enzyme extracts was determined with the Bradford (1976) assay. The method of Beaudoin-Eagan and Thorpe (1985) was used to estimate PAL and TAL activities. The reaction mixture, at a final volume of 3 mL, consisted of 1.9 mL of 50 mM Tris-HCl buffer (pH 8.0), 100 μL of enzyme preparation, and either 1.0 mL of 15 mM L-phenylalanine for PAL or 1.0 mL of 15 mM L-tyrosine for TAL. Assays were started by the addition of enzyme extract after an initial incubation for 60 min at 40°C; reactions were stopped by the addition of 200 μL of 6 N HCl. The amounts of trans-cinnamic and p-coumaric acids that formed were determined by measuring absorbance at 290 and 330 nm, respectively, against an identical mixture in which D-phenylalanine was substituted for L-phenylalanine or D-tyrosine for L-tyrosine. Enzyme activity was expressed in nmoles (cinnamic or coumaric acid) mg $protein^{-1}$ min^{-1}, where 1 unit is defined as 1 nmole (cinnamic or coumaric acid) mg $protein^{-1}$ min^{-1}.

Determination of Total Phenolic Content

Total phenolic content was evaluated by the Folin-Ciocalteu method (Singleton and Rossi, 1965). The assay mixture contained 50 μL of sample with 0.475 mL of 0.25 N Folin-Ciocalteu reagents (Sigma Chemical Co., St. Louis, MO, USA). After 3 min, 0.475 mL of 1 mol L^{-1} Na_2CO_3 was added and absorbance was measured after 1 h. Phenolic content was estimated using a standard curve prepared with gallic acid. The total content was expressed as gallic acid equivalents (GAE) in mg g^{-1} FW.

Determination of POD and SOD Activities

Activity of peroxidase (POD) was assessed according to the method of Chance and Maehly (1955). The reaction mixture comprised 50 μL of 20 mM tetraguaiacol, 2.8 mL of 50 mM Tris-HCl buffer (pH 8.0), and 0.1 mL of extract. The reaction was started with the addition of 20 μL of 40 mM H_2O_2, and the change in absorbance at 470 nm was recorded for 1 min. POD activity was calculated using an extinction coefficient for tetraguaiacol of 26.6 mM^{-1} cm^{-1} at 470 nm. One unit of activity was defined as the amount of enzyme required for the formation of 1 μmol of tetraguaiacol per minute. Activity of superoxide dismutase (SOD) was determined by measuring its ability to inhibit the photoreduction of nitroblue tetrazolium (NBT) following the method of Giannopolitis and Ries (1977). The reaction mixture (3.0 mL) consisted of 63 μM NBT, 1.3 μM riboflavin, 13 mM methionine, 0.1 mM EDTA, 50 mM Tris-HCl (pH 8.0), and 50 μL of extract. This mixture was held in a test tube and placed for 20 min under light at 78 μmol photons s^{-1} m^{-2}. Absorbance was then recorded at 560 nm. A non-illuminated reaction mixture that did not develop color served as the control, and its absorbance was subtracted from the A_{560} of the reaction solution. One unit of activity was defined as the amount of enzyme required to inhibit 50% of NBT photoreduction, in comparison with tubes lacking plant extract.

Detection of Antioxidant Enzymes

For active staining of POD after separation through 12.5% polyacrylamide gel electrophoresis (PAGE) (30 μg of protein), the gels were soaked for 10 min in 50 mM Tris buffer (pH 8.0), then incubated with 0.46% (v/v) guaiacol and 13 mM H_2O_2 in the same buffer at room temperature (RT). After red bands appeared, they were fixed in water:methanol:acetic acid (6.5:2.5:1.0, v:v:v; Caruso et al., 1999). To stain for catalase activity (CAT) after 12.5% PAGE (30 μg of protein), the gel was incubated with 3.2 mM H_2O_2 for 20 min, then treated with a solution containing 1% $FeCl_3$ and 1% $K_3Fe(CN)_6$ for 10 min, as described by Racchi et al.

(2001). SOD activity was analyzed after 12.5% PAGE (30 μg of protein) to determine any change in the activity of SOD isozymes. The gel was soaked for 25 min at RT in 50 mM Tris-HCl (pH 8.0) containing 2.5 mM NBT. Cu/Zn-SODs were inhibited with KCN and H_2O_2 while Fe-SODs were inhibited with H_2O_2; Mn-SODs are resistant to both inhibitors (Fridovich, 1989). The gel was rinsed in distilled water, then incubated for 30 min in the same buffer, which contained 28 mM TEMED and 28 μM riboflavin. Afterward, the gel was placed under an illuminator for 30 min to develop the purple color, except for areas where SOD was localized.

RESULTS AND DISCUSSION

In our soybean leaf tissues, chitin hexamer elicited increases in PAL, TAL, total phenolic compounds, and POD and CAT activities, but not that of SOD. Likewise, thuricin 17 induced PAL, TAL, total phenolic compounds, POD, and SOD, but not CAT.

Changes in lignification-related enzymes were apparent by 72 h after the leaves were treated with chitin hexamer and/or thuricin 17 (Fig. 1). PAL activity in the latter tissues rose until 60 h after treatment and thereafter decreased (Fig. 1A). Activity of PAL in chitin hexamer-treated leaves increased continuously throughout the experimental period, while PAL in the chitin hexamer + thuricin 17-treated leaves did not increase above the control level. At 60 h, PAL activity was 61.8% higher in thuricin 17-treated leaves and 8.4% higher in chitin hexamer-treated leaves, compared with the control. Finally, at 72 h, PAL activity was 11.5 and 18.1%, respectively, greater than the control in thuricin 17- and chitin hexamer-treated leaves. Induction of PAL activity by chitin oligosaccharides has been reported previously for soybean (Khan et al., 2003), although that earlier study showed that maximum activity occurred at 24 and 36 h after chitin treatment. In contrast, our results agree with those of Vander et al. (1998), who found that chitin oligomers (degree of polymerization, 4 to 10) did not elicit PAL activities at 24 h after being injected into the intercellular spaces of wheat leaves.

TAL activity in thuricin 17-treated leaves increased until 48 h after treatment and thereafter slightly decreased (Fig. 1B). In chitin hexamer-treated leaves, such activity increased continuously throughout the experimental period whereas, in chitin hexamer + thuricin 17-treated leaves, TAL levels remained low and unaffected by treatment. At 48 h, this activity was increased by 57.0% in thuricin 17-treated leaves but by only 18.8% in chitin hexamer-treated leaves, as compared with the control. By 72 h, TAL activity was increased by 5.0% because of thuricin 17 and by 23.8% in leaves from chitin hexamer-treated plants, compared with the control. In agreement with our results, Khan et al. (2003) have reported that chitin oligomers induce TAL activity in soybean leaves.

The concentration of total phenolic compounds in soybean leaves was determined at 12, 36, and 72 h (Fig. 2). At 36 h, that level had increased by 15.3% in chitin hexamer-treated leaves, by 8.0% following thuricin 17 treatment, and by 19.3% in chitin hexamer + thuricin 17-treated leaves, compared with the control. By 72 h, total phenolics had risen by 23.2% in thuricin 17-treated leaves and by 19.0% in chitin hexamer + thuricin 17-treated leaves, but by only 1.4% in chitin hexamer-treated leaves. Chitin-induced accumulation of phenolic compounds in soybean plants has also been reported by Khan et al. (2003). Moreover, Kurosaki et al. (1986) have shown that the insoluble mycelial walls of the fungus *Chaetomium globosum* stimulate the induction of

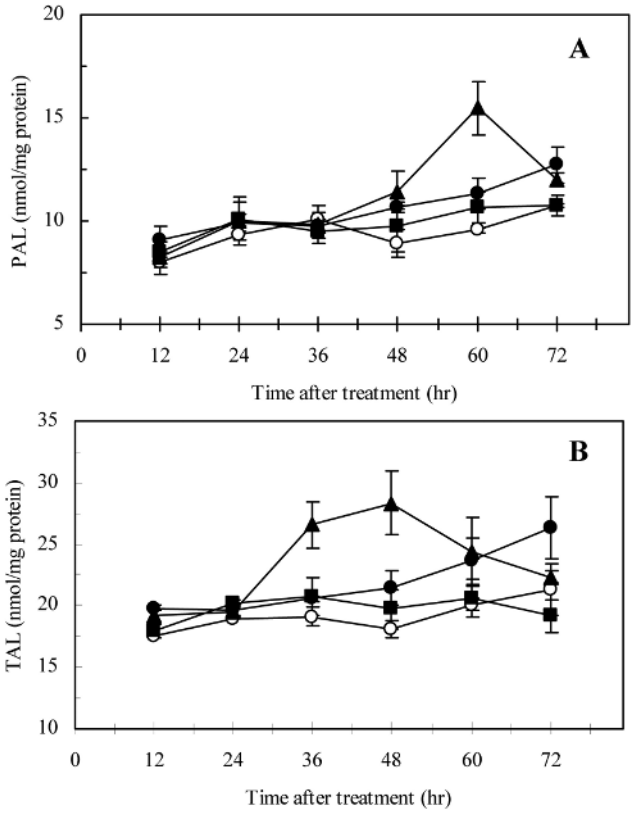

Figure 1. Changes in PAL (**A**) and TAL (**B**) activities in soybean leaves after treatment with chitin hexamer [0.5 mL (100 μmol L^{-1})] and thuricin 17 (1 x 10^8 M). Control (○), Chitin hexamer [$(GlcNAc)_6$] (●), Thuricin 17 (▲), Chitin hexamer + thuricin 17 (■). Each point represents mean ± s.e. (n=3).

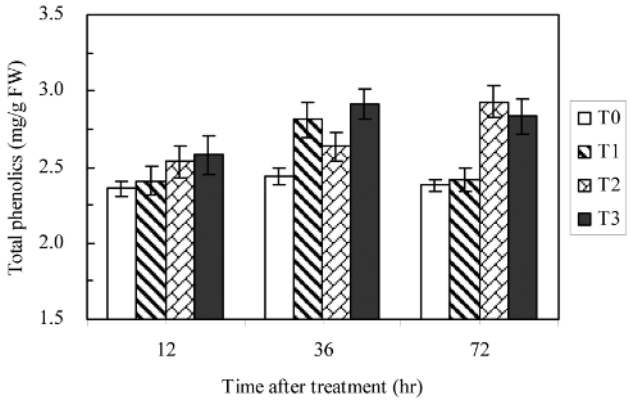

Figure 2. Changes in total phenolic contents from soybean leaves after treatment with chitin hexamer and thuricin 17. T0: Control , T1: Chitin hexamer [$(GlcNAc)_6$], T2: Thuricin 17, T3: Chitin hexamer + thuricin 17. Each bar represents mean ± s.e (n=3).

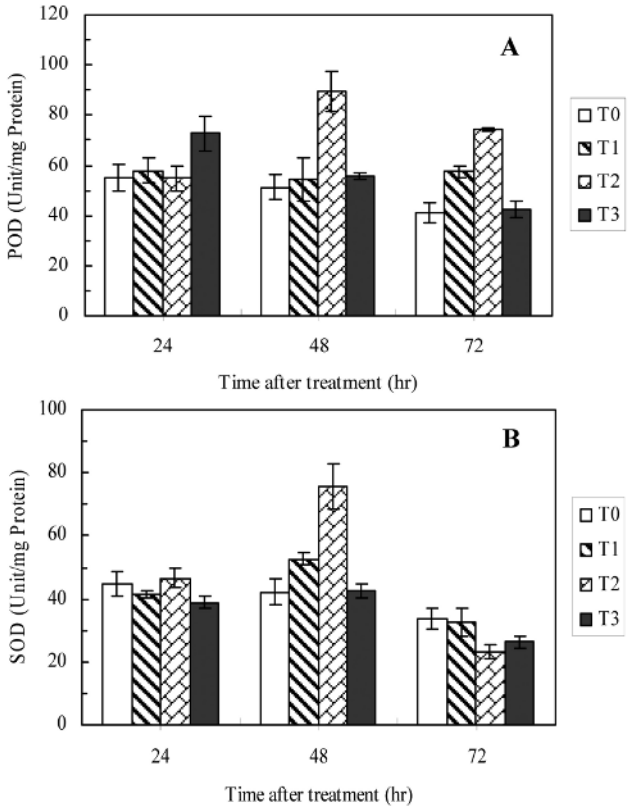

Figure 3. Changes in peroxidase (**A**) and superoxide dismutase (**B**) activities in soybean leaves after treatment with chitin hexamer and thuricin 17. T0: Control, T1: Chitin hexamer [$(GlcNAc)_6$], T2: Thuricin 17, T3: Chitin hexamer + thuricin 17. Each bar represents mean ± s.e (n=3).

PAL and the accumulation of phenolic acids in cultured carrot cells. In fact, these authors postulate that chitin may be the fungal cell wall factor most able to induce PAL and the accumulation of phenolic acids in carrot.

POD activity increased by 31.9% in chitin hexamer + thuricin 17-treated leaves at 24 h (Fig. 3A), and by 74.6% in thuricin 17-treated leaves at 48 h. After 72 h, this activity had risen by 40.3% with chitin hexamer and by 81.2% with thuricin 17, but by only 3.4% in chitin hexamer + thuricin 17-treated leaves, compared with the control. At 48 h, SOD activity also had increased by 24.9% in chitin hexamer- and by 79.9% in thuricin 17-treated leaves, relative to the control (Fig. 3B). These results suggest that chitin plays an important role in inducing antioxidant enzymes in soybean. Chitin and chitosan are effective elicitors in the hypersensitive lignification response by plants, both intact (Vander et al., 1998) and wounded (Pearce and Ride, 1982; Barber et al., 1989), Chitin oligomers (degree of polymerization, 7 to 10) also can induce POD activities at 24 h after injection into the intercellular spaces of wheat leaves (Vander et al., 1998). This elicitation of lignification-related enzyme activity depends on the chain length and concentration in solution (Pearce and Ride, 1982).

After polyacrylamide gel electrophoresis (PAGE), we measured the activities of POD, CAT, and SOD to detect possible changes in the isozyme levels in soybean (Fig. 4). At 60 h, two bands (40 and 31 kDa) exhibited staining for POD activity in leaves treated with thuricin 17 (Fig. 4A). Activity of the 31-kDa isoenzyme was greater in thuricin 17-treated leaves than in those that received the control treatment. One band (59 kDa) from leaves treated with the chitin hexamer also stained for CAT activity (Fig. 4B). The electrophoretic pattern of SODs in leaves showed six bands of activity (25, 23, 20, 18, 15, and 13 kDa). These were identified as Fe-SODs because they were inhibited only by H_2O_2 (Fig. 4C-b). Compared with the control, two major Fe-SOD bands were induced to higher levels in leaves treated with

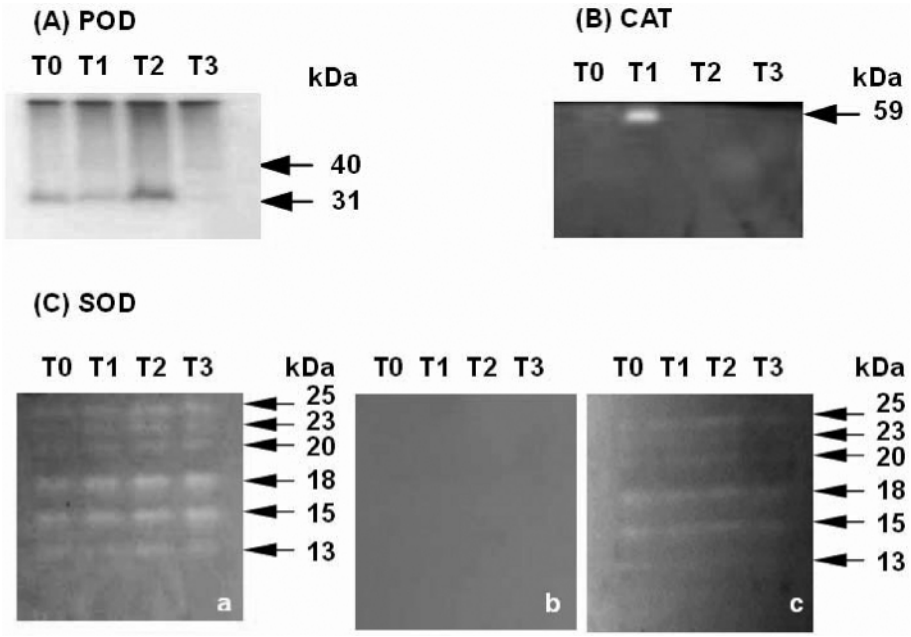

Figure 4. Active staining of (**A**) peroxidase (POD), (**B**) catalase (CAT), and (**C**) superoxide dismutase (SOD) [a) PAGE, b) H_2O_2 treatment, c) KCN treatment] in soybean leaves after treatment with chitin hexamer and thuricin 17. T0: control, T1: chitin hexamer [$(GlcNAc)_6$], T2: Thuricin 17, T3: chitin hexamer + Thuricin 17.

thuricin 17 and chitin hexamer + thuricin 17 (Fig. 4C-a). Plants generally contain Fe-SOD and Cu/Zn-SOD in their chloroplasts, Cu/Zn-SOD in the cytosol, and Mn-SOD in the mitochondrial matrix and proxisomes (Bower et al., 1994).

In conclusion, our results demonstrated that both chitin hexamer and thuricin 17 induce phenyl ammonia lyase (PAL) and tyrosine ammonia lyase (TAL) activities in soybean leaves. This is associated with changes in total phenol metabolism. Following treatment with either one, the concentration of phenolic compounds increased. These elicitors were also effective in triggering changes in the activity levels of antioxidant enzymes (peroxidase, superoxide dismutase, and catalase). In fulfilling our study objective, we have now shown that it is possible for thuricin 17 to provoke defense responses in an agronomic plant such as soybean. These findings have potential use in sustainable management because they allow for crop production under conditions of limited stress, e.g., reduced availability of irrigation water or lower application rates of chemical pesticides. Thus, our examination utilizing a generalized set of stress responses should be further followed by research focused on more specific effects.

ACKNOWLEDGEMENTS

This work was supported by an NSERC grant to Dr. Donald L Smith.

Received December 20, 2007; accepted February 11, 2008.

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