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Postharvest Chitosan Treatment Induces Resistance in Potato Against *Fusarium sulphureum*

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Abstract

The effects of chitosan treatment and inoculation on dry rot in tubers and slices of potato were studied. The results showed that chitosan treatment significantly reduced the lesion diameter of potato inoculated with *Fusarium sulphureum*. The treatment at 0.25% showed the best effect. Chitosan at 0.25% increased the activities of peroxidase and polyphenoloxidase, and the contents of flavonoid compounds and lignin in tissues. Increased activities of β-1,3-glucanase, and phenylalanine ammonialyase were observed, but there were no significant differences between the treated and the control. These findings suggested that the effects of chitosan could be associated with the induced resistance against *Fusarium* dry rot in potato.

Key words: chitosan, potato, decay, induced resistance

INTRODUCTION

Fusarium dry rot is one of the most important diseases of potato, affecting tubers in storage and seed pieces after planting. *Fusarium sulphureum* is found to be one of the major pathogens that can cause dry rot in the Gansu Province, the largest potato production area of China (He *et al*. 2004). It is reported that 27.59% of the tubers can be infected by various pathogens during the storage, and dry rot occupies 88.5% of the infection (Wei *et al*. 2006). Postharvest treatment with fungicide such as thiabendazole (Greyerbiehl 2002) has been successful in controlling *Fusarium* dry rot of potato. However, because of problems related to fungicide toxicity, new strategies for control have been proposed (Tian and Fan 2000); because of development of fungicide resistance by pathogens, potential harmful effects on the environment and human health, as well as the necessity to reduce losses, the use of fungicides is reduced. Chitosan, deacetylated chitin, is a kind of macromolecule cation polysaccharide. The chemical can significantly reduce disease incidence in apples, kiwis, and pears (Bautista-Banos *et al*. 2006). Chitosan not only inhibits the growth of the pathogens, but also induces morphological changes, structural alterations, and molecular disorganization of the fungal cells (Ait *et al*. 2004). The chemical also increases the activities of the peroxidase (POD), chitinase (CHT), and β-1,3-glucanase (GLU) in tomatoes, strawberries, and raspberries (Liu *et al*. 2007; Zhang and Quantick 1998). Besides, it has been reported that the activities of lipoxygenase (LOX) and phenylalanine ammonialyase (PAL), and the content of lignin have been elicited in

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wheat leaves treated with chitosan (Vander *et al*. 1998). The objective of this study is to determine the induced effect of chitosan treatment on potato tuber resistance to dry rot caused by *Fusarium sulphureum* and to investigate its possible mechanism.

MATERIALS AND METHODS

Plant materials

Potato (*cv*. Atlantic) was harvested from Zhongchuan Farm, Yongdeng County in Gansu Province. The tubers based on size and the absence of physical injuries or disease infection were packed in net bags (15 kg per bag), transported to the laboratory, and stored at (16±2)°C. Before treatment, the tubers were surface-disinfected with 2% sodium hypochlorite for 20 min, and then rinsed with tap water and air-dried.

Pathogen

Fusarium sulphureum Schlechlendah, causal microorganism of dry rot of potato tuber, was provided by the Institute of Plant Protection, Gansu Academy of Agricultural Sciences. The spores of the pathogens were removed from the two-week-old PDA, and suspended in 5 mL of sterile distilled water containing 0.05% (v/v) Tween 80. The suspensions were filtered through four layers of sterile cheese cloth to remove the adhering mycelia. The desired spore concentration was adjusted with the aid of a hemocytometer, prior to use.

Chitosan

Chitosan, edible level with 90.2%, de-acetyl degree and granularity with 80 mesh, was obtained from the Ocean Bioengineering Limited Company, Haidebai, Jinan City, China.

Effect of chitosan on lesion diameters of tuber inoculated with *F. sulphureum*

Following the method of Bi *et al*. (2006), with some modifications, the tuber was dipped in 0.25, 0.5, and 1% chitosan (pH 5.6±0.2), which was predissolved in 0.5% lactic acid for about 10 min, and then dried in air for 2 h. A uniform wound (3 mm deep and 3 mm wide) was made at the equator of each tuber using a sterile dissecting needle after 72 h of treatment, and then a 20-μL conidial suspension of *F. sulphureum* was inoculated into each wound after 1 h of wounding. Inoculated tubers were incubated in the boxes, covered with plastic film at room temperature (25±2)°C. Lesion diameters were evaluated after 15 days of inoculation. Each treatment was applied to three replicates of 10 tubers.

Effect of chitosan on lesion diameters of slices inoculated with *F. sulphureum*

According to the method of Ray and Hammerschmidt (1998), slices (20 mm in diameter and about 5 mm in thickness) were made with a sterile knife, rinsed in sterile water, sterilized with 75% ethanol and flame, placed on wet sterile filter paper, and incubated for 4 h in darkness. A 200-μL chitosan solution at 0.25, 0.5, or 1% was scrawled on discs. The control was treated with water. The pathogen was inoculated after 24, 48, 72, and 96 h of treatment. The inoculums, 4 mm in diameter, were obtained from the 7-day-old *F. sulphureum* grown on PDA, and then placed hyphae side down on slices. The slices were incubated for 3 days at 23-25°C in the dark. The lesion diameter was recorded. Each treatment was applied to three replicates of five slices.

Assay of defense enzymes

Tubers were removed after 1, 2, 3, 4, and 5 days of treatment with 0.25% chitosan. Approximately 3 g of tissue samples were taken from 1-2 mm below the treated side with a stainless steel cork borer. Tissue samples inoculated with *F. sulphureum* were taken from 3-4 mm below the treated side with a stainless steel cork borer, and were removed at 3, 4, and 5 days, respectively. Each sample was packed and frozen in liquid nitrogen, and finally kept at -80°C prior to crude enzyme extraction.

Extraction of the crude enzyme

All extraction procedures were conducted at 4°C. Samples (3 g) were ground with 1% polyvinyl polypyrrolidone (PVPP), with different buffers to extract different enzymes: 3 mL sodium phosphate buffer (50 mM, pH 7.5) for POD, 3 mL sodium phosphate buffer (0.1 M, pH 6.4) for PPO, 3 mL sodium acetate buffer (50 mM, pH 5.0) for β-1,3-glucanase, and 3 mL of 0.05 M sodium borate buffer (pH 8.8, containing 5 mM β-mercaptoethanol) for PAL. The samples were centrifuged at 15000×g at 4°C for 30 min. The supernatants were used as crude enzyme sources to assay enzymatic activities.

Determination of enzyme activity

POD The POD activity was determined according to the methods of Venisse *et al*. (2001) with some modifications. Guaiacol was used as the substrate. Oxidation of guaiacol to tetraguaiacol was monitored spectrophotometrically at 470 nm for 2 min at 30°C. The enzyme activity was expressed in unit (U) per microgram protein. The enzyme unit was defined as ΔA470 of 0.01 per minute.

PPO The PPO activity was determined according to the method of Jiang *et al*. (2002), by adding 0.5 mL of enzyme preparation to 3 mL of 500 mM catechol (100 mM sodium phosphate, pH 6.4) as a substrate. An absorbance was measured at 420 nm. The activity was expressed in the unit (U) per microgram protein. One unit was defined as ΔA420 of 0.01 per minute.

β-1,3-Glucanase It was assayed by measuring the amount of reducing sugar released from the substrate using the method of Ippolito *et al*. (2000) with some modifications. Enzyme preparation (0.25 mL) was incubated with 250 μL of 0.5% laminarin (w/v) for 60 min at 37°C and 50 μL reactive mixture was then taken out. The reaction mixture was diluted 1:4 with the sterile distilled water. For the control, the same mixture was similarly diluted at zero incubation time. The reaction was stopped by adding 250 μL of 3,5-dinitrosalicylate and boiling for 5 min on a water bath. The solution was diluted with 4 mL of distilled water, and the amount of reducing sugars was measured spectrophotometrically at 630 nm using a UV-160 spectrophotometer (Shimadzu, Japan). Specific activity was expressed as the formation of 1 μmol glucose equivalent per hour per milligram protein.

PAL The PAL activity was assayed referring to the method of Assis *et al*. (2001), with some modifications. 1 mL of enzyme extract was incubated with 2 mL of borate buffer (50 mM, pH 8.8) and 1 mL of L-phenylalanine (20 mM) for 60 min at 37°C. The reaction was stopped with 1 mL of 1 M HCl. PAL activity was determined by the production of cinnamate, which was measured at 290 nm absorbance. The control mixture was stopped by adding 1 mL of 1 M HCl immediately after mixing the crude enzyme preparation with L-phenylalanine. Specific enzyme activity was defined as nanomoles cinnamic acid per hour per milligram protein.

Flavonoid compounds Flavonoid compounds were measured according to the methods of Pirie and Mullins (1976). 1 g of frozen tissue was homogenized with 5 mL of 1% HCl-methanol ice-cold solution and extracted for 2 h and then centrifuged at 4°C for 30 min at 14000 ×g. The supernatant was collected and absorbance measured at 325 nm, with flavonoid compounds expressed as OD_{325} g^{-1} FW.

Lignin Lignin was assayed according to the method of Morrison (1972) with some modifications. Frozen tissue of 0.5 g was homogenized with 5 mL of 95% ice-cold ethanol solution and then centrifuged at 4°C for 30 min at 14000×g. The collected pellet was washed with 95% ethanol thrice, and then washed thrice with ethanol:hexane = 1:2 (v/v). Pellets were collected for drying (24 h at 60°C) and transferred to tubes, and then 1 mL of 25% bromized acetyl-acetic acid was added. The tubes were incubated in a water bath at 70°C for 30 min, and then 1 mL of 2 mol L^{-1} NaOH was added to stop the reaction. 2 mL of ice acetic acid and 0.1 mL of 7.5 M hydroxylamine hydrochloric acid were added to each tube and centrifuged. 0.5 mL of supernatant solution was removed. The absorbance of the supernatant was measured at 280 nm with a spectrophotometer. Lignin compounds were expressed as OD_{280} g^{-1} FW.

Protein content The protein content was determined according to the method of Bradford (1976) using bovine serum albumin as a standard.

Statistical analysis

All statistical analyses were performed using SPSS. To test the effect of the treatment, the data were analyzed by one-way analysis of variance (ANOVA). Mean separations were performed using the least significant difference method. The data were also expressed as the means (± SE).

RESULTS

Effect of chitosan on lesion diameters of tuber and slices inoculated with *F. sulphureum*

Chitosan treatment at 0.25% significantly reduced the lesion diameter of a tuber inoculated with *F. sulphureum* (Fig.1-A). However, no difference was found between high concentration treatment (at 1%) and the control.

Lesion diameters of slices inoculated with *F. sulphureum* varied with chitosan concentration and incubation time (Fig.1-B). Chitosan at 0.25% showed an effective reduction of lesion diameters at 24, 48, and 72 h of inoculation time. The treatment at 0.5 and 1% reduced lesion diameters at 48 and 72 h, and 24 and 72 h of inoculation time, respectively. The smallest lesion diameters after treatments were observed at 72 h of inoculation time. No significant difference was found between the treatments and the control when the pathogen was inoculated 96 h after treatment.

Effect of chitosan treatment and inoculation on POD and PPO activities

Chitosan at 0.25% enhanced POD and PPO activities in slices (Fig.2). No significant difference of POD was observed during the 2 days of treatment. The activity of POD increased gradually after 2 days of treatment. The maximum POD activity was found 5 days after treatment, with the activity 3.8 times higher in the slices treated with chitosan than in the control (Fig.2-A). However, the activity of PPO kept mostly stable after treatment (Fig.2-B). Chitosan treatment at 0.25% caused a more progressive and significant increase in POD activity on tissues challenged by *F. sulphureum*. The maximum POD activity was found 5 days after inoculation, with the activity 1.5 times higher in the slices treated with chitosan than in the control (Fig.2-A). A little increase of PPO activity was observed in the treated slices challenged by *F. sulphureum* (Fig.2-B).

Effect of chitosan treatment and inoculation on GLU and PAL activities

Chitosan at 0.25% enhanced GLU and PAL activities in the slices (Fig.3). The activity of GLU increased gradually after a one-day treatment (Fig.3-A). However, the activity of PAL kept stable after treatment (Fig.3-B). Chitosan treatment caused a more progressive and significant increase in GLU and PAL activities on tissues challenged by *F. sulphureum*. The maximum GLU and PAL activities were found 4 days after inoculation, with the activities 1.3 and 1.5 times higher in treated slices than in the control, respectively (Fig.3).

Effect of chitosan treatment and inoculation on the contents of flavonoid compounds and lignin in tissue

Chitosan at 0.25% increased the contents of flavonoids

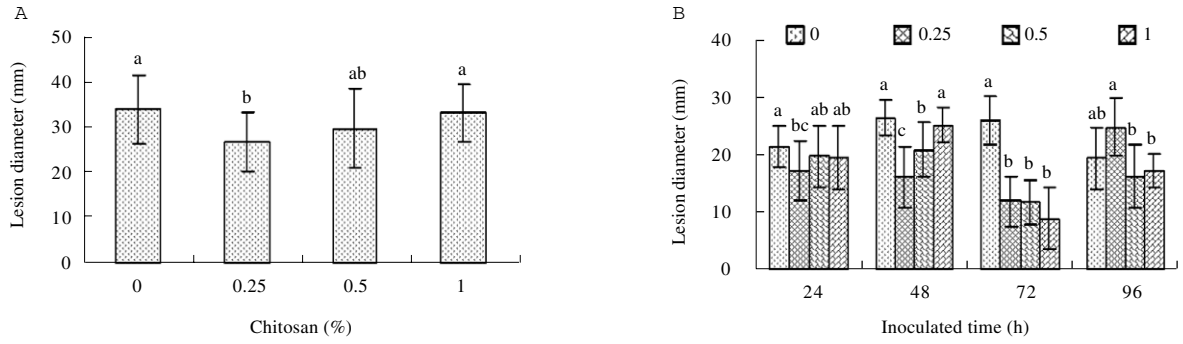

Fig. 1 Effect of chitosan on lesion diameters of tuber (A) and slices (B) inoculated with *F. sulphureum*. Different letters show significant levels at 5%. Vertical bars represent the standard error.

compounds and lignin in slices (Fig.4). The content of flavonoids enhanced gradually after 2 days of treatment (Fig.4-A). However, the content of lignin increased continually during the period of experiment (Fig. 4-B). The maximum contents of flavonoids and lignin were found 5 days after treatment, with the contents 1.2 times and 1.3 times higher in treated slices than in the control, respectively. Chitosan caused a progressive and significant increase in lignin content in tissues challenged by *F. sulphureum*. The maximum content of lignin was found 5 days after inoculation with the activity 1.1 times higher in treated slices than in the control (Fig.4-A). A little increase of flavonoids was observed in treated slices challenged by *F. sulphureum* (Fig.4-B).

DISCUSSION

Postharvest chitosan treatment provided a significant suppression of *Fusarium* dry rot in tubers and slices. This suppression was clearly correlated with the activation of defense enzymes and accumulation of antifungal compounds. These data provided the evidence that this chemical induced the resistance in potato. A similar result had been obtained in potato by Greyerbiehl (2002). There were also evidences that chitosan could significantly reduce postharvest diseases of strawberries, pimientos, cucumbers, pears, peaches, and litchis (Yourman and Jeffers 1999).

Different induced effects were found at different times, after treatment with the same concentration of chitosan. The result suggested that defense response induced by chitosan was associated with incubation time, which may be needed for host reception, signal transduction, and related gene repression. It was also found that the high concentration treatments have worse induced effect in this experiment. However, an opposite result was reported in tomato fruits by Liu *et al*. (2007), who found that chitosan at 0.5 and 1% could significantly decrease the gray mould and blue mould caused by *Botrytis cinerea* and *Penicillium expansum* in tomato fruit ($P<0.05$) and the controlled effects of chitosan on both diseases were enhanced when the concentration of chitosan was increased from 0.5 to 1%. The phenomenon was that the high chitosan con-

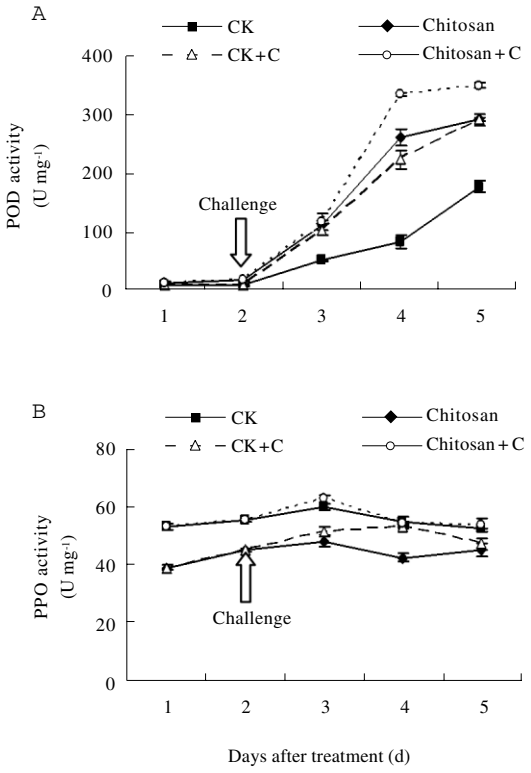

Fig. 2 Effect of chitosan treatment and inoculation on POD (A) and PPO (B) activities. Bar shows mean±SE. The same as below.

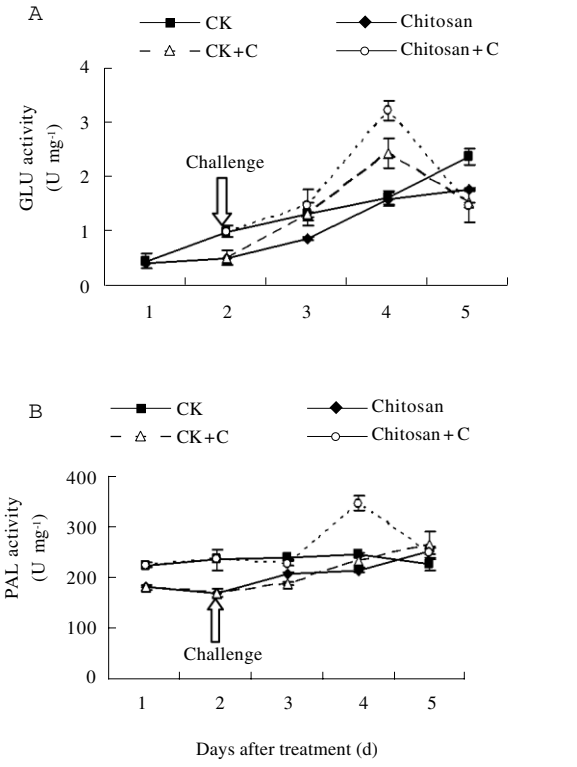

Fig. 3 Effect of chitosan treatment and inoculation on GLU (A) and PAL (B) activities.

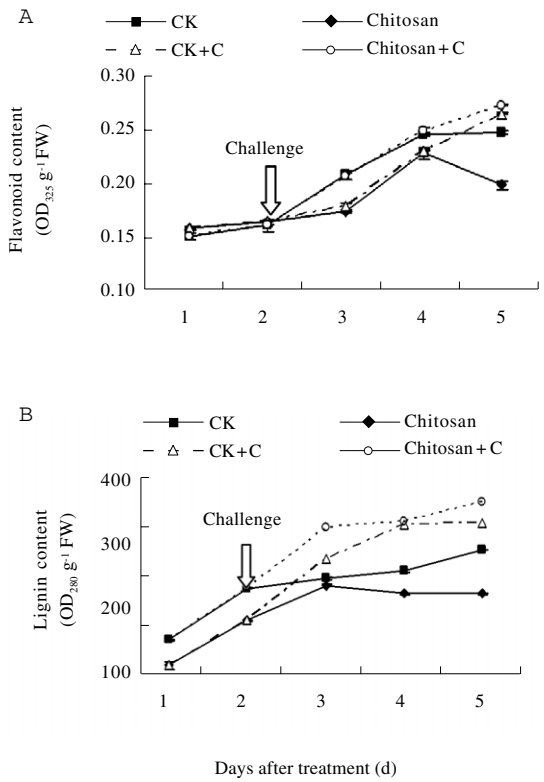

Fig. 4 Effect of chitosan treatment and inoculation on content of flavonoids (A) and lignin (B).

centration could stimulate the sensitivity of potato tissue to pathogen.

Induced defense reactions in plants were highly correlated with enzymatic responses. The enzymes participated in the first defensive line and inhibited the incidence of pathogens.

It has been reported that chitosan induces the occurrence of defense marks. In this experiment, chitosan treatment and inoculation has increased the activities of POD and PPO in potato tissue. This supports the reports of chitosan-enhanced activities of POD and PPO in tomato fruit (Liu *et al*. 2007). POD participates into the cell wall reinforcement and is involved in the final steps of lignin biosynthesis and in the cross-linking of cell-wall protein (Graham and Graham 1991). When inoculated with the pathogen, the structural barriers strengthen or limit the activities of the pathogen. PPO is involved in the oxidation of polyphenols into quinines, which can restrict the growth of the plant pathogen. It has also been found that activities of GLU and PAL in inoculated tuber tissue treated with chitosan have been increased. Several studies have demonstrated that chitosan treatment induces activities of GLU and PAL, and the formulation of lignin (Smith 1996; Greyerbiehl 2002). The increased activity of GLU has been thought to be a common phenomenon in the control of plant disease in resistance systems (Venisse *et al*. 2002). No significant induction of GLU activity by chitosan can be because of other factors such as plant variety or the method of the experiment. PAL is the first enzyme of the phenylpropanoid pathway and is involved in the biosyntheses of phenolics, phytoalexins, and lignin (Pellegrini *et al*. 1994). Therefore, increased PAL activity can increase disease resistance of plants. In the present study, the levels of flavonoid compounds and lignin in the tuber tissue with chitosan treatment increase, suggesting that flavonoid compounds, as a type of phytoalexins, can directly kill the fungal pathogen (Shadle *et al*. 2003). In other cases lignin deposition may actually contain the pathogen by the lignification of the pathogen cell wall surrounding the pathogen or by the deposition of lignin around the penetrating pathogen (Greyerbiehl 2002).

In conclusion, postharvest treatment with chitosan has proved effective for controlling dry rot of tubers and slices inoculated with *F. sulphureum*. This is, to the authors' knowledge, the first report linking the beneficial role of chitosan against dry rot in potato. This suggests that chitosan is promising as a natural fungicide, to partially substitute for the utilization of synthetic fungicides in fruits and vegetables. However, further study is needed on the mechanism of chitosan against fungal pathogens, as well as, appropriate application of postharvest disease control.

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