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Effect of chitosan solution on the inhibition of *Acidovorax citrulli* causing bacterial fruit blotch of watermelon

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Abstract

BACKGROUND: The production of watermelon in China has been seriously hampered by fruit blotch disease and limited control measures are now applied. Chitosan has been employed to control a variety of plant diseases and is considered to be the most promising biochemical to control this disease.

RESULTS: The *in vitro* antibacterial effect of chitosan and its ability in protection of watermelon seedlings from bacterial fruit blotch were evaluated. Results showed that three types of chitosan, in particular, chitosan A at 0.40 mg mL^{-1} significantly inhibited the growth of *Acidovorax citrulli*. The antibacterial activity of chitosan A was affected by chitosan concentration and incubation time. The direct antibacterial activity of chitosan may be attributed to membrane lysis evidenced by transmission electron microscopic observation. The disease index of watermelon seedlings planted in soil and the death rate of seedlings planted in perlite were significantly reduced by chitosan A at 0.40 mg mL^{-1} compared to the pathogen control. Fresh and dry weight of watermelon seedlings planted in soil was increased by chitosan seed treatment, but not by chitosan leaf spraying.

CONCLUSION: The results indicated that chitosan solution may have a potential in controlling bacterial fruit blotch of watermelon.
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Keywords: antibacterial activity; bacterial fruit blotch; watermelon; chitosan; *Acidovorax citrulli*

INTRODUCTION

Watermelon is a versatile food. Today, China is the world's largest watermelon producer.[1–3] However, production of watermelon in China has been seriously hampered by a variety of bacterial pathogens, in particular, fruit blotch pathogen *Acidovorax citrulli*, which is a devastating disease of cucurbit plants and has been responsible for up to 90% losses of marketable yield in some watermelon fields.[4–7] Since there are no resistant commercial cultivars, control of bacterial fruit blotch depends on the availability of uncontaminated watermelon seed.[6,7] Hot water and bleach treatments are not effective in eliminating the contamination from infested seed.[4–7] Furthermore, biocontrol is often affected by changing environmental conditions although several potential antagonistic microorganisms against fruit blotch pathogen of cucurbit plants have been reported.[8,9] In addition, watermelon fruit blotch cannot be managed with pesticides,[6,7] although periodic applications of copper sprays may result in a reduced rate of spread of the disease, if field spray programmes are begun prior to fruit-set, but they may be of minimal value under conditions highly favourable for disease development. Thus, alternative disease measures are needed.

Recently, some biochemicals, in particular, the natural non-toxic biopolymer chitosan, have been employed to suppress a variety of bacterial pathogen and control several plant diseases, for example tomato bacterial wilt and broccoli bacterial head rot in our previous studies.[10–17] Chitosan has several advantages over other types of antimicrobial agents for its higher antimicrobial activity, a broader spectrum of activity, a higher killing rate, and lower toxicity toward mammalian cells.[12–17] Currently, chitosan and its derivatives have been attracting more and more attention in sustainable agriculture and food safety.[13,15,17] However, there is little information about the effect of chitosan on bacterial pathogens of watermelon. The

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aim of this study is to examine the antibacterial effect of chitosan solution against *A. citrulli* both *in vitro* and *in vivo*.

MATERIALS AND METHODS

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 (St Louis, MO, USA) and named as A and B, respectively. Water-soluble chitosan was purchased from Yuan-ye Biotechnology Company Ltd (Shanghai, China) and named as C. Stock solution of chitosan (5 mg mL^{-1}) was prepared in 1% acetic acid with pH being adjusted to 6.0 with NaOH.[12] After stirring (160 rpm) for 24 h at room temperature, the stock solution was autoclaved at 121 °C for 20 min. Sterile deionised water of pH 6.0 was used as a control.

Cultivation of bacteria

The virulent strain ZJU1106 of *A. citrulli* was isolated from diseased watermelon 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[11,12] at 28 °C. After incubation, a bacterial suspension was prepared in sterilised water, and the initial concentration of bacteria was adjusted to approximately 10^9 colony forming units (CFU) mL^{-1}.

Counting surviving cells

Bacterial suspensions were 10-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 mean value of the cells at the lowest dilution was calculated. Each experiment was carried out in duplicate and repeated twice.

Molecular weight and deacetylation degree

The molecular weight (MW) of the three types of chitosan were measured by the Center of Analysis & Measurement of Zhejiang University using gel permeation chromatography, while the deacetylation degree (DD) of the three types of chitosan was determined as described by Lou *et al*.[17]

In vitro antibacterial activity of chitosan

Effect of chitosan type

Chitosan A, B and C with different molecular weights and degrees of N-deacetylation were used in this study to evaluate the antibacterial effect of chitosan type. The antibacterial activity of three types of chitosan against *A. citrulli* strain ZJU1106 was determined at 6 h of incubation at 0.40 mg mL^{-1}, while the bacterial suspension was at 10^8 CFU mL^{-1}.

Effect of chitosan concentration

Chitosan A solutions of 5 mL in volume were prepared by adding chitosan stock to sterile deionised water to give a final concentration of 0.05, 0.10, 0.20 and 0.40 mg mL^{-1}. Bacterial suspension was added to 5 mL of chitosan solution to give a final bacterial suspension 10^8 CFU mL^{-1} and then the mixture was incubated at 28 °C on a rotary shaker (Hualida Company, Taicang, China) at 160 rpm while in the control treatment chitosan stock was replaced with sterile deionised water of pH 6.0 in order to maintain the same pH. Six hours later, samples were collected from each cell suspension and the viable bacterial cells were counted as indicated above.

Effect of incubation time

Chitosan A solution of 5 mL in volume was prepared by adding 200 μL chitosan stock to 4.80 mL sterile deionised water to give a final concentration of 0.40 mg mL^{-1}. *A. citrulli* strain ZJU1106 was inoculated into chitosan solution as indicated above. In the control treatment, chitosan stock was replaced with sterile deionised water of pH 6.0 in order to maintain the same pH. Antibacterial activity of chitosan solution on the growth of *A. citrulli* was determined after 0, 2, 4, 6, 12 and 24 h of incubation, respectively.

Transmission electron microscopy

Strain ZJU1106 of *A. citrulli* was prepared for transmission electron microscopy analysis after treating with chitosan for 4 and 12 h as described by Lou *et al*.[17] One millilitre of bacterial culture (approximately 10^8 CFU mL^{-1}) was added into the chitosan solution to give a final chitosan concentration of 0.40 mg mL^{-1} that showed higher antibacterial activity compared to the other concentrations tested. After incubation on a rotary shaker (160 rpm) at 30 °C for 4 h, the suspension was centrifuged. The cells were washed twice with 0.1 mol L^{-1} sodium phosphate buffer solution [pH 7.2, phosphate-buffered saline (PBS)] and fixed with 2.5% (v/v) glutaraldehyde in 0.1 mol L^{-1} PBS. The samples were post-fixed with 1% (w/v) OsO_4 in 0.1 mol L^{-1} PBS for 1 h at room temperature, and washed three times with the same buffer, dehydrated separately at 4 °C for 15 min in a graded series of ethanol solutions (70, 80, 90, 95 and 100%, v/v), then embedded in Epon 812 a low-viscosity embedding medium. Thin sections of the specimens were cut with a diamond knife on an Ultracut Ultramicrotome (Super Nova; Reichert-Jung Optische Werke, Vienna, Austria) and the sections were double-stained with saturated uranyl acetate and lead citrate. The grids were examined with a JEM-1230 transmission electron microscope (Hitachi, Tokyo, Japan) at an operating voltage of 75 kV.

In vivo antibacterial activity of chitosan

A bioassay was carried out to determine the ability of chitosan to protect watermelon seedlings from bacterial fruit blotch both in soil and in perlite. The inhibitory effect of chitosan A at 0.40 mg mL^{-1} against fruit blotch of watermelon planted in soil was evaluated by using either leaf spraying or seed treatment methods as described by Algam *et al*.[10] while the inhibitory effect of chitosan A at 0.40 mg mL^{-1} against fruit blotch of watermelon planted in perlite was evaluated by using leaf spraying. Pre-germinated seeds of watermelon (cv. Zhejiang honey-2) were sown in pots (10 cm diameter × 10 cm height) containing either unsterilised natural vegetable soil or unsterilised perlite. At the three leaf stage, seedlings were inoculated with bacterial suspension of 10^8 CFU mL^{-1} by leaf spraying according to the method of Li *et al*.[14] Control plants were sprayed with distilled water. Plants were maintained in a temperature-controlled glasshouse with Osram daylight lamps providing supplementary light for a 12 h photoperiod, about 70–80% humidity and 28 ± 2 °C. The pots were arranged in a randomised block design with four replicates and four plants per pot. After 60 days, the disease index was determined according to the method described by Algam *et al*.[10]

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Enzyme assays

The leaves of watermelon seedlings (0.1 g fresh weight per pot) were sampled at 0, 2, 4, 6 and 8 days after bacterial inoculation and were ground to a fine powder in liquid nitrogen and used for extraction of phenylalanine ammonia lyase (PAL), peroxidase (POD) and polyphenol oxidase (PPO) by homogenisation in different buffers at 4 °C. The homogenates were centrifuged at 10 000 × *g* for 15 min at 4 °C (Kubota, Tokyo, Japan) and the supernatants were used for three enzymes assays. PAL was extracted in 25 mmol L^{-1} sodium borate buffer (pH 8.8) containing 32 mmol L^{-1} β-mercaptoethanol and was assayed as described by Lisker *et al.*[18] by measuring the absorbance at 290 nm (Hitachi U 2000, Tokyo, Japan). POD was extracted in 100 mmol L^{-1} sodium phosphate buffer (pH 7.0) and was assayed as described by Hammerschimidt *et al.*[19] by measuring the absorbance at 470 nm. PPO was extracted in Tris-HCl buffer (pH 7.0) containing 0.1 mol L^{-1} KCl, 1% (v/v) Triton X-100, 1 mmol L^{-1} EDTA and 5% (w/v) polyvinylpolypyrrolidone and was assayed as described by Li and Steffens[20] by measuring the absorbance at 420 nm.

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 the main treatments and their interactions were calculated by analysis of variance after testing for normality and variance homogeneity.

RESULTS AND DISCUSSION

Molecular weight and deacetylation degree of chitosan

Results from this study indicated that the MW of water-soluble chitosan C was about 521 kDa, while the DD of chitosan C was 93.4%. In addition, the difference in MW and DD values between the two types of acid-soluble chitosan was observed. Indeed, the MWs of chitosan A and B were about 1129 and 607 kDa, respectively, while the DDs of chitosan A and B were 85.3% and 72.0%, respectively.

In vitro antibacterial activity of chitosan

Effect of chitosan type

Results from this study revealed that the antibacterial activity of chitosan was affected by the type of chitosan; however, the three types of chitosan significantly inhibited the growth of *A. citrulli* strain ZJU1106. The viable bacterial cells decreased by 0.84 Log CFU mL^{-1} in chitosan C solution of 0.40 mg mL^{-1}, 5.44 Log CFU mL^{-1} in chitosan B solution of 0.40 mg mL^{-1}, and 5.90 Log CFU ml^{-1} in chitosan A solution of 0.40 mg mL^{-1} compared to the control after 6 h of incubation (Fig. 1). These results indicated that application of chitosan, in particular chitosan A, seems to be a promising method to control bacterial fruit blotch of watermelon seedlings. The difference in antibacterial activity of chitosan may be attributed to the composition of chitosan, which is consistent with the result of Li *et al.*[13,14] who reported that the antibacterial activity of chitosan depend on degree of deacetylation and molecular weight.

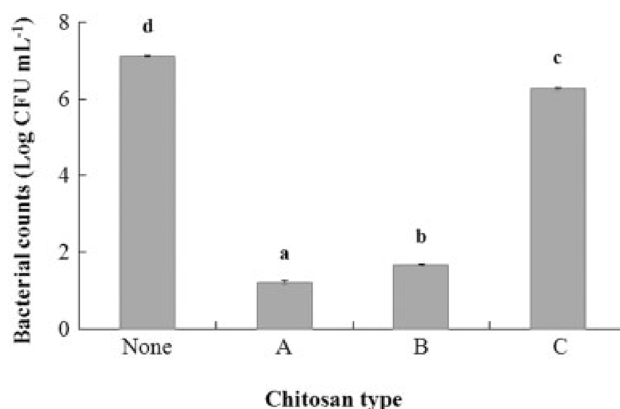

Figure 1. Effect of chitosan type on the antibacterial activity of chitosan against *Acidovorax citrulli* strain ZJU1106. The density of chitosan is about 0.40 mg mL^{-1} while bacterial concentration is approximately 10^8 CFU mL^{-1}. Columns with the same letters are not significantly different ($P < 0.05$). Error bars represent the standard error of the mean ($n = 6$). Data are from a representative experiment repeated twice with similar results.

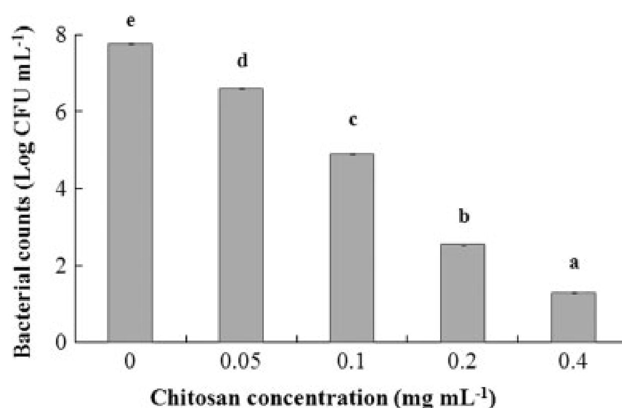

Figure 2. Effect of chitosan A at different concentration on the antibacterial activity of *Acidovorax citrulli* strain ZJU1106. Initial concentration of bacteria is approximately 10^8 CFU mL^{-1}. Columns with the same letters are not significantly different ($P < 0.05$). Error bars represent the standard error of the mean ($n = 6$). Data are from a representative experiment repeated twice with similar results.

Effect of chitosan concentration

This study indicated that chitosan A at four different concentrations had effective antibacterial activity against *A. citrulli* strain ZJU1106 compared to the control after 6 h of incubation (Fig. 2). In addition, the antibacterial activity of chitosan solution increased with the increase of chitosan concentration. The viable bacterial counts in chitosan A of 0.05 mg mL^{-1} decreased by 1.17 Log CFU mL^{-1} while the viable bacterial counts in chitosan A solution of 0.40 mg mL^{-1} decreased by 6.48 Log CFU mL^{-1} compared to the control (Fig. 2). These results are consistent with the result of Li *et al.*,[11–15] who reported that the antibacterial activity of chitosan solution against a variety of plant pathogen was influenced by its concentration in the solution.

Effect of incubation time

In the absence of chitosan, the viable bacterial counts in sterile deionised water decreased 0.13 Log CFU mL^{-1} after 2 h of incubation compared to the starting value. With the increase in incubation time, the viable bacterial counts remain stable (data not shown). In the presence of chitosan A, the viable bacterial counts were significantly decreased within 24 h of incubation compared to the starting value (Fig. 3). The viable bacterial counts decreased 6.36, 6.66, 6.41, 6.31 and 5.96 Log CFU mL^{-1} after 2, 4, 6,

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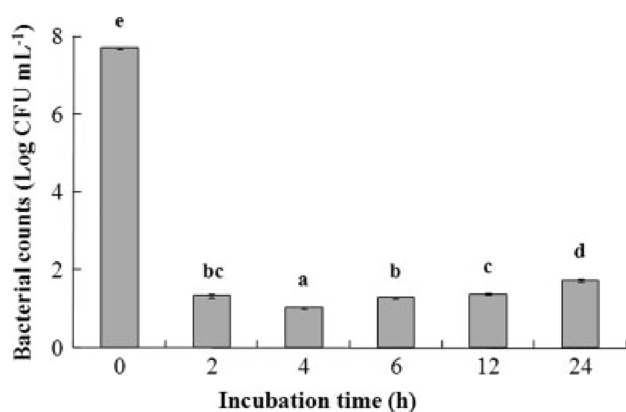

Figure 3. Effect of incubation time on the antibacterial activity of chitosan A against Acidovorax *citrulli* strain ZJU1106. The density of chitosan is about 0.40 mg mL^{-1} while bacterial concentration is approximately 10^{8} CFU mL^{-1}. Columns with the same letters are not significantly different ($P < 0.05$). Error bars represent the standard error of the mean ($n = 6$). Data are from a representative experiment repeated twice with similar results.

12 and 24 h of incubation, respectively, while the reduction in the viable bacterial counts after 4 h of incubation was higher than that of the other treatments (Fig. 3). In general, this study indicated that the antibacterial activity of chitosan A solution was affected by the incubation time, which is consistent with the result of Li *et al.*,[11–15] who found that a certain incubation time is required for the chitosan solution to inhibit the bacterial growth.

Transmission electron microscopy

In transmission electron microscopy micrographs, the control showed an intact and apparent cell membrane (Fig. 4A, C and E). However, strain ZJU1106 of *A. citrulli* treated with 0.40 mg mL^{-1} of chitosan A for 12 h showed badly damaged and altered of cell membrane (Fig. 4B and F). Chitosan A-treated *A. citrulli* cells for 4 h had a separation of the cytoplasma membrane from the cell envelope and coagulation of the cytosolic components compared to the control (Fig. 4D), and the bacterial cells were markedly degraded and formed vacuole-like structure (Fig. 4B and F). Moreover, most of the cell walls were badly broken which may increase the leaching out of a mass of nutrient and nucleic materials. Therefore, the direct antibacterial activity of chitosan may be attributed to membrane lysis based on transmission electron microscopy observations.

In vivo plant experiments

Seedlings planted in soil

Result from this study indicated that the seedlings of watermelon inoculated with the pathogen alone resulted in 0.97 diseases index (Table 1). However, the disease index of watermelon seedlings were significantly reduced compared to the pathogen control in the presence of chitosan A solution of 0.40 mg mL^{-1}, which were applied by either leaf spraying or seed treatment (Table 1). Chitosan applied by leaf spraying had 37.1% reduction in disease index, while chitosan applied by seed treatment caused 33.0% reduction (Table 1). The height of watermelon seedlings was unaffected by chitosan compared to the pathogen control regardless of the application method (Table 1). There was no significant difference in fresh and dry weight of watermelon seedlings between chitosan leaf spraying and the pathogen control, while chitosan seed treatment increased the fresh and dry weight compared to the pathogen control (Table 1). Watermelon seedlings uninoculated with *A. citrulli* strain ZJU1106 were free of symptoms.

Seedlings planted in perlite

This result indicated that watermelon seedlings planted in perlite had a very high death rate compared to those planted in soil when inoculated with *A. citrulli* strain ZJU1106. Therefore, we used the death rate instead of disease index to evaluate the effect of chitosan on the control of watermelon fruit blotch. Indeed, watermelon plants inoculated with the pathogen alone resulted in 73.7% death of seedling in perlite (Fig. 5). However, the death rate of watermelon seedlings was 26.7% in the presence of chitosan A solution of 0.40 mg mL^{-1}, which applied by leaf spraying (Fig. 5). Watermelon seedlings uninoculated with *A. citrulli* strain ZJU1106 were free of symptoms.

In agreement with *in vitro* experimental results, chitosan solution not only significantly reduced the disease index of watermelon seedlings planted in soil, but also significantly reduced the disease death rate in perlite compared to the pathogen control, which can be attributed, at least in part, to the direct antibacterial activity of chitosan solution. This result revealed that the antibacterial activity of chitosan A against *A. citrulli* was unaffected by various environmental conditions, indicating that application of chitosan seems to be a promising method to control bacterial fruit blotch on watermelon seedlings.

Changes in enzyme activities

The POD activities of the negative control kept almost stable while the POD activities of the pathogen control increased with the increase of time up to 6 days (Fig. 6). In general, the POD activities were increased by chitosan compared to the negative control, but reduced by chitosan compared to the pathogen control regardless of the method of application except that the POD activities of watermelon seedlings from chitosan treated seeds were slightly higher than that of the pathogen control within about 2 days after inoculation (Fig. 6). In addition, the POD activities of watermelon seedlings from chitosan treated seeds were higher than that of seedlings sprayed with chitosan regardless of the time after inoculation (Fig. 6). However, no obvious regular patterns were observed for the enzyme activities of PPO and PAL (data not shown).

Plants are able to defend themselves against pathogens by producing a wide spectrum of enzymes such as PAL, POD and PPO. As previous reports in the literature,[21,22] the defence responses were clearly observed by the accumulation of PAL, POD and PPO in seedlings treated with chitosan. But in this study, the results showed that chitosan treatments had limited effect on the enzyme activities, which may be attributed to the complexity of antibacterial mechanism of chitosan. Indeed, the direct killing effect on the bacteria may play the main role in the prevention and control of watermelon fruit blotch.

Overall, our results clearly demonstrate that the antibacterial activity of chitosan was dependent on chitosan type and concentration. However, the three types of chitosan solutions, in particular chitosan A solution, had strong *in vitro* antibacterial activity against *A. citrulli* strain ZJU1106 under various environmental conditions, while transmission electron microscopy observations revealed that the direct antibacterial activity of chitosan may be attributed to membrane lysis. To the best of our knowledge, this

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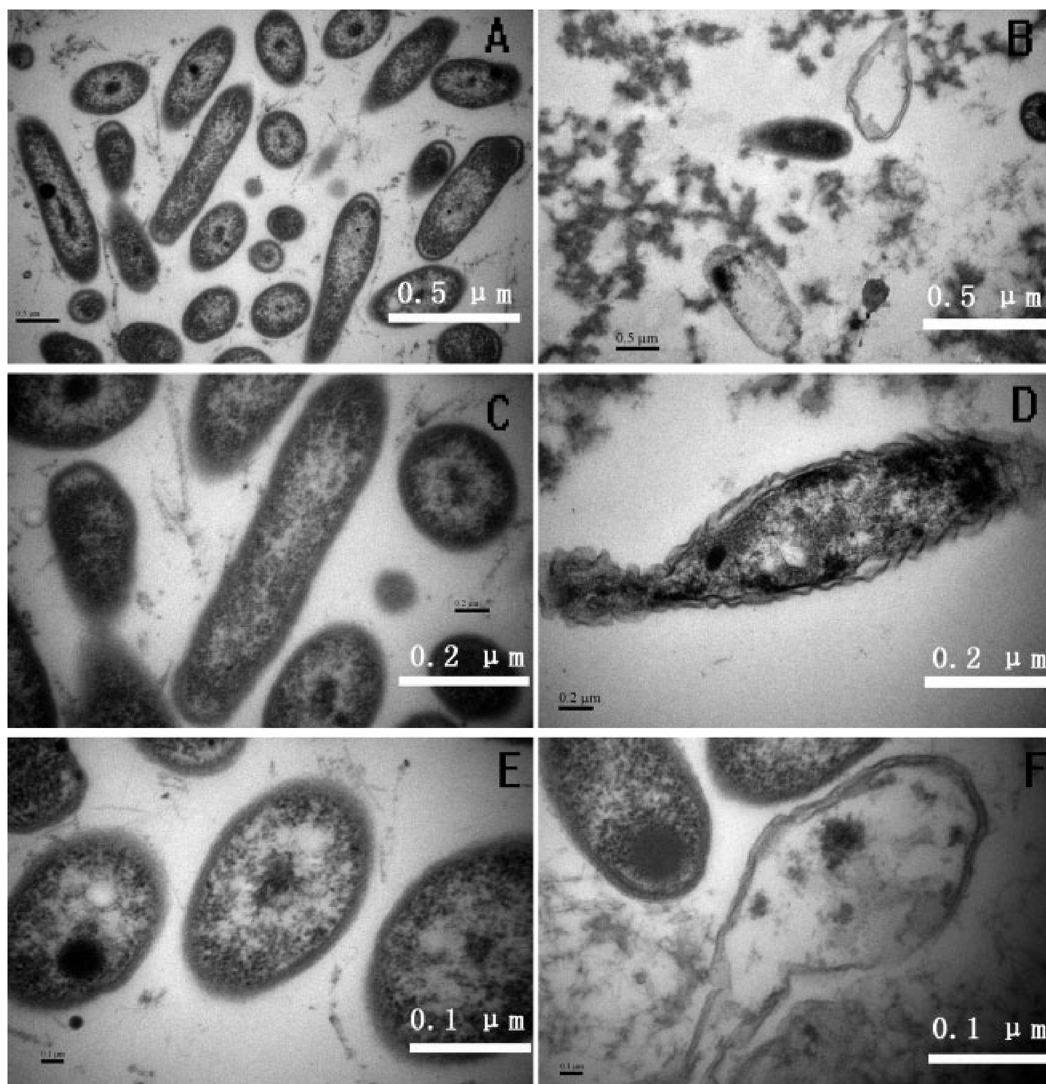

Figure 4. Transmission electron microphotographs of *Acidovorax citrulli* strain ZJU1106 treated with buffer (A, C, E) and with 0.40 mg mL^{-1} chitosan A for 4 h (D) and for 12 h (B, F). Bar in (A) and (B) = 0.5 μm; in (C) and (D) = 0.2 μm; in (E) and (F) = 0.1 μm.

Table 1. Effect of chitosan application method on the inhibition of bacterial fruit blotch and the growth promotion of watermelon seedlings planted in soil

Chitosan	Disease index	Height (cm)	Fresh weight (*g*)	Dry weight (*g*)
None	0.97 ± 0.01^{b}	12.43 ± 1.43^{a}	1.63 ± 0.23^{a}	0.19 ± 0.02^{a}
Leaf spraying	0.61 ± 0.02^{a}	12.53 ± 1.28^{a}	1.70 ± 0.17^{a}	0.23 ± 0.01^{ab}
Seed treatment	0.65 ± 0.02^{a}	12.80 ± 1.46^{a}	1.99 ± 0.22^{b}	0.29 ± 0.01^{b}

The concentration of chitosan is 0.40 mg mL^{-1} while the concentration of bacterial inoculum is approximately 10^{8} CFU mL^{-1}.
Data are from a representative experiment repeated twice with similar results.
Means in a column followed by the same letter are not significantly different ($P < 0.05$).

is the first report about antibacterial activities of chitosan on bacterial pathogen of watermelon. In addition, it is evident that chitosan A solution has a potential in the prevention and control of bacterial fruit blotch of watermelon seedlings both in soil and in perlite. Considering the absence of any sort of remedial measures for bacterial fruit blotch of watermelon, the present investigation revealed that chitosan seems to be a promising candidate to control bacterial fruit blotch of watermelon.

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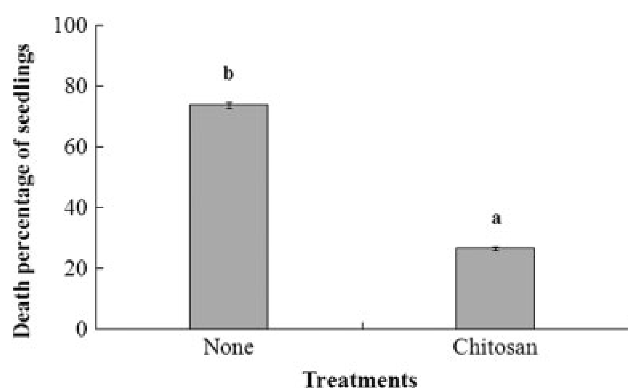

Figure 5. Effect of chitosan solution on the protection of watermelon seedlings from death caused by *Acidovorax citrulli* in perlite. The density of chitosan is about 0.40 mg mL^{-1} while initial concentration of bacterial inoculum is approximately 10^8 CFU mL^{-1}. Columns with the same letters are not significantly different ($P < 0.05$). Error bars represent the standard error of the mean ($n = 4$).

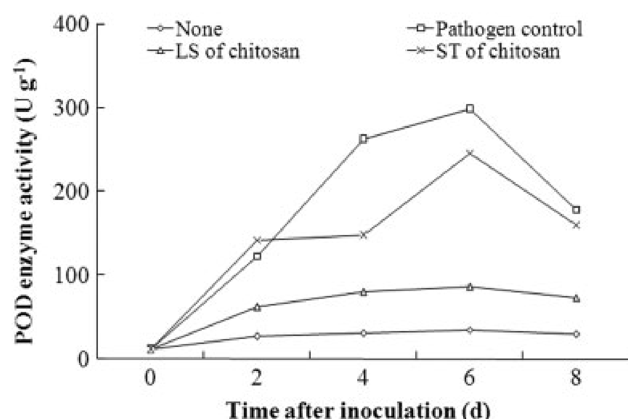

Figure 6. Effect of chitosan on the peroxidase enzyme activities of watermelon seedlings after inoculation of *Acidovorax citrulli*. POD, peroxidase; LS, leaf spraying; ST, seed treatment. The density of chitosan is about 0.40 mg mL^{-1} while the initial concentration of bacterial inoculum is approximately 10^8 CFU mL^{-1}.

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