Notes

Effect of Chitosan Solution on the Inhibition of *Pseudomonas fluorescens* Causing Bacterial Head Rot of Broccoli

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The *in vitro* antibacterial properties of two kinds of chitosan solutions and their effect in protection of broccoli from bacterial head rot disease were evaluated. Results showed that the two kinds of chitosan solution at different concentrations exhibited strong antibacterial activity against *Pseudomonas fluorescens*. However, the antibacterial activity of chitosan A solution increased with the increase of chitosan concentration up to 0.10 mg/ml while the antibacterial activity of chitosan B solution increased with the increase of chitosan concentration up to 0.05 mg/ml. In addition, the antibacterial activity of chitosan A and chitosan B solution of 0.10 mg/ml increased with the incubation time within 12 h and 24 h, respectively. The disease incidence and the lesion diameter of broccoli inoculated with *P. fluorescens* were significantly reduced when plants were either pre-treated or post-treated with six different combinations of chitosan solutions. Overall, the results indicated that the two kinds of chitosan solutions had a potential in controlling bacterial head rot of broccoli.

Keywords : antibacterial activity, bacterial head rot, broccoli, chitosan, *Pseudomonas fluorescens*

Broccoli (*Brassica oleracea* L. var. *italica*) is one of the many important economic crops grown in the world and has been marketed as a nutritious dietary supplement due to its high fiber, vitamin C, vitamin A and mineral content including calcium and iron (Vasanthi et al., 2009; Wang et al., 2008). In China, planted acreage of broccoli has been increasing in recent years and, in 2007, over 20,000 hectares were grown, of which around 60 percent were mainly

exported to Japan, Korea, Malaysia and Canada (Su, 2008). However, bacterial head rot has been recently observed on broccoli in China and the causal bacterium was attributed to *Pseudomonas fluorescens* (Li et al., 2009a). Broccoli production in China has been seriously hampered by the disease, which reduced the quality and marketability of broccoli. In severe cases, the incidence of disease was between 65% and 81% in some fields (Li et al., 2009a). In addition, this disease has been reported in many other countries, which lead to crops losses of between 30% and 100% in some fields (Cui and Harling, 2006; Darling et al., 2000; Hildebrand, 1989). However, there are very few bactericidal chemicals available for controlling this disease and most of them are copper formulations with limited effectiveness (Pajot and Silué, 2005). Therefore, new measures should be developed to prevent the infection and further spread of the disease.

Some biochemicals in particular chitosan have been attempted to control plant disease (Bell et al., 1998; Ben-Shalom and Fallik, 2003). Treating tomato plants with chitosan solution reduced mycelial growth, sporangial production, and release of zoospores and germination of cysts of *Phytophthora infestans* which resulted in significant disease protection (Atia et al., 2005). Furthermore, chitosan seed treatment could reduce *Colletotrichum* sp. infection and improve performance of chili seedling (Photchanachai et al., 2006). In addition, Li et al. (2008b) found that chitosan solution has strong *in vitro* antibacterial activity against *Xanthomonas* strains that cause leaf spot disease of *Euphorbia pulcherrima*. Interestingly, the effects of chitosan as an antimicrobial preservative for fruit, vegetables and food have also been evaluated (Devlieghere et al., 2004; Fujimoto et al., 2006). However, few investigations have been generated about inhibitory ability of chitosan on bacterial diseases in broccoli. The objective of this research was to evaluate the antibacterial activity of two kinds of chitosan solutions against the *P. fluorescens* pathogen that causing head rot disease of broccoli under different environments.

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Preparation of chitosan stock. Chitosan (degree of N-deacetylation no less than 85%, practical grade, from crab shells) and chitosan (degree of N-deacetylation 75%, from crab shells) were obtained from Sigma-Aldrich (USA) and named as chitosan A and chitosan B, respectively. Stock solution of chitosan A or chitosan B (5 mg/ml) was prepared in 1% acetic acid with pH being adjusted to 6.0 with NaOH (Li et al., 2008b). After stirring (160 rpm) for 24 h at room temperature, the stock solution was autoclaved at 121°C for 20 min. Sterile deionized water of pH 6.0 was used as a control.

Cultivation of bacteria. The virulent strain PFB-01 of *P. fluorescens* was isolated from diseased broccoli in Zhejiang province and deposited in the culture collection of the Institute of Biotechnology, Zhejiang University, China. The bacterial strain was cultured for 48 h on nutrient agar medium (Fang et al., 2009; Li et al., 2008a) at 28°C. After incubation, bacterial suspension was prepared in sterilized water, and the initial concentration of bacteria was adjusted to approximately 10^9 colony forming units (CFU)/ml.

Counting surviving cells. Bacterial suspensions were ten-fold serially diluted and 10 µl samples were inoculated on nutrient agar medium in hexaplicate for each dilution and were incubated for 48 h at 28°C. After incubation, the surviving cells on the agar were counted based on the colony forming units and then the mean value of the cells at the lowest dilution was calculated. Each experiment was carried out in duplicate and was replicated twice.

Statistical analysis. The software STATGRAPHICS Plus, version 4.0 (Copyright Manugistics Inc., Rockville, Md., USA) was used to perform the statistical analysis. Levels of significance ($p < 0.05$) of main treatments and their interactions were calculated by analysis of variance after testing for normality and variance homogeneity.

Effect of chitosan concentration on the antibacterial activity. Chitosan solutions of 5 ml in volume were prepared by adding chitosan stock to sterile deionized water to give a final chitosan concentration of 0.01, 0.05 and 0.10 mg/ml. Bacterial solution was added to 5 ml of chitosan solution to give a final bacterial concentration of 10^8 CFU/ml and then the mixture was incubated at 28°C on a rotary shaker (Hualida Company, China) at 160 rpm. In the control treatment chitosan stock was replaced with sterile deionized water of pH 6.0 in order to obtain the same pH. Two hours later, samples were collected from each cell suspension and bacterial counting was followed as indicated above. The surviving cell numbers in chitosan A solution of 0.01 mg/ml decreased 0.73 Log CFU/ml while the surviving

Fig. 1. Effect of chitosan concentration on the antibacterial activity of *Pseudomonas fluorescens* strain PFB-01. (A) Chitosan A; (B) Chitosan B. Initial concentration of bacteria is approximately 10^8 CFU/ml. Columns with the same letters are not significantly different ($p < 0.05$). Error bars represent the standard error of the mean ($n = 6$). Data are from a representative experiment repeated twice with similar results.

cell numbers in chitosan A solution of 0.10 mg/ml decreased 4.98 Log CFU/ml compared to the control (Fig. 1A). However, the surviving cell numbers in chitosan B solution of 0.01 mg/ml decreased 1.90 Log CFU/ml while the surviving cell numbers in chitosan B solution of 0.05 mg/ml decreased 3.42 Log CFU/ml compared to the control (Fig. 1B). Concentrations higher than 0.05 mg/ml were not significantly different (Fig. 1B).

Both chitosan A and chitosan B at three different concentrations showed effective antibacterial activity against *P. fluorescens* strain PFB-01 compared to the control after 2 h of incubation (Fig. 1A, 1B). Although *P. fluorescens* have long been identified as biological control agents (Cho et al., 2009; Choi et al., 2006; Ipper et al., 2005; Kong et al., 2009), several studies revealed that *P. fluorescens* has wide range under natural field infection (Cui and Harling, 2006; Lee et al., 2009; Li et al., 2009b; Saygili et al., 2004). Interestingly, this study indicated that the two kinds of chitosan solutions at different concentrations had strong

antibacterial activity against the pathogenic *P. fluorescens* compared to the control. Therefore, application of chitosan seems to be a promising method to inhibit the strains of *P. fluorescens* from different host plants. In addition, in agreement with the result of Li et al. (2008b), this result indicated that the antibacterial activity of chitosan A and chitosan B against *P. fluorescens* was influenced by its concentration in the solution. However, the antibacterial activity of chitosan A solution increased with the increase of chitosan concentration up to 0.10 mg/ml while the antibacterial activity of chitosan B solution increased with the increase of chitosan concentration up to 0.05 mg/ml. This difference may be ascribed to the composition of chitosan, which is consistent with the result of Seyfarth et al. (2008), who found that the antifungal activity of chitosan depend on degree of deacetylation and molecular weight.

Effect of incubation time on the antibacterial activity.

Chitosan solutions of 5 ml in volume were prepared by adding 100 µl chitosan stock to 4.90 ml sterile deionized water to give a final chitosan concentration of 0.10 mg/ml. *P. fluorescens* strain PFB-01 was inoculated into chitosan solution as indicated above. In the control treatment, chitosan stock was replaced with sterile deionized water of pH 6.0 in order to obtain the same pH. Antibacterial activity of chitosan solution on the growth of *P. fluorescens* was determined after 0, 2, 4, 6, 12 and 24 h of incubation, respectively. This result indicated that in the absence of chitosan, the surviving cell numbers in sterile deionized water decreased 0.20 Log CFU/ml after 2 h of incubation compared to the starting value. With the increase in incubation time, the surviving cell numbers remain stable (Table 1). In the presence of chitosan A or chitosan B, the

surviving cell numbers were significantly decreased compared to the starting value (Table 1). After 2 h of incubation, the surviving cell numbers in the chitosan A solution decreased 4.43 Log CFU/ml while the surviving cell numbers in the chitosan B solution decreased 2.60 Log CFU/ml compared to the control (Table 1). However, the surviving cell numbers in the chitosan A solution decreased 5.55 Log CFU/ml while the surviving cell numbers in the chitosan B solution decreased 3.29 Log CFU/ml compared to the control after 12 h of incubation (Table 1). Result from this study indicated that the antibacterial activity of both chitosan A and chitosan B solution were affected by the incubation time, which is consistent with the result of Li et al. (2008b), who found that a certain incubation time is required for the chitosan solution to inhibit the bacterial growth. However, the antibacterial activity of chitosan A solution of 0.10 mg/ml increased with the incubation time within 12 h while the antibacterial activity of chitosan B solution of 0.10 mg/ml increased with the incubation time within 24 h.

Effect of chitosan pre-treatment and post-treatment on head rot disease of broccoli.

A bioassay was used to determine the ability of chitosan to suppress the incidence and severity of bacterial head rot disease of broccoli during growth and storage. Healthy heads of pre-harvested broccoli (cultivar Sijilv) were treated with either the bactericide (Zinc thiazole) or chitosan solutions before and after pathogen inoculation. Bacteria was cultured in nutrient broth and adjusted to 10^8 CFU/ml. Heads of broccoli was inoculated using a 100 µl tip dipped in a 50 µl suspension of *P. fluorescens* strain PFB-01. Six different combinations of chitosan solutions were applied in this study, which included chitosan A at 0.05 and 0.10 mg/ml, chitosan B at 0.05 and 0.10 mg/ml, chitosan A + B at 0.05 and 0.10 mg/ml. The bactericide (100 µg/ml) or six different combinations of chitosan solutions were sprayed until runoff onto the heads. Control plants were sprayed with distilled water. Each treatment had three plants. Infiltrated plants were incubated in a growth chamber at 28°C and 90% relative humidity with 16 h of light and 8 h of dark. Ten inoculation sites per plant were used for scoring of disease symptoms. One week after inoculation, the disease incidence and the average radius of the lesion were observed and scored.

The heads of broccoli inoculated with the pathogen alone resulted in 83.3% and 76.7% disease incidence in pre-treatment and post-treatment of chitosan, respectively (Table 2), while the average diameter of the lesion was 6.1 mm and 5.4 mm, respectively (Table 3). However, the disease incidence and the lesion diameter of broccoli heads inoculated with *P. fluorescens* were significantly reduced when plants were either pre-treated or post-treated with six

Table 1. Effect of incubation time on the antibacterial activity of chitosan solution against *Pseudomonas fluorescens* strain PFB-01^a

Incubation time (h)	Cell numbers (Log ₁₀ CFU/ml)		
	Control	Chitosan A ^b	Chitosan B
0	8.60 ± 0.19 b ^c	8.60 ± 0.19 d	8.60 ± 0.19 e
2	8.40 ± 0.25 ab	4.17 ± 0.29 c	6.00 ± 0.14 d
4	8.43 ± 0.28 ab	3.70 ± 0.10 b	5.70 ± 0.15 c
6	8.36 ± 0.23 ab	3.76 ± 0.21 b	5.48 ± 0.16 b
12	8.44 ± 0.14 ab	3.05 ± 0.12 a	5.31 ± 0.17 b
24	8.25 ± 0.20 a	4.27 ± 0.14 c	4.95 ± 0.13 a

^a Initial concentration of bacteria is approximately 10^8 CFU/ml.

^b Concentration of chitosan solution is 0.10 mg/ml.

^c The data are shown as means ± standard error from a representative experiment repeated twice with similar results (n = 6). Means in a column followed by the same letter are not significantly different ($p < 0.05$).

Table 2. Effect of chitosan pre-treatment and post-treatment on the disease incidence of broccoli heads inoculated with *Pseudomonas fluorescens* strain PFB-01^a

Treatments	Disease incidence (%)	
	Pre-treatment	Post-treatment
Pathogen control	83.3 g ^c	76.7 e
Chitosan A (0.05 mg/ml)	30.0 cd	43.3 bcd
Chitosan A (0.10 mg/ml)	16.7 b	33.3 b
Chitosan B (0.05 mg/ml)	50.0 ef	53.3 d
Chitosan B (0.10 mg/ml)	53.3 f	46.7 cd
Chitosan A + B (0.05 mg/ml)	23.3 bc	36.7 bc
Chitosan A + B (0.10 mg/ml)	50.0 ef	50.0 d
Bactericide (Zinc thiazole) ^b	40.0 de	36.7 bc
None	0.0 a	0.0 a

^a The inoculum concentration of bacteria is approximately 10⁸ CFU/ml.

^b The concentration of the bactericide is 100 µg/ml.

^c Data are from a representative experiment repeated twice with similar results. Means in a column followed by the same letter are not significantly different ($p < 0.05$).

Table 3. Effect of chitosan pre-treatment and post-treatment on the lesion diameter of broccoli heads inoculated with *Pseudomonas fluorescens* strain PFB-01^a

Treatments	Lesion diameter (mm)	
	Pre-treatment	Post-treatment
Pathogen control	6.1 e ^c	5.4 g
Chitosan A (0.05 mg/ml)	1.2 bc	2.2 cde
Chitosan A (0.10 mg/ml)	0.4 ab	1.3 b
Chitosan B (0.05 mg/ml)	2.8 d	3.2 f
Chitosan B (0.10 mg/ml)	2.5 d	2.6 ef
Chitosan A + B (0.05 mg/ml)	1.0 abc	1.4 bc
Chitosan A + B (0.10 mg/ml)	1.9 cd	2.3 de
Bactericide (Zinc thiazole) ^b	1.7 cd	1.7 bcd
None	0.0 a	0.0 a

^a The inoculum concentration of bacteria is approximately 10⁸ CFU/ml.

^b The concentration of the bactericide is 100 µg/ml.

^c Data are from a representative experiment repeated twice with similar results. Means in a column followed by the same letter are not significantly different ($p < 0.05$).

different combinations of chitosan solutions compared to the respective control (Table 2, 3). In pre-treatment of chitosan, there was no significant difference in disease incidence between three different chitosan solutions (A at 0.05 mg/ml, B at 0.05 mg/ml, A + B at 0.10 mg/ml) and the bactericide, while two chitosan solutions (A at 0.10 mg/ml, A + B at 0.05 mg/ml) and one chitosan solution (B at 0.10 mg/ml) reduced and increased the disease incidence, respectively, compared to the bactericide (Table 2). In addition, there was no significant difference in lesion diameter between five different chitosan solutions (A at 0.05 mg/ml,

B at 0.05 and 0.10 mg/ml, A + B at 0.05 and 0.10 mg/ml) and the bactericide, while one chitosan solutions (A at 0.10 mg/ml) reduced the lesion diameter compared to the bactericide (Table 3). In post-treatment of chitosan, there was no significant difference in disease incidence between four different chitosan solutions (A at 0.05 and 0.10 mg/ml, B at 0.10 mg/ml, A + B at 0.05 mg/ml) and the bactericide, while two chitosan solutions (B at 0.05 mg/ml, A + B at 0.10 mg/ml) increased the disease incidence compared to the bactericide (Table 2). In addition, there was no significant difference in lesion diameter between four different chitosan solutions (A at 0.05 and 0.10 mg/ml, A + B at 0.05 and 0.10 mg/ml) and the bactericide, while two chitosan solutions (B at 0.05 and 0.10 mg/ml) increased the lesion diameter compared to the bactericide (Table 3). Broccoli heads uninoculated with *P. fluorescens* strain PFB-01 were free of symptoms.

In agreement with *in vitro* experiment results, both pre-treatment and post-treatment of plants with different combinations of the two kinds of chitosan solutions significantly reduced the disease incidence and the lesion diameter of broccoli heads compared to the control, which can be attributed, at least in part, to the direct antibacterial activity of chitosan solution. However, in general, the disease incidence and the lesion diameter were more reduced by the pre-treatment than the post-treatment of plants with chitosan solutions. In particular, plants pre-treated with chitosan A solution at 0.10 mg/ml showed more reduction on the disease incidence and the lesion diameter than that of the bactericide. Therefore, chitosan A coating seems to be a promising method to prevent the disease from broccoli heads.

Overall, our results clearly demonstrate that the two kinds of chitosan solutions have strong antibacterial activity against *P. fluorescens* strain PFB-01 under various environmental conditions. To the best of our knowledge, this is the first report about antibacterial activities of chitosan on the pathogenic *P. fluorescens*. In addition, it is evident that the two kinds of chitosan solutions have a potential in the prevention and control of bacterial head rot disease of broccoli. Considering the absence of any sort of remedial measures for bacterial head rots disease of broccoli the present investigation may prove helpful in a field which is largely dominated by economically handicapped broccoli growers.

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References

- Atia, M. M. M., Buchenauer, H., Aly, A. Z. and Abou-Zaid, M. I. 2005. Antifungal activity of chitosan against *Phytophthora infestans* and activation of defence mechanisms in tomato to late blight. *Biol. Agric. Hortic.* 23:175-197.
- Bell, A. A., Hubbard, J. C. and Li, L. 1998. Effects of chitin and chitosan on the incidence and severity of Fusarium yellows of celery. *Plant Dis.* 82:322-328.
- Ben-Shalom, N. and Fallik, E. 2003. Further suppression of *Botrytis cinerea* disease in cucumber seedlings by chitosan-copper complex as compared with chitosan alone. *Phytoparasitica* 31:99-102.
- Cho, S., Lee, S. H., Park, S., Choi, K. U., Cho, J., Hur, J. H., Shrestha, A. and Lim, C. 2009. Identification of a genetic locus related to antiviral production in *Pseudomonas fluorescens* strain Gpf01 against cucumber mosaic virus. *Plant Pathol. J.* 25:77-85.
- Choi, G. J., Kim, J. C., Park, E. J., Choi, Y. H., Jang, K. S., Lim, H. K., Cho, K. Y. and Lee, S. W. 2006. Biological control activity of two isolates of *Pseudomonas fluorescens* against rice sheath blight. *Plant Pathol. J.* 22:289-294.
- Cui, X. H. and Harling, R. 2006. Evaluation of bacterial antagonists for biological control of broccoli head rot caused by *Pseudomonas fluorescens*. *Phytopathology* 96:408-416.
- Darling, D., Harling, R., Simpson, R. A., McRoberts, N. and Hunter, E. A. 2000. Susceptibility of broccoli cultivars to bacterial head rot: in vitro screening and the role of head morphology in resistance. *Eur. J. Plant Pathol.* 106:11-17.
- Devlieghere, F., Vermeulen, A. and Debevere, J. 2004. Chitosan: antimicrobial activity, interactions with food components and applicability as a coating on fruit and vegetables. *Food Microbiol.* 21:703-714.
- Fang, Y., Li, B., Wang, F., Liu, B. P., Wu, Z. Y., Su, T., Qiu, W. and Xie, G. L. 2009. Bacterial fruit rot of apricot caused by *Burkholderia cepacia* in China. *Plant Pathol. J.* 25:429-432.
- Fujimoto, T., Tsuchiya, Y., Terao, M., Nakamura, N. and Yamamoto, M. 2006. Antibacterial effects of chitosan solution® against *Legionella pneumophila*, *Escherichia coli*, and *Staphylococcus aureus*. *Int. J. Food Microbiol.* 112:96-101.
- Hildebrand, P. D. 1989. Surfactant-like characteristics and identity of bacteria associated with broccoli head rot in Atlantic Canada. *Can. J. Plant Pathol.* 11:205-214.
- Ipper, N. S., Kim, J. E., Koo, J. H., Hur, J. H. and Lim, C. K. 2005. Inhibitory effects of a Korean strain Gpf01 identified as *Pseudomonas fluorescens* on cucumber mosaic virus. *Plant Pathol. J.* 21:262-269.
- Kong, H. G., Choi, K. H., Heo, K. R., Lee, K. Y., Lee, H. J., Moon, B. J. and Lee, S. W. 2009. Generation of a constitutive green fluorescent protein expression construct to mark biocontrol bacteria using P43 promoter from *Bacillus subtilis*. *Plant Pathol. J.* 25:136-141.
- Lee, Y. S., Han, H. S., Kim, G. H., Koh, Y. J., Hur, J. S. and Jung, J. S. 2009. Causal agents of blossom blight of Kiwifruit in Korea. *Plant Pathol. J.* 25:220-224.
- Li, B., Wang, G. L., Wu, Z. Y., Qiu, W., Tang, Q. M. and Xie, G. L. 2009a. First report of bacterial head rot of broccoli caused by *Pseudomonas fluorescens* in China. *Plant Dis.* 93:1219.
- Li, B., Wang, X., Chen, R. X., Huangfu, W. G. and Xie, G. L. 2008b. Antibacterial activity of chitosan solution against *Xanthomonas* pathogenic bacteria isolated from *Euphorbia pulcherrima*. *Carbohydr. Polym.* 72:287-292.
- Li, B., Xu, L. H., Lou, M. M., Li, F., Zhang, Y. D. and Xie, G. L. 2008a. Isolation and characterization of antagonistic bacteria against bacterial leaf spot of *Euphorbia pulcherrima*. *Lett. Appl. Microbiol.* 46:450-455.
- Li, B., Yu, R. R., Yu, S. H., Qiu, W., Fang, Y. and Xie, G. L. 2009b. First report on bacterial heart rot of garlic caused by *Pseudomonas fluorescens* in China. *Plant Pathol. J.* 25:91-94.
- Pajot, E. and Silué, D. 2005. Evidence that DL-3-aminobutyric acid and acibenzolar-S-methyl induce resistance against bacterial head rot disease of broccoli. *Pest Manag. Sci.* 61:1110-1114.
- Photchanachai, S., Singkaew, J. and Thamthong, J. 2006. Effects of chitosan seed treatment on *Colletotrichum* sp. and seedling growth of chili cv. "Jinda". *Acta Horticulturae* 712:585-590.
- Saygili, H., Aysan, Y., Sahin, F., Ustun, N. and Mirik, M. 2004. Occurrence of pith necrosis caused by *Pseudomonas fluorescens* on tomato plants in Turkey. *Plant Pathol.* 53:803.
- Seyfarth, F., Schliemann, S., Elsner, P. and Hipler, U. C. 2008. Antifungal effect of high- and low-molecular-weight chitosan hydrochloride, carboxymethyl chitosan, chitosan oligosaccharide and N-acetyl-D-glucosamine against *Candida albicans*, *Candida krusei* and *Candida glabrata*. *Int. J. Pharm.* 353:139-148.
- Su, Y. J. 2008. Spread and prospect of broccoli cultivar with high yield. *Chin. Countryside Well-off Technol.* 8:42-44.
- Vasanthi, H. R., Mukherjee, S. and Das, D. K. 2009. Potential health benefits of broccoli- a chemico-biological overview. *Mini Rev. Med. Chem.* 9:749-759.
- Wang, X. M., Cui, K. and Lu, Y. L. 2008. Overview of applicable value and production and export prospect of Chinese broccoli. *Chin. Agri. Sci. Bull.* 24:478-480.