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Activity and Differential Induction of Chitinase Isozymes in Soybean Cultivars Resistant or Susceptible to Root-knot Nematodes

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Abstract: Host physiological events in relation to infestation by parasitic nematodes are not well documented. Soybean plant responses to *Meloidogyne incognita* infestation were compared to resistant (Bryan) and susceptible (Brim) cultivars at 0, 1, 3, 10, 20, and 34 days after infestation (DAI). The resistant cultivar had higher chitinase activity than the susceptible cultivar at every sample time beginning at 3 DAI. Results from isoelectric focusing gel electrophoresis analyses indicated that three acidic chitinase isozymes with isoelectric points (pIs) of 4.8, 4.4, and 4.2 accumulated to a greater extent in the resistant compared to the susceptible cultivar following challenge. SDS-PAGE analysis of root proteins revealed that two proteins with molecular weights of approximately 31 and 46 kD accumulated more rapidly and to a higher level in the resistant than in the susceptible cultivar. Additionally, three major protein bands (33, 22, and 20 kD) with chitinase activity were detected with a modified SDS-PAGE analysis in which glycolchitin was added into the gel matrix. These results indicate that higher chitinase activity and early induction of specific chitinase isozymes may be associated with resistance to root-knot nematode in soybean.

Key words: chitinase, *Glycine max*, isozymes, *Meloidogyne incognita*, nematode, pathogenesis-related protein, resistance, soybean.

Root-knot nematodes (*Meloidogyne* spp.) are a major cause of crop losses by plant-parasitic nematodes, and crop damage is high compared with other disease-causing agents (Rodríguez-Kábana et al., 1990; Atkinson, 1995; Roberts, 1995). These losses are due to the world-wide distribution and broad host range of these nematodes, and to the severity and type of damage they cause, which often can increase infection by other pathogens (Mai, 1985). Root-knot nematode was the fourth leading cause of economic losses due to soybean disease in the southern United States during 1974 through 1994 (Wrather et al., 1995). The use of resistant cultivars is the primary means of managing root-knot nematode in soybean (Fassuliotis, 1987; Weaver et al., 1988; Luzzi et al., 1994). Resistance usually is associated with fewer egg masses or less gall formation in the host root system (Hussey and Boerma, 1981; Bouton et al., 1989) and is controlled by multiple genes (horizontal resistance) in many crop species, including soybean (Luzzi et al., 1994), common bean (Omwega et al., 1989), alfalfa (Griffin et al., 1990), white clover (Pederson and Windham, 1992), sugarbeet (Yu, 1995), and cotton (McPherson et al., 1995). The mechanisms of horizontal resistance to pathogenic organisms or pests are poorly understood (Atkinson, 1995; Roberts, 1995; Tuzun et al., 1996), and the essential physiological and molecular events leading to nematode resistance in plants are not well characterized (Opperman et al., 1994; Atkinson, 1995; van der Eycken et al., 1996). The elucidation of such events will greatly enhance our understanding of complex plant-nematode interactions and facilitate plant breeding and genetic engineering efforts.

Root-knot nematodes spend most of their active lives within plant roots. The infective stage is the second-stage juvenile (J2), which penetrates the root and migrates to the vascular tissue to establish a permanent feeding site (Williamson and Hussey, 1996). Plants generally respond to nematode invasion by activation of a series of local and systemic

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defense mechanisms (Bouton et al., 1989; Trudgill, 1991; Lindgren et al., 1992). Inducible defenses against nematodes include accumulation of peroxidases (Ibrahim, 1991), polyphenol oxidases and superoxide dismutase (Zacheo and Bleve-Zacheo, 1995), proteinase inhibitors (Bowles et al., 1991; Gheysen et al., 1996), and perhaps chitinases (Punja and Zhang, 1993). Additionally, activation of pathways involved in phytoalexin biosynthesis occurs, resulting in a hypersensitive response that is characterized by rapid, localized necrosis of root tissue (Sijmons et al., 1994).

Chitinase is a hydrolytic enzyme that degrades chitin (a polymer of β-1,4-linked *N*-acetylglucosamine), a structural component of some nematode egg shells, insect and crustacean exoskeletons, and many phytopathogenic fungi (Muzzarelli, 1977). Plant chitinases have been proposed to play an important role in defense against parasitic nematodes (Cohn and Spiegel, 1991; Mercer et al., 1992; Punja and Zhang, 1993). Chitinases from common bean (*Phaseolus vulgaris*) caused an increase in nematode egg mortality and premature hatch in vitro (Mercer et al., 1992). Like many other pathogenesis-related enzymes, chitinases have multiple isoforms with various isozymes associated with particular physiological functions (Dumas-Gaudot et al., 1994; Graham and Sticklen, 1994); however, little is known of their involvement in root-knot nematode resistance in plants (Punja and Zhang, 1993). Elucidation of the physiological roles of these enzymes may give insight into their expression in relation to nematode disease resistance. The main objectives of this study were to compare total chitinase activities and induction patterns of specific chitinase isozymes between resistant and susceptible soybean cultivars in response to *M. incognita* infestation.

Materials and Methods

Experimental design and data analysis: Experiments were conducted in a greenhouse with temperatures ranging from 25 °C (day) to 20 °C (night) and a 12-hour light period. A randomized complete-block design with split-plot arrangement of treatments was used for each experiment. Main plots consisted of two soybean cultivars (susceptible Brim and resistant Bryan) with and without *M. incognita;* subplots consisted of sampling dates. In the first experiment, six sampling dates of 0, 1, 3, 10, 20, and 34 days after soil infestation (DAI) with *M. incognita* were used. In the second experiment, only the first four sampling dates were included to verify early-event results from the first experiment. All treatments were replicated four times. Data from each experiment were subjected to analysis of variance with SAS software (SAS Institute, Cary, NC).

Sample preparation and disease evaluation: Four soybean seeds per cultivar were planted into 500-cm^3 plastic pots filled with sterilized sand. Seedlings were thinned to 2 plants/pot after emergence and were fertilized weekly with 20 ml Peter's fertilizer solution (20-20-20, Scott-Sierra, Maryville, OH). At the two trifoliolate-leaf stage, the soil in which the seedlings were planted was infested with 1,300 J2 and 400 eggs. Nematodes were placed 1 cm from the stem base at a 1-cm depth. *Meloidogyne incognita* inoculum was prepared from eggs extracted from galled tomato (*Lycopersicon esculentum* cv. Rutgers) roots with an NaOCl technique (Hussey and Barker, 1973). The egg suspension was poured over 45-μm and 25-μm-aperture sieves. Eggs from the 25-μm-aperture sieve were collected in a 1-liter glass bottle and agitated in water for 5 days to remove remaining NaOCl and induce egg hatching. The final concentration of eggs and J2 was adjusted with tap water. At the final sampling date (34 DAI), root and shoot fresh weights as well as numbers of galls and egg masses were recorded.

Extraction and quantification of root proteins: Roots and shoots of four soybean plants were sampled at each date. Roots were thoroughly washed with tap water, flash-frozen in liquid nitrogen, and stored at −80 °C. Approximately 1 g of frozen root tissue was ground in a mortar containing 4.0 ml of 0.1 M Na-acetate buffer, pH 5.2, and a small amount of sea sand to increase protein ex-

traction efficiency. Root homogenate was centrifuged for 20 minutes at 12,000*g*. The supernatant was collected, and protein was quantified with the Bio-Rad protein assay procedure (Bio-Rad Laboratories, Richmond, CA) and IgG (Sigma Chemical, St. Louis, MO) as a standard.

Determination of total chitinase activities: A colorimetric assay for chitinase activity in roots, with CM-chitin-RBV (carboxyl methylchitin-remazol brilliant violet) as substrate, was performed as described by Wirth and Wolf (1990). The reaction mixture (total volume 800 µl) contained 550 µl distilled and deionized H_2O, 200 µl CM-chitin-RBV, and 50 µl root supernatant. All assays were performed in triplicate. After incubation at 37 °C for 5 minutes, 200 µl of 1 N HCl was added, placed on ice for 10 minutes, and centrifuged for 5 minutes at 12,000*g*. Absorbance of the resulting supernatant (900 µl) was measured spectrophotometrically at 550 nm. Chitinase activity was calculated according to Wirth and Wolf (1990) and expressed as international units per milligram protein. One international unit (U) is defined as the amount of enzyme required to catalyze the formation of 1 nmol product (GlcNAc) per minute.

In-gel activity assay of chitinase isozymes following isoelectric focusing (IEF): Proteins isolated from roots were separated in 2.0-mm-thick 7.5% polyacrylamide gels, (pI 3.0–10.0) with an LKB 2117 Multiphor IEF system (Pharmacia, Piscataway, NJ). The method of Pan et al. (1991) was used to determine the number of protein bands with chitinolytic activity. A 10% polyacrylamide gel containing 0.04% glycol-chitin as the substrate for chitinases was prepared and tightly adhered to the separating gel following IEF. Both gels were sealed together with plastic wrap to prevent desiccation and incubated at 37 °C overnight. The overlaid gel was stained with 500 ml of freshly prepared 0.01% (w/v) fluorescent brightener 28 (Sigma Chemicals, St. Louis, MO) in 500 mM Tris-Cl (pH 8.0) for 5 minutes, and destained with deionized water overnight at room temperature. Chitinase isozymes were visualized as cleared zones in gels placed on a UV transilluminator and photographed.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses: SDS-PAGE of root proteins was performed with the Bio-Rad Mini-Protein apparatus (Bio-Rad Laboratories, Richmond, CA) to separate root proteins by their relative molecular weights. A 14% (w/v) slab gel containing SDS with a 5% (w/v) stacking gel was used. Samples containing 15 µg protein were denatured at 100 °C for 3 minutes in 1% SDS containing 100 mM-mercaptoethanol and Tris-glycine buffer at pH 6.8. Electrophoresis was conducted at 100 V through the stacking gel and 200 V through the separating gel. Following electrophoresis, protein bands were visualized with a silver staining procedure (Dunn, 1993).

To detect protein bands with chitinolytic activity after electrophoresis, a modified SDS-PAGE assay (Zhang and Punja, 1994) was conducted. Protein samples (15 µg each) were mixed with loading buffer (1% SDS containing 100 mM-mercaptoethanol and tris-glycine buffer at pH 6.8), incubated at 37 °C for 15 minutes, and electrophoresed on 14% polyacrylamide gels containing 0.04% glycol-chitin (as the substrate for chitinases). After electrophoresis, the gel was incubated twice (30 minutes each) to remove SDS with a 'renature solution' containing 25% isopropanol in 0.1 M sodium phosphate buffer pH 6.0, followed by two incubations with 0.1 M sodium phosphate buffer (30 minutes each time). Chitinase isozymes were visualized following the procedure described above for the IEF in-gel activity assay.

Results

Root-knot nematode disease evaluation: Disease incidence, measured by number of galls and egg masses at 34 DAI (Table 1), showed that the resistant cultivar Bryan had fewer galls and egg masses per gram of tissue than the susceptible cultivar Brim ($P < 0.05$). Fresh shoot weight of non-challenged soybeans was higher for Bryan than for Brim ($P < 0.05$). Infestation with *M. incognita* did not

TABLE 1. *Meloidogyne incognita* reproduction and plant growth of the susceptible soybean cultivar Brim and the resistant cultivar Bryan 34 days after infestation.

Treatments	Total galls	Galls per gram roots	Total eggs	Egg masses per gram roots	Shoot weight (g)	Root weight (g)
Brim					4.85b	5.01a
Bryan					6.50a	5.61a
Brim + *M. incognita*	38.5a	6.70a	15.5a	2.69a	5.71ab	5.75a
Bryan + *M. incognita*	4.3b	0.85b	1.7b	0.34b	6.34a	5.04a

Data are means of four replicates. Means within a column followed by the same letter are not different according to Fisher's least significant difference ($P \leq 0.05$).

significantly affect fresh shoot weight of either cultivar. There was no significant difference in root weight between cultivars with or without *M. incognita*.

Total chitinase activity assay: Total chitinase activity was similar between resistant and susceptible soybean cultivars under control conditions (Fig. 1A). Similar responses were observed during the first 3 DAI (Fig. 1B); however, the resistant cultivar had higher chitinase activity than the susceptible cultivar 10 DAI ($P < 0.05$). Chitinase activity reached its maximum at 20 DAI in the resistant cultivar and at 34 DAI in the susceptible cultivar.

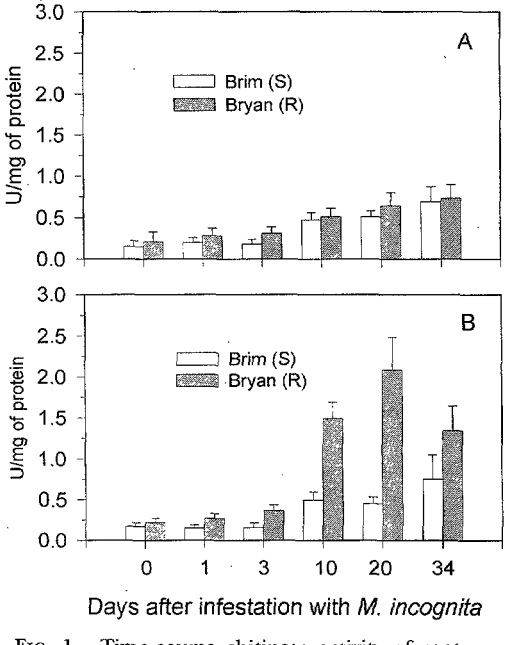

FIG. 1. Time-course chitinase activity of root proteins from the susceptible (S) soybean cultivar Brim and the resistant (R) cultivar Bryan. A)Without infection with *Meloidogyne incognita*. B) With *M. incognita* infection. Chitinase activity was measured colorimetrically with carboxyl methyl-chitin-remazol brilliant violet as substrate.

In-gel activity of chitinase isozymes following isoelectric focusing (IEF): A wide range IEF (pI 3–10) analysis was used initially to detect the presence of both acidic and basic chitinase isozymes in resistant and susceptible cultivars before and after nematode infestation. No differences in accumulation patterns of basic chitinase isozymes were observed between resistant and susceptible cultivars regardless of the nematode treatment. Five acidic (pI < 5.0) chitinase isozymes, which were further separated by using a narrow range of IEF (pI 3–6), were constitutively present in both cultivars (Fig. 2). The accumulation patterns of several acidic isozymes appeared to be time-dependent regardless of the nematode and cultivar treatments. For example, in the uninfested plants, accumulation of three acidic chitinases with respective pIs of 3.5, 3.9 and 4.2 increased with time in both resistant and susceptible cultivars. However, two chitinases with pIs of 4.2 and 4.4 accumulated to a greater extent in the resistant cultivar than in the susceptible cultivar starting at 10 DAI. Similar trends also were observed in treatments with *M. incognita*. Moreover, a chitinase isozyme (pI 4.8) accumulated to a greater extent at 10 and 20 DAI in resistant plants than in susceptible plants.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analyses: SDS-PAGE analysis (Fig.

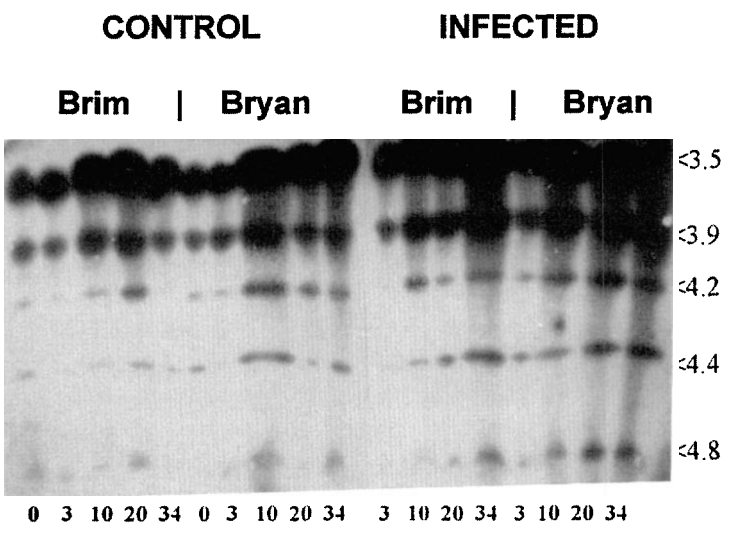

FIG. 2. Time-course accumulation patterns of acidic chitinase isozymes from the susceptible cultivar Brim and the resistant cultivar Bryan without (control) and with (infected) *Meloidogyne incognita* following isoelectrofocusing (IEF) gel electrophoresis (7.5%). Each lane contained 15 μg protein extracted from roots collected at 0, 3, 10, 20, and 34 days after infection, as indicated. Chitinase isozyme patterns were detected with an overlay gel containing 0.04% glycol-chitin after IEF gel electrophoresis (pI 3-6).

3) revealed the induction of many major protein bands in both susceptible and resistant cultivars. Protein patterns observed in total root homogenates revealed that similar amounts of proteins were induced except for the 31-kD protein, which accumulated to a higher level in the resistant cultivar Bryan starting at 3 DAI. In addition, a 46-kD protein was found to be present only in Bryan 10 DAI.

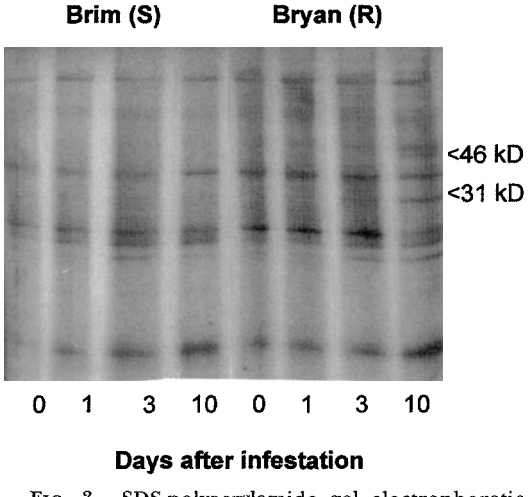

FIG. 3. SDS-polyacrylamide gel electrophoretic analysis of root proteins of the susceptible soybean cultivar Brim and the resistant cultivar Bryan before and after infection with *Meloidogyne incognita*. Each lane contained 15 μg root protein collected at 0, 1, 3, and 10 days after infection, as indicated. Molecular weights (in kD) of target fragments are shown.

The modified SDS-PAGE analysis revealed three major protein bands, with molecular weights of approximately 33, 22, and 20 kD, that showed chitinase activity (Fig. 4). The activity of chitinases, as shown by the intensity of bands, appeared to be greater in Bryan than in Brim at 10 DAI. Four additional weak bands (see arrows in Fig. 4) with respective molecular weights of approximately 31, 27, 26, and 25 kD also showed some chitinase activity. Among them, the 31-kD band appeared to be induced only in Bryan 10 DAI.

DISCUSSION

There has been a great deal of speculation about the function of defense-related proteins in pathogen-infected plants (Linthorst, 1991; Tuzun et al., 1996). Ex-

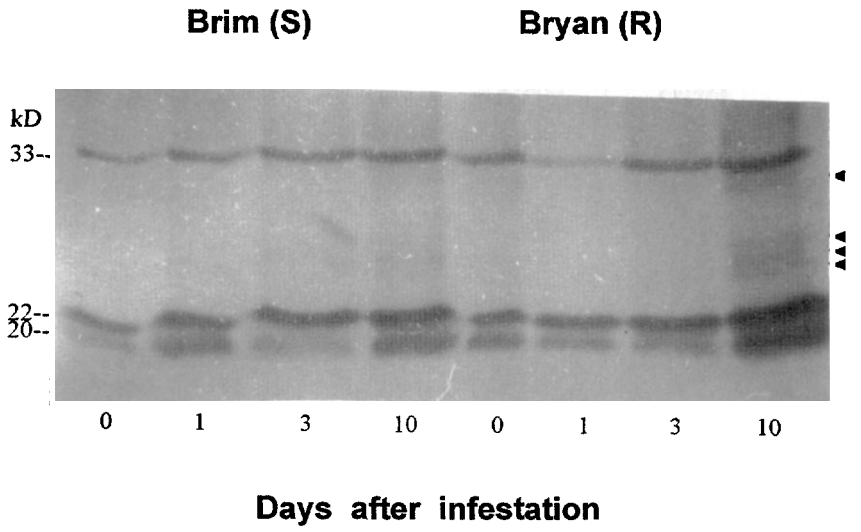

FIG. 4. In-gel chitinase activity of proteins from root homogenates of the susceptible (S) soybean cultivar Brim and the resistant (R) cultivar Bryan before and after infection with *Meloidogyne incognita*. This assay was based on a modified SDS-polyacrylamide gel electrophoretic technique in which 0.04% glycol-chitin was added as substrate for chitinases in the separating gel matrix. Each lane contained 15 µg protein extracted from roots collected at 0, 1, 3, and 10 days after infection, as indicated.

pression of genes encoding for these defense proteins generally does not occur in healthy developing plants but is triggered in response to infection by a wide range of pathogens and by abiotic stresses such as chemical treatment and wounding (Zacheo and Bleve-Zacheo, 1995; Zhang and Punja, 1994). Several studies indicate that chitinases may play a defensive role in fungal disease resistance (Graham and Sticklen, 1994; Lawrence et al., 1996; Punja and Zhang, 1993). However, studies on the role of chitinases in resistance to infection by root-knot nematodes have been preliminary and limited (Punja and Zhang, 1993). The present study was therefore conducted to determine if chitinases are associated with root-knot nematode resistance in soybean. Time-course studies of chitinase activity during nematode infestation showed that the resistant cultivar had higher chitinase activity than the susceptible cultivar starting 3 DAI. Based on disease incidence data in which the resistant cultivar had fewer galls and egg masses than the susceptible one, we speculate that the higher chitinase activity detected in the resistant cultivar Bryan could be associated with root-knot nematode resistance in soybean. Further studies utilizing better-defined genetic material such as near isogenic lines or recombinant inbred lines are required.

Induction patterns and the relative importance of specific chitinase isozymes vary among different host-pathogen interactions (Dumas-Gaudot et al., 1994; Lawrence et al., 1996; Zacheo and Bleve-Zacheo, 1995; Zhang and Punja, 1994). We found that three acidic chitinases (CH-4.8, CH-4.4, and CH-4.2) accumulated to a greater extent in the resistant cultivar upon nematode infection. Because acidic forms of chitinases are generally targeted extracellularly, whereas basic forms usually accumulate in the central vacuole (Dore et al., 1991; Lawrence et al., 1996), we speculate that these acidic isozymes may be located in the extracellular spaces in the soybean root. According to Mercer et al. (1992), chitinases from common bean caused an increase in nematode egg mortality and premature hatch in vitro. Future experiments are needed to purify these chitinase isozymes and determine if they have the ability to degrade the chitin layer of nematode egg shells, which may result in premature hatching of juveniles or reduced viability.

Early induction of pathogenesis-related

proteins that range in size from 14–45 kD has been shown to be an important defense-related phenomenon in potato-*Globodera pallida* interactions (Rahimi et al., 1993). In our study, a 31-kD protein was found to accumulate to a higher level in the resistant cultivar Bryan than in the susceptible cultivar Brim. In addition, a 46-kD protein was detected only in the resistant cultivar 10 DAI. However, the physiological roles of these proteins remain to be determined. Western-blot analyses, with antibodies raised against specific pathogenesis-related proteins, would improve our understanding of the nature and role of these target proteins.

We demonstrated that in-gel chitinase activity in soybean root homogenates can be achieved with IEF and (or) SDS-PAGE procedures in conjunction with the addition of glycol-chitin in the respective gel matrix system. IEF gel analysis has the advantage of not denaturing proteins compared with the modified SDS-PAGE procedure, thus allowing detection of all isozymes according to their pls. However, this technique requires use of an overlay gel to detect chitinase isozymes (Pan et al., 1991) and is cumbersome to perform. In contrast, the modified SDS-PAGE technique is easy to conduct and allows direct detection of chitinases of various molecular weights in a single gel system. Because proteins can be separated based on their pls via IEF or based on their molecular weights via SDS-PAGE, these two techniques, when used together, can give complementary information on the temporal induction of chitinases during pathogenesis and should be applicable to other host-pathogen systems without the use of a 2-D protein gel system. In summary, we provide evidence that the early induction of specific chitinase isozymes may be associated with root-knot nematode resistance in soybean. The 31-kD chitinase, which was detected only in the resistant cultivar 10 DAI, may have a role in defending plants from root-knot nematode infection. Future work will be conducted to purify the chitinases from soybean and to test their nematicidal activity to *M. incognita* and other root-knot nematodes in vitro and in vivo.

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