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ScienceDirect
Bioresource Technology 98 (2007) 1353–1358
BIORESOURCE TECHNOLOGY

The role of chitosan in protection of soybean from sudden death syndrome caused by *Fusarium solani* f. sp. *glycines*

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Received 12 September 2005; received in revised form 18 May 2006; accepted 21 May 2006
Available online 7 July 2006

Abstract

The *in vitro* antifungal properties of chitosan and its role in protection of soybean from a sudden death syndrome (SDS) were evaluated. Chitosan inhibited the radial and submerged growth of *F. solani* f. sp. *glycines* with a marked effect at concentrations up to 1 mg/ml indicating antifungal property and at 3 mg/ml was able to delay SDS symptoms expression on soybean leaves for over three days after fungal inoculation when applied preventively. Chitosan was able to induce the level of chitinase activity in soybean resulting in the retardation of SDS development in soybean leaves. However, the SDS symptoms gradually appeared and were associated with the reduction of chitinase activity level after five days of infection period. These results suggested the role of chitosan in partially protecting soybeans from *F. solani* f. sp. *glycines* infection.

Keywords: *Fusarium solani*; Chitosan; Sudden death syndrome; Soybean

1. Introduction

Sudden death syndrome (SDS), caused by the soil-borne fungus *F. solani* f. sp. *glycines*, is an economically harmful disease of soybean (Rupe, 1989). SDS causes rapid defoliation of soybean, resulting in reducing both the quality and quantity of soybean product (Roy et al., 1989; Rupe, 1989). The development of SDS is favored by cool and wet rhizospheric conditions through the growing season (Scherm and Yang, 1996). There is no total elimination of this disease because *F. solani* f. sp. *glycines* as mycelium and chlamydospores can survive in the soil and tolerate to the unfavorable conditions (Rupe and Gbur, 1995). The use of chemical substances for controlling *Fusarium* pathogen, mainly methyl bromide as a broad spectrum disinfectant, has been found to be effective (Allen et al., 2004). However, the excessive application of chemical fungicides led to increase in fungicide resistance in pathogens and a continued presence of the pathogens in other areas of the field (Bourbos et al., 1997) as well as contamination of the environment. Additionally, the fungicides contaminated in the environment tend to accumulate in agricultural products and human body via the food chain.

Chitosan (poly-β-(1,4)-D-glucosamine), a transformed oligosaccharide, is obtained by alkaline deacetylation of chitin, one of the most abundant natural biopolymers, that is extracted from the exoskeleton of crustaceans such as shrimps and crabs, as well as the cell walls of some fungi (Sandford, 1989; Roller and Covill, 1999; Domard and Domard, 2002). Thus, chitosan has attracted tremendous attention as a potentially important biological resource due to its biological properties including biocompatibility, nontoxicity and biodegradability (Kurita, 1998). It has been widely applied in the fields of agriculture, environment,

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doi:10.1016/j.biortech.2006.05.029

pharmaceuticals, medicines and industrial food processing (Sandford, 1989; Shahidi et al., 1999; Liu et al., 2001).

The interest in the antimicrobial properties of chitosan has focused on its possible role in plant protection. Chitosan has been found to interfere with the growth of several plant pathogenic fungi e.g., *Fusarium solani*, *F. oxysporum*, *Puccinia arachidis, Botrytis cinerea, Colletotrichum gloeosporioides* (Shimosaka et al., 1993; Bell et al., 1998; Sathiyabama and Balasubramanian, 1998; Ben-Shalom et al., 2003; Bautista-Baños et al., 2003). Chitosan caused morphological changes, structural alterations and molecular disorganization of the fungal cells reflecting its fungistatic or fungicidal potential (Hadwiger et al., 1986; Benhamou, 1996). The potential of chitosan to protect fungal diseases of various horticultural plants has been studied in various investigations (Benhamou et al., 1994; Lafontaine and Benhamou, 1996; Ben-Shalom et al., 2003; Bautista-Baños et al., 2003). Chitosan has also been found to activate several biological processes of plant defense responses such as enzymatic activities. Plant defense-related enzymes were known to participate in early defense mechanisms and to prevent pathogen infections (Ben-Shalom et al., 2003; Bautista-Baños et al., 2006).

This work describes the potential of chitosan as an antifungal agent on the growth of *F. solani* f. sp. *glycines*. Consequently, chitosan was evaluated as an effective biological substance for the soybean protection from SDS symptoms expression.

2. Methods

2.1. Materials

Chitosan from crab shell was obtained from Seafresh Chitosan (Lab) Co. Ltd., Thailand. The degree of deacetylation of chitosan was 85% and the molecular weight was 2×10^5 daltons. The viscosity of 1% chitosan solution in 1% acetic acid and moisture content were 149 centipoise and 8.97%, respectively. The purified chitosan was prepared as described by Benhamou (1992). Soybean (*Glycine max* (L.) Merr.) seeds (SJ5 cultivar) were obtained from Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand. All cultural media were purchased from Difco Laboratories, USA. Chemicals were obtained from Sigma–Adlrich (USA).

2.2. Fungal culture and growth

F. solani f. sp. *glycines* was maintained on Potato Dextrose Agar (PDA) medium. It was aerobically cultivated in Potato Dextrose Broth (PDB) at 28 °C with continuous shaking at 150 rpm.

Antifungal assay of chitosan was conducted for both the radial and submerged growth determination of *F. solani* f. sp. *glycines*. Purified chitosan was dissolved in 0.25 N HCl under continuous stirring, and the pH was adjusted to 5.6 with 2 N NaOH and then sterilized as previously described (Bell et al., 1998). For the radial growth determination, the sterile chitosan solution was added into PDA at concentrations of 1, 3 or 5 mg/ml. Each PDA plate was seeded with 6-mm-diameter mycelial plugs of *F. solani* f. sp. *glycines* and incubated at 28 °C in the dark. The fungal growth was measured daily for seven days (Bell et al., 1998). Growth inhibition was expressed as the percentage of inhibition of radial growth relative to the control.

For the submerged growth determination, the sterile chitosan solution was added into PDB to obtain the same chitosan concentrations of the radial growth determination. Spore suspension of *F. solani* f. sp. *glycines* was inoculated in chitosan-supplemented PDB to give a final volume of 1×10^4 spores/ml and incubated for one day. The fungal growth was monitored daily by dry weight determination for 10 days (Yonni et al., 2004).

2.3. Evaluation of the role of chitosan in protection of soybean from SDS development

The use of chitosan as a natural antifungal agent against SDS in soybeans was investigated as described previously (Sathiyabama and Balasubramanian, 1998) with some modification. Soybean seeds (SJ5 cultivar) were grown with autoclaved soil and usually watered until being at V1 growth stage (14-day-old). The experiment used a Completely Randomized Design (CRD) which was divided into six treatments with five replications. Both chitosan and *F. solani* f. sp. *glycines* were not applied in T_1 as negative control. The surface of soybean leaf was sprayed with 1 mg/ml of benomyl as chemical antifungal agent for T_3. Soybean leaves of other treatments including T_2, T_4, T_5 and T_6 were sprayed with 100 µl of chitosan solution at concentrations of 0, 1, 3 and 5 mg/ml on the abaxial surface, respectively. After 24 h, all treatments, except T_1, were inoculated with 100 µl of spore suspension (1×10^3 spores/ml) of *F. solani* f. sp. *glycines* on the abaxial surface. All inoculated soybean plants were covered with water-sprayed polyethylene bags for 24 h. The visible symptoms appearance of all soybean plants was observed daily for 9 days. Finally at the 14-day, all soybean plants were harvested for growth determination of root length, stem height and dry weight.

2.4. Chitinase activity assay in soybean leaves

After fungal inoculation, chitosan-untreated and 3 mg/ml of chitosan-treated leaves were collected for chitinase activity assay at 1, 2, 3, 4, 5, 6, 8, 10, 12 and 14-day. The intercellular fluid of soybean leaves was prepared by grinding leaf tissues and collecting by centrifugation for total protein and chitinase activity assay. The total protein concentration was determined for the cleared intercellular fluid prior to their use in enzyme activity assays. Total protein was determined by Coomassie Blue Protein Assay (BioRad, USA) according to the sensitive method of Bradford (1976). The chitinase activity assay was quantitative detection by measuring the amount of reducing sugars (*N*-acetyl-

D-glucosamine, GlcNAc) liberated during the hydrolysis of chitin solution as previously described (Shimosaka et al., 1993). One unit of chitinase enzyme was defined as the amount of enzyme catalyzing the turnover of 1 μmol of GlcNAc per minute under the assay conditions. All experiments were independently repeated at least three times and representative data are shown.

2.5. Statistical analysis

The means and standard deviation of radial growth, submerged growth and chitinase activity were calculated. Data from soybean plant growth were statically analyzed by using the analysis of variance (ANOVA) and DUNCAN multiple range tests if a significant difference was detected ($p < 0.05$). SPSS, version 10.0 was used for statistical analysis.

3. Results and discussion

3.1. Effects of chitosan as a natural antifungal agent on inhibition of the radial and submerged growth of *F. solani f. sp. glycines*

There was no halo formation of *F. solani* f. sp. *glycines* cultivated on 0 and 1 mg/ml of chitosan but the growth on 3 and 5 mg/ml chitosan-amended plates was restricted relative to that of the control (Fig. 1) and the percentages of radial growth inhibition were 38.2 and 54.6, respectively. Furthermore, they also formed a halo around the colony on the agar surface (data not shown). The halo-forming property was used for testing the chitosanolytic activity in the screening of *Fusarium* species, especially *F. splendens* and *F. solani*. *F. solani* f. sp. *phaseoli* formed halo around the colony on the 2.5 mg/ml of chitosan-containing agar plates (Shimosaka et al., 1993).

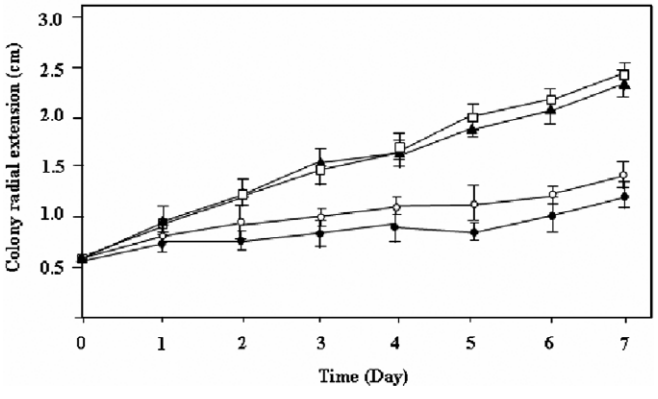

Fig. 1. The radial growth of *F. solani* f. sp. *glycines* on chitosan-supplemented PDA plate. *F. solani* f. sp. *glycines* was cultivated on PDA plates amended with 0, 1, 3 and 5 mg/ml of chitosan at 30 °C at 7-day of incubation period. The diameters of fungal colonies that grew on 0 (□), 1 (▲), 3 (○) and 5 (●) mg/ml of chitosan-supplemented PDA plates were measured daily for 7 days of incubation period. Values presented are means and standard deviation of triplicate assays.

Allan and Hadwiger (1979) suggested that the presence of chitosan within the cell walls of some fungi rendered those strains more resistant to the antifungal property of externally-amended chitosan. Roller and Covill (1999), however, found that chitosan reduced the growth rate of *Mucor racemosus* at 1 mg/ml and at 5 mg/ml completely prevented the growth of *Byssochlamys* spp. Benhamou (1992) found that chitosan at 3 to 6 mg/ml inhibited the radial growth of *F. oxysporum* f. sp. *radicis-lycopersici*, the causative agent of tomato crown and root rot. The decrease in growth inhibition was obtained with chitosan at concentrations less than 3 mg/ml. Based on the results from the *in vitro* studies, inhibition of the radial growth of *F. solani* f. sp. *glycines* was possibly due to the antifungal property of chitosan. Several mechanisms for the antifungal action of chitosan have been proposed. Two models had been proposed to explain the antifungal activity of chitosan. Firstly, the activity of chitosan was related to its ability to directly interfere with the membrane function (Stössel and Leuba, 1984). Secondly, the interaction of chitosan with fungal DNA and mRNA is the basis of its antifungal effect (Hadwiger et al., 1986).

Studies on the effect of chitosan on submerged growth of *F. solani* f. sp. *glycines* using dry weight measurements over a period 10 days at 28 °C showed complete inhibition of the growth of *F. solani* f. sp. *glycines* at all concentrations of chitosan (Fig. 2). However, an abnormal mycelial morphology including hyphal swelling and cytoplasm aggregation of *F. solani* f. sp. *glycines* was observed with 3 and 5 mg/ml of chitosan. But none of these abnormal shapes were exhibited in 1 mg/ml of chitosan-treated cells (data not shown). Chitosan at concentrations ranging from 1 to 6 mg/ml induced morphological changes in *F. oxysporum* f. sp. *radicis-lycopersici* (Benhamou, 1992). These alterations could

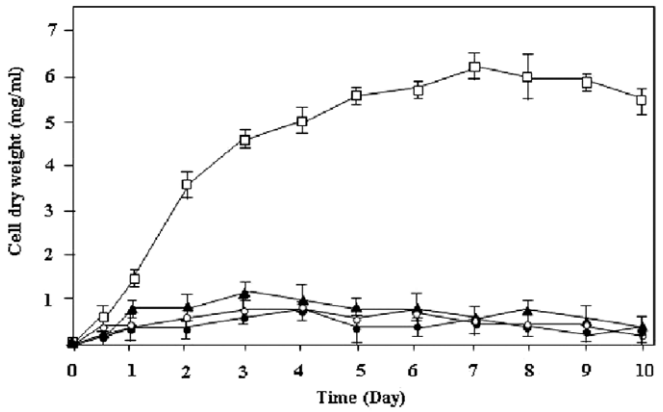

Fig. 2. Effect of chitosan on the submerged growth of *F. solani* f. sp. *glycines F. solani* f. sp. *glycines* was cultivated in PDB amended with 0 (□), 1 (▲), 3 (○) and 5 (●) mg/ml of chitosan to give final volume of 1×10^4 spores/ml and incubated at 30 °C with continuous shaking at 150 rpm. The fungal growth was monitored by dry-weight determination at 0, 12-h, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10-day of incubation period. Growth was expressed as mg of cell dry weight per ml of cell sample. The values presented are the mean and standard deviation of three independent experiments.

be related with damages in the cell membrane structural integrity due to chitosan presence, leading to the release of some macromolecules caused by an increment of membrane permeability (Stössel and Leuba, 1984).

3.2. Preventive application of chitosan on SDS symptoms expression in soybeans

The visible foliar symptoms of soybean SDS appeared only one day after fungal inoculation in chitosan-untreated leaves (T_2). A number of small brown blotches developed on leaves and rapidly became necrotic within three days after fungal inoculation. Some necrotic blotches became larger and changed to pale brown. Then, the symptoms developed daily with the increase in dead tissue until the leaves turned to yellow and finally dropped off, leaving the petioles attached to the stem. No significant retardation of SDS development was observed at 1 mg/ml of chitosan (T_4) and even 1 mg/ml of benomyl-treated leaves (T_3). Their foliar symptoms still appeared similar to that of chitosan-untreated leaves. The third day after inoculation, the foliar symptoms obviously appeared in 5 mg/ml of chitosan-treated leaves (T_6). Although T_6 showed a slightly retardant effect on the expression of SDS symptom, the number of necrotic blotches was greater than that of 3 mg/ml of chitosan-treated leaves (T_5).

The foliar symptoms on T_5 were clearly visible five days after inoculation. Furthermore, the number of necrotic blotches formed on 3 mg/ml of chitosan-treated leaves was reduced relative to chitosan-untreated leaves. The symptom appearance also increased slightly with time; however, the symptom severity was less than that of chitosan-untreated leaves. The results clearly indicated that an effective dose of chitosan at 3 mg/ml could retard SDS symptom expression on soybean leaves over three days after fungal inoculation.

In a fungal-plant interaction, chitosan could activate the defense response mechanisms in plant cells and completely inhibit all RNA synthesis of some fungi and finally reduce cell viability as well as suppress the fungal growth (Hadwiger et al., 1986). Chitosan might enter the plant cells through wounds on the leaf surface (Sathiyabama and Balasubramanian, 1998). Chitosan in plant cells could be localized in the nucleus of plant leaves and actually interact with the cellular DNA leading to biochemical reactions in the plant cells (Hadwiger et al., 1981; Hadwiger et al., 1986). Thus, chitosan could induce resistance in pea against *F. solani* f. sp. *pisi* by accumulating defense response proteins (Kendra et al., 1989). Additionally, Sathiyabama and Balasubramanian (1998) found that chitosan at 1 mg/ml could reduce uredospores of *P. arachidis*. However, chitosan could not absolutely protect the soybean from SDS because the foliar symptoms still appeared later. This was possibly due to either the severity of *F. solani* f. sp. *glycines* invasion or a reduction of the defense response components in soybeans.

Table 1
Effects of chitosan on the growth of soybean plants

Treatment[A]	Means ± SD		
	Root length[B] (cm)	Stem height[B] (cm)	Dry weight[C](g)
T_1	25.0 ± 2.2	64.4 ± 7.5	0.992 ± 0.109[d]
T_2	22.9 ± 4.7	72.4 ± 11.4	0.442 ± 0.082[ab]
T_3	25.6 ± 6.2	77.8 ± 16.3	0.528 ± 0.084[bc]
T_4	24.0 ± 2.7	71.6 ± 16.3	0.498 ± 0.080[ab]
T_5	22.6 ± 4.2	76.7 ± 18.3	0.634 ± 0.087[c]
T_6	21.3 ± 5.1	72.0 ± 16.4	0.446 ± 0.083[ab]

[A] The in vivo experiment was divided into 6 treatments.
T_1 = Negative control (without *F. solani* f. sp. *glycines*)
T_2 = Positive control (inoculated with *F. solani* f. sp. *glycines*)
T_3 = Treated with 1 mg/ml of benomyl and *F. solani* f. sp. *glycines*
T_4 = Treated with 1 mg/ml of chitosan and *F. solani* f. sp. *glycines*
T_5 = Treated with 3 mg/ml of chitosan and *F. solani* f. sp. *glycines*
T_6 = Treated with 5 mg/ml of chitosan and *F. solani* f. sp. *glycines*.
[B] Means were not significantly different ($p < 0.05$) according to the analysis of variance.
[C] Means followed by the same letter within column were not significantly different ($p < 0.05$) according to Duncan's multiple range test.

3.3. Effect of chitosan on the growth of soybean plant

After 14 days of fungal inoculation, soybean plants of all treatments were harvested for growth determination of root length, stem height and dry weight. No significant differences ($p < 0.05$) in means of root length and stem height of soybean plants were found in all treatments (Table 1). In contrast, the significant difference ($p < 0.05$) in mean was found on dry weight of soybean plants. There was maximum increase per gram of dry weight in 3 mg/ml of chitosan-treated leaves (0.634 g) (T_5) as compared to chitosan-untreated leaves (T_2). As a result, chitosan at 3 mg/ml could provide the higher soybean growth than other chitosan-treated leaves and 1 mg/ml of benomyl-treated leaves (T_3) due to its role in protecting soybeans against SDS symptom development.

3.4. The level of chitinase activity in infected soybean leaves

To investigate the level of chitinase activity in infected soybean leaves, chitosan-untreated and 3 mg/ml of chitosan-treated leaves were collected for chitinase activity assay after fungal inoculation. The level of chitinase activity in 3 mg/ml of chitosan-treated leaves was drastically increased from 12.4 to 17.9 U/mg protein after three days of fungal inoculation (Fig. 3). The low level of chitinase activity was probably responsible for the earlier observed SDS symptom expression in chitosan-untreated leaves. In addition to chitosan-treated leaves, there were almost no macroscopic foliar symptoms of SDS on leaves during the high level of chitinase activity period. Then, chitinase activity in chitosan-treated and chitosan-untreated leaves sharply decreased from 6 to 14 days after fungal inoculation. The symptoms seemed to gradually appear and be associated with the decrease of chitinase activity level after 5 days of

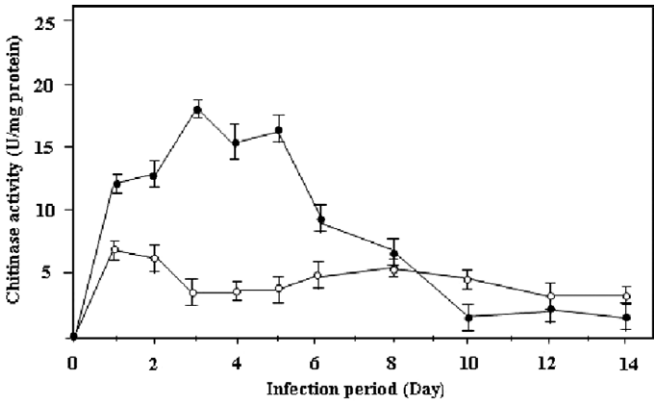

Fig. 3. The level of chitinase activity in fungal infected soybean leaves Both chitosan-untreated and 3.0 mg/ml of chitosan-treated soybean leaves were inoculated with 100 μl of fungal spore suspension (1×10^3 spores/ml) on the abaxial surface. Soybean leaves were harvested daily until 14 days for chitinase activity assay. The extraction of intercellular fluid from chitosan-untreated leaves (○) and 3 mg/ml of chitosan-treated leaves (●) and chitinase activity assay were performed as previously describes (Shimosaka et al., 1993). Values presented are means and standard deviation of triplicate experiments.

fungal inoculation. The results could imply that the application of chitosan might sensitize the soybean plant responses in protecting themselves from the phytopathogenic fungal invasion by elaboration of chitinase activity.

Higher plants have the ability to initiate various defense mechanisms, when they are infected either by phytopathogens or after treatment with biotic and abiotic elicitors. Chitosan had been shown to act as a potent oligosaccharide elicitor which can induce defense response mechanisms in several plants, mostly dicots. Chitinase, a hydrolytic enzyme, was one of the pathogenesis-related proteins which might be implicated in plant defense system against pathogenic fungi (Shibuya and Minami, 2001). Chitinase and β-1,3-glucanase are defense response proteins that are produced by *F. solani* f. sp. *pisi* when cells were induced with chitosan (Kendra et al., 1989). Furthermore, chitinase and β-1,3-glucanase are effective in inhibiting the *in vitro* growth of several fungi (Mauch et al., 1988). Celery, *Apium graveolens*, treated with chitosan showed an increase in chitinase activity of 20-fold compared to that of chitosan-untreated plants and exhibited a delay in symptom expression caused by *F. oxysporum* (Krebs and Grumet, 1993). Similarly, chitosan stimulated chitinase production in cucumber plant and protected this plant from root rot disease caused by *Pythium aphanidermatum* (Ghauoth et al., 1994).

The evidence suggested that chitosan could induce active defense responses just as chitinase enzyme in soybean induces the resistance against *F. solani* f. sp. *glycines*. Chitosan, a potent elicitor, could induce resistance components as endogenous salicyclic acid, intercellular chitinase and β-1,3-glucanase activity in *Arachis hypogaea* against leaf rust caused by *P. arachidis* (Sathiyabama and Balasubramanian, 1998).

4. Conclusions

Chitosan played an important role in the growth suppression of *F. solani* f. sp. *glycines* and the protection of soybean plant against SDS. The radial and submerged growth of *F. solani* f. sp. *glycines* were reduced by chitosan concentration up to 1 mg/ml. The effective dose of chitosan (3 mg/ml) although could retard the SDS symptom expression in soybean leaves over three days after fungal inoculation, it could not absolutely protect the soybean from disease incidence however; the foliar symptoms still appeared later. Chitinase activity in soybean could increase the resistance in soybean against *F. solani* f. sp. *glycines* because this enzyme was able to degrade the fungal cell walls inhibiting the fungal growth and symptom expression.

Acknowledgements

The authors thank the Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand for providing a strain of *F. solani* f. sp. *glycines* and Dr. Edward A. Grand for a critical reading of the manuscript. This research work was partially supported by the grant from the Post-Graduate Education, Training and Research Program in Environmental Science, Technology and Management under Higher Education Development Project of the Commission on Higher Education, Ministry of Education, Thailand.

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