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Use of Chitosan to Control Crown Gall Disease

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ABSTRACT
The antibacterial activity of chitosan solution against *Agrobacterium tumifaciens* was investigated in this study. The *in vitro* antibacterial effect of chitosan against *A. tumifaciens* was affected by chitosan concentrations, pH value, concentration of acetic acid used to dissolve chitosan and incubation time. Chitosan concentrations 2.5 mg mL^{-1} and 5 mg mL^{-1} exhibited strong antibacterial activity at the pH 6.6 and 5.6, respectively. The gall diameter and gall weight of tomato seedlings at dipping time 10 and 20 min was significantly reduced by chitosan concentrations 2.5 and 5 mg mL^{-1} at pH 6.6 and 5.6, respectively. The viable bacterial counts after 8 h of incubation in chitosan concentration 5 mg mL^{-1} at pH 5.6 was 0.0 log CFU mL^{-1}.

Key words: Crown gall, *Agrobacterium tumifaciens*, chitosan concentrations, pH value, incubation time, adsorptive properties, tomato

INTRODUCTION

Chitosan is a natural polysaccharide derived from chitin, a major component of the shells of crustaceans such as crab, shrimp and crawfish. In recent years, many investigations showed that chitosan could play important roles in plant protection; it can inhibit the growth of bacteria, fungi, viruses and other pests (El Hadrami *et al*., 2010). Chitosan has several advantages over other antimicrobial substances. It has a higher antimicrobial activity, a broader spectrum of activity, a higher killing rate and a lower toxicity toward mammalian cells (Luo and Wang, 2013). Several investigations have been recently done on use of chitosan to control plant bacterial disease (Li *et al*., 2008, 2010, 2013b; Algam *et al*., 2010; Wang *et al*., 2012, 2014; Coqueiro *et al*., 2011). Concentrations of chitosan that inhibit growth of bacteria differ according to bacterial species and the type of chitosan. Chitosan showed potential applicant to control of different bacterial plant diseases in greenhouse experiments. It inhibited bacterial head rot of broccoli (Li *et al*., 2010), rice leaf blight and leaf streak (Li *et al*., 2013a) and bacterial fruit blotch of watermelon (Li *et al*., 2013b). Badawy and Rabea (2013) found that O-(benzoyl) chitosan derivatives inhibit *Agrobacterium tumefaciens*. Furthermore, chitosan used to inhibit fungal diseases; it used to inhibit tomato root rot under greenhouse conditions (Abd-El-Kareem *et al*., 2006). The time of contact between chitosan and bacteria, affect the viability of the bacterial cells. When the incubation time of contact between bacterial cells and chitosan increases the bacterial cell number decreases (Li *et al*., 2010, 2013a).

Chitosan is adsorbed on gram-negative bacteria resulted in changes of cell wall and in the permeability of cell membrane leading to the death of the bacteria (Chung *et al*., 2004).

Agrobacterium tumefaciens is a gram-negative soiborne bacterium has worldwide distribution. It causes crown gall disease on a wide range of plants (Islam *et al*., 2010).

This study aimed to investigate the inhibitory effect of chitosan against *A. tumefaciens in vitro* and *in vivo*, also evaluate the effect of incubation time of *A. tumefaciens* with chitosan solution and the absorption of chitosan on the bacterial cell of tested bacteria.

MATERIALS AND METHODS

Source of pathogenic bacteria and chitosan: A virulent isolate of plant pathogenic bacterium of *Agrobacterium tumefaciens* was obtained from; Bacterial Dis. Res. Dept., Plant Pathol. Res. Inst., Agric. Res. Center, Giza, Egypt.

Chitosan extracted from crab shells (Poly-(l.4-B-D-glucopyranose mine), 2-Amino-2-deoxy-(l->4)-B-D-glucopyran, was obtained from-ROTH-Bestellen sie zum Nulltarif) was used in this experiments.

Effect of chitosan concentrations at different pH values on the growth of *A. tumifaciens in vitro*

Preparation of chitosan concentrations: High concentrations of chitosan (5, 10 and 15 mg mL^{-1}) were dissolved in 1% acetic acid solution, while low concentrations of chitosan (1 and 2.5 mg mL^{-1}) were dissolved in lower concentration of acetic acid (0.5 and 0.25% acetic acid) after shaking (160 rpm) for 24 h at room temperature. The dissolved solutions were alkalized with NaOH (Li *et al*., 2008) to different pH values (4.0, 5.6, 6.6, 7.6 and 8.6) for high concentrations of chitosan, while the pH values for low concentrations were adjusted to 5.6 and 6.6. Aqueous acetic acid solutions (0.25, 0.5 and 1.0%) alkalized by using NaOH and adjusted to pH 4.0, 5.6, 6.0, 7.6 and 8.6 to use as controls. Chitosan and control solutions were autoclaved at 121°C for 20 min.

Antibacterial activity of chitosan: The inhibitory effect of different concentrations of chitosan against the growth of *A. tumefaciens* was evaluated by using filter paper disc method (Algam *et al*., 2010). After 48 h of incubation at 28°C, bacterial growth of *A. tumefaciens* was harvested into saline solution (0.85% NaCl) to prepare bacterial suspension that was adjusted to 10^8 CFU mL^{-1} approximately. Add 3 mL of the suspension to 500 mL of melted King's B agar (KBA), then poured in sterilized petri dishes and allowed to solidify.

Autoclaved 5 mm diameter of filter paper discs (Whatman No.1, Whatman international, UK) were soaked in the chitosan concentration (high and low concentrations) for 5 min and then one disc were placed on the center of each inoculated plate. Negative controls were done with alkalized acetic acid in the same way. Plates were incubated for 48 h at 28°C and then the diameters of the inhibition zones were measured. Each treatment was replicated three times.

Pots experiment: Chitosan concentrations (5 mg mL^{-1} at pH 5.6, 1 and 2.5 mg mL^{-1} at pH 6.6) were used in this experiment. Tomato seedlings-cv. Peto 86 (*Lycopersicon esculentum*, Mill) 3 weeks old obtained from Agricultural Green House, Ministry of Agriculture, Doki, Giza, Egypt, were used as test plants. Tomato seedlings were wounded (1-2 mm depth) by sterilized scalpel at the crown region. The wounded crown regions of tomato seedlings were dipped in the chitosan solution at different dipping times; just dipping, l0 and 20 min. Tomato seedlings were soaked in diluted acetic acid solution (0.5% at pH 5.6 and 1.0% at pH 5.6) for the same dipping times and used as control. Every soaked tomato seedling was transplanted in pot (20 cm diameter×20 cm height) containing new mixed soil (clay-sandy soil in ratio 1:1 in weight) previously inoculated with 50 mL suspension of the pathogen (10^8 CFU mL^{-1}). Five pots were inoculated by only the pathogen and used as positive control. In addition, five pots treated only with water and served as negative control. The pots were arranged in a randomized block design with five replicates.

The plants were examined for gall formation after 3-4 weeks. Disease severity of crown gall was estimated as average of gall diameter (mm)/plant and average of gall weight (g)/plant. Percentage of Disease Reduction (PDR) was calculated form diameter and weight of galls (Tawfik *et al*., 2005) as following:

$$PDR = \frac{C - E}{C} \times 100$$

where, C is average of gall diameter or weight in positive control treatment. E is average of gall diameter or weight in treatments.

The experiment was conducted in a greenhouse (Cereal Dis., Res. Dept., Plant Pathol. Res. Inst., ARC, Giza, Egypt).

Effect of incubation time and adsorptive characteristics of chitosan on bacterial cell: Chitosan concentration 5 mg mL^{-1} in 1% acetic acid at pH 5.6 was prepared as mentioned before. Chitosan solution was inoculated with *A. tumifaciens* to obtain a final concentration of bacterial cells 10^8 CFU mL^{-1} approximately. In the control treatment, chitosan stock was replaced with sterile water of pH 5.6. The mixture was shacked at 100 rpm min^{-1} for 10 min. There were two flasks of bacterial suspension; first one to study the effect of incubation time while, second one for study the adsorptive characteristics of chitosan on bacterial cell. For the first flask, tenfold serial dilution was applied after 0, 2, 4, 6, 8 and 24 h of incubation time under shaking and 0.1 mL of each dilution was spread on KBA plates and after 48 h the number of Colony Forming Units (CFU) were determined and Log CFU mL^{-1} were calculated (Li *et al*., 2010, 2013b). The second flask of bacteria-chitosan suspension mixture, the same incubation times and techniques mentioned before were applied except that the chitosan-bacteria mixture was allowed to settle for 10 min before take 0.1 mL of upper layer to spread on the plates (Chung *et al*., 2004). The number of CFU was determined and Log_{10} CFU mL^{-1} was calculated as mention before. The same measured was applied in the water control flasks.

Statistical analysis: Collected data was firstly subjected to analysis of variance (ANOVA) as completely randomized design according to Gomez and Gomez (1984). Comparisons among treatment means were made using Duncan multiple range test.

RESULTS

Effect of chitosan concentrations at different pH values on the growth of *A. tumifaciens in vitro*: The diameter of inhibition zone on growth of *A. tumifaciens* was used as a parameter to evaluate the antibacterial activity of different concentrations of chitosan at different pH values. Data in Table 1 revealed that chitosan at different concentrations (5, 10 and 15 mg mL^{-1}) at pH 6.6, 7.6 and 8.6 dissolved in 1% acetic acid had no significant inhibitory effect the growth of

Table 1: Effect of different concentrations of chitosan dissolved in 1% acetic acid on the growth of *A. tumifaciens*

Chitosan concentrations (mg mL^{-1})	Inhibition zone diameter (cm) pH values 4	5.6	6.6	7.6	8.6
Control (0)	1.07^{bc}	0.00^{d}	0.00^{d}	0.00^{d}	0.00^{d}
5	1.93^{a}	1.10^{bc}	0.83^{c}	0.00^{d}	0.00^{d}
10	1.40^{b}	1.03^{bc}	0.00^{d}	0.00^{d}	0.00^{d}
15	1.17^{bc}	0.97^{bc}	0.00^{d}	0.00^{d}	0.00^{d}

Means followed by the same letter are not significantly different according to LSD test ($p \leq 0.05$)

Table 2: Effect of different concentrations of chitosan dissolved in 0.25 and 0.5% concentrations of acetic acid on growth of *A. tumifaciens*

Chitosan concentrations (mg mL^{-1})	Acetic acid concentration (%)	Inhibition zone (cm) pH 5.6	pH 6.6
Control (0)	0.25	0.00^{d}	0.00^{d}
	0.50	0.00^{d}	0.00^{d}
1	0.25	0.00^{d}	0.00^{d}
	0.50	2.50^{c}	5.67^{b}
2.5	0.25	0.00^{d}	0.00^{d}
	0.50	3.17^{c}	7.40^{a}

Means followed by the same letter are not significantly different according to LSD test ($p \leq 0.05$)

A. tumifaciens. The inhibition zones of the three aforementioned pH and the three tested concentrations were equal to that of control treatment (0.0 cm), except the concentration 5 mg mL^{-1} of chitosan at pH 6.6 where the inhibition zone was 0.83 cm that had significant increase in the inhibitory effect.

Different concentrations of chitosan varied in their antibacterial activities against *A. tumifaciens* according to their pH. Whereas, pH 5.6 was the most favorable pH for antibacterial activity to all tested chitosan concentrations (5, 10 and 15 mg mL^{-1}, respectively) where, the inhibition zones were 1.10, 1.03 and 0.97 cm, respectively compared to control treatment that had no inhibitory effect. All the three tested concentrations had significant difference with control treatment, while there was no significant difference between these three tested concentrations in their inhibitory effect on tested bacteria.

The control treatment of alkalinized acetic acid having pH 4 caused inhibition zone 1.07 cm. Only the concentration 5 mg mL^{-1} of pH 4 had inhibition zone diameter 1.93 cm had significant difference when compared with control and had 0.86 cm increment than control. On the other hand, there were no significant increase on inhibition zone of the other two concentrations (10 and 15 mg mL^{-1}) than the control treatments. These two treatments had inhibition diameter 1.17 and 1.40 cm and caused increase in the inhibition zone by 0.33 and 0.10 cm.

Therefore, the best treatment in Table 1 was 5 mg mL^{-1} of chitosan concentration at pH 5.6 dissolved in 1% acetic acid that caused 1.10 cm increase in inhibition zone in growth of *A. tumifaciens*.

Data in Table 2 indicated that the effect of low concentrations of chitosan (1 and 2.5 mg mL^{-1}) that dissolved in low concentrations of acetic acid (0.25 and 0.5%) on growth of *A. tumifaciens* varied according to pH values and chitosan concentrations. Chitosan at concentrations 1 and 2.5 mg mL^{-1} that dissolved in acetic acid concentration 0.25% had no effective antibacterial activity where the inhibition zones were (0.0 cm). While, chitosan soluble in 0.5% acetic acid causing inhibition the growth of *A. tumifaciens*. Furthermore, the inhibition zone diameter gave significant increment with increase of pH value from 5.6-6.6; it increase from 2.5-5.67 cm at dissolved chitosan of 1 mg mL^{-1} concentration in 0.5% acetic acid and significant increase from 3.17-7.4 cm with chitosan concentration 2.5 mg mL^{-1} dissolved in 0.5% acetic acid.

With pH 5.6 and acetic concentration 0.5%, the increase of soluble chitosan concentration from 1-2.5 mg mL^{-1} led to non-significant increase in inhibition zone from 2.5-3.17 cm. On the other hand, with pH 6.6 and acetic concentration 0.5%, the increase of soluble chitosan concentration from 1-2.5 mg mL^{-1} lead to significant increase in inhibition zone from 5.67-7.4 cm. Therefore, the best results in this table were recorded with the last two mentioned treatments.

Pot experiment: Results in Table 3 indicate that all tested concentrations of chitosan significantly reduced the tomato crown gall severity comparing with untreated plants. Data also showed that the severity of crown gall decreased significantly by increasing concentrations of chitosan. Chitosan concentration 5.0 mg mL^{-1} gave the highest percentage disease reduction (100%) and completely reduced the disease severity to 0.0 at any tested dipping times followed by chitosan concentrations 2.5 and 1.0 mg mL^{-1}, respectively where the percentage disease reductions in gall diameter or in gall weight were 52.0, 40.0% and 65.3, 62.5%, respectively at just dipping time.

Dipping the tomato seedlings in the chitosan concentrations at three different times (just dipping, 10 and 20 min) before sowing in the infested soil led to different degrees of plant protection.

Dipping tomato seedlings for 10 and 20 min before sowing gave the most effective results where the percentages disease reduction in gall diameter or gall weight were 100% at all tested chitosan concentrations, while just dipping was the lowest effective one at chitosan concentrations 2.5 and 1 mg mL^{-1}, respectively. Tomato seedlings in the negative control were free form symptoms while that in the positive control gave 100% disease severity.

Effect of incubation time of chitosan on bacterial cell count: Data in Table 4 showed that in the absence of chitosan, the viable bacterial counts in sterile water (immediately treatment) significantly decreased after 6 h of incubation compared to the presence of chitosan (immediately treatment), the viable bacterial counts were significantly decreased after 2 h of incubation where the viable bacterial counts decreased

Table 3: Effect of different concentrations of chitosan in control of crown gall disease on tomato seedlings

Treatments and dipping time (min)	Average of gall diameter (mm)	Average of gall weight (g)	Reduction of gall diameter (%)	Reduction of gall weight (%)
Negative control (without bacteria)				
-	0.00^{e}	0.00^{d}	-	-
Positive control (bacteria only)				
-	10.00^{a}	0.49^{a}	-	-
Aqueous acetic acid 1% (pH 5.6)				
Just dipping	7.60^{b}	0.36^{b}	24.00	26.5
10	7.00^{bc}	0.33^{b}	30.00	32.7
20	6.00^{cd}	0.25^{bc}	40.00	49.0
Aqueous acetic acid 0.5% (pH 6.6)				
Just dipping	10.00^{a}	0.48^{a}	0.00	2.0
10	7.00^{bc}	0.34^{b}	30.00	30.6
20	5.60^{cd}	0.24^{bc}	44.00	62.5
1 mg mL^{-1} Chitosan dissolved in 0.5% acetic acid (pH 6.6)				
Just dipping	6.00^{cd}	0.26^{bc}	40.00	46.9
10	0.00^{e}	0.00^{d}	100.00	100.0
20	0.00^{e}	0.00^{d}	100.00	100.0
2.5 mg mL^{-1} Chitosan dissolved in 0.5% acetic acid (pH 6.6)				
Just dipping	4.80^{d}	0.17^{c}	52.00	65.3
10	0.00^{e}	0.00^{d}	100.00	100.0
20	0.00^{e}	0.00^{d}	100.00	100.0
5 mg mL^{-1} Chitosan dissolved in 1% acetic acid (pH 5.6)				
Just dipping	0.00^{e}	0.00^{d}	100.00	100.0
10	0.00^{e}	0.00^{d}	100.00	100.0
20	0.00^{e}	0.00^{d}	100.00	100.0

Means in a column followed by the same letter are not significantly different according to LSD test ($p \leq 0.05$)

Table 4: Effect of incubation time and adsorptive characteristics of chitosan concentration 5 mg mL^{-1} against *A. tumefaciens* cells

	(Log_{10} CFU mL^{-1})			
Incubation time (h)	Water immediately	Water 10 (min)	Chitosan immediately	Chitosan 10 (min)
0	7.97^{a}	7.91^{a}	7.16^{ef}	6.79^{g}
2	7.93^{a}	7.89^{a}	6.61^{gh}	6.51^{h}
4	7.78^{ab}	7.73^{ab}	6.12^{i}	6.04^{i}
6	7.58^{bc}	7.36^{cde}	4.98^{j}	4.86^{j}
8	7.48^{cd}	7.34^{de}	2.37^{k}	0.00^{m}
24	7.33^{de}	7.05^{f}	1.90^{l}	0.00^{m}

Means followed by the same letter are not significantly different according to LSD test ($p \leq 0.05$), CFU: Colony forming unit

by 0.39 and 0.55 log CFU mL^{-1}, respectively compared to the starting value. In general, this study indicated that the antibacterial activity of chitosan was affected by the incubation time.

Effect of adsorptive characteristics of chitosan on bacterial cell count: Data in Table 4 showed that in the absence of chitosan, the viable bacterial counts in sterile water (10 min settle treatment) significantly decreased after 6 h of incubation compared to the presence of chitosan (10 min settle treatment), the viable bacterial counts were significantly decreased after 2 h of incubation, where the viable bacterial counts decreased by 0.55 and 0.28 log CFU mL^{-1}, respectively compared to the starting value.

Also, the viable bacterial counts was 0.00 log CFU mL^{-1} after 8 h of incubation in presence of chitosan (10 min settle treatment) compared with water (10 min settle treatment) when bacterial counts was 7.34 log CFU mL^{-1}.

DISCUSSION

As shown in Table 1, chitosan at high concentrations (5, 10 and 15 mg mL^{-1}) showed effective antibacterial activity against *A. tumefaciens*. Chitosan concentration 5 mg mL^{-1} recorded stronger antibacterial activity compared with others, this result is consistent with the result of Liu *et al*. (2006) and Li *et al*. (2008, 2010, 2013a, 2013b) who found that the antibacterial activity of chitosan was influenced by its concentration in the solution. The effective antibacterial activity of chitosan solution 5 mg mL^{-1} was due to increased solubility and higher charge density at lower pH (Sekiguchi *et al*., 1994). Therefore, the solubility of most chitosan preparations decreases sharply as the solution pH rises above 6.0-6.5 (Varum *et al*., 1994).

Data in Table 1 also indicated that, the antibacterial activity of chitosan solutions was reduced with increasing pH and dropped suddenly when the pH was greater than 6.6, where the inhibition zones in bacterial growth were 0.0 cm. At or below pH 6.6, the antibacterial activity of chitosan increased where the inhibition zone in bacterial growth was 1.10 cm at pH 5.6 at chitosan concentration 5 mg mL^{-1} compared with control treatment that gave 0.0 cm. These results are in agreement with that of Chung *et al*. (2003), who found that the inhibition percentage of chitosan solution decrease with increasing pH and dropped dramatically when the pH was greater than 6.0. In addition, the antibacterial activity of chitosan has been associated with the pH value, with higher activity at lower pH value (Fujimoto *et al*., 2006). On the contrast, data in Table 2 revealed that the inhibitory

effect of chitosan solution increased with the increment of pH value from 5.6 to 6.6 with 0.5% acetic acid concentration for both studied chitosan concentrations (1 and 2.5 mg mL^{-1}). These results may be due to that the solvent (acetic acid) was different from the other cases; it was 0.5% in results of Table 2 while it was 1% in those of Table 1 and the other studies mentioned before.

Data in Table 4 showed that the antibacterial activity of chitosan was affected by the incubation time. Where, the viable bacterial counts were significantly decreased after 2 h of incubation at chitosan 5 mg mL^{-1} (immediately treatment) compared to that in sterile water (immediately treatment). These results are consistence with the result of Li *et al*. (2008, 2013a). In addition, these results are in agreement with that of (Fujimoto *et al*., 2006), who found that the cells of *Staphylococcus aureus* were decreased more than 2.46 log CFU mL^{-1} after 1 day of incubation, not only in the chitosan solutions but also in phosphate buffer solution as a control.

Several studies have been showed that the interactions between positively charged chitosan molecules and negatively charged residues on the bacterial cell surface play an important role on the inhibitory effect of chitosan on gram-negative bacteria where, the positive surface charges at acidic condition. Chitosan interacts with anionic components on bacteria surface, such as negatively charged lipopolysaccharide in outer membrane of gram-negative bacteria and peptidoglycan and teichoic acid in cell wall of gram-positive bacteria (Helander *et al*., 2001; Luo and Wang, 2013) and that explain the significant effect of chitosan on bacterial count after settle for 10 min compared with control. Since, chitosan molecules do not carry positive charges at alkaline pH which may explain the absence of inhibition zones in growth of *A. tumefaciens* in chitosan concentrations of pH (7.6-8.6) in our study.

Li *et al*. (2010) found that the rot incidence and the lesion diameter of broccoli heads inoculated with *Pseudomonas fluorescens* were more reduced by the pre-treatment than the post-treatment of plants with chitosan solution. In our study, as shown in Table 3, dipping the tomato seedlings in chitosan concentrations for three different times ((just dipping,10 and 20 min) before transplanting in the infested soil led to different degrees of plant protection.

All chitosan concentrations tested in this experiment significantly reduced the tomato crown gall severity. When the tomato seedlings were dipped in the chitosan solutions before transplanting in infested soil with *A. tumifaciens* which can be attributed, at least in part, to the direct antibacterial activity of chitosan solution. Therefore, chitosan coating seems to be promising method to control of crown gall disease.

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