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Antimicrobial Activity of Different Molecular Weight Chitosans to Inhibit Some Important Plant Pathogenic Fungi

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ABSTRACT

The efficacy of different molecular weight (MW) chitosans to inhibit plant pathogenic fungi was investigated *in vitro* and *in vivo*. The first experiment studied the antimicrobial activity of different MW chitosans to inhibit the fungus *Sphaceloma ampelinum* in a liquid medium. The results revealed that a natural polymer chitosan (chitosan III), with a higher MW, exhibited more efficiency than oligomer and irradiated chitosans, with lower MWs, to inhibit the activity of the fungus. The efficiency of inhibition was increased with an increase in the concentrations of the chitosans. A concentration of 10,000 ppm was the most effective to inhibit the fungus *S. ampelinum in vitro*. The second experiment investigated the antimicrobial activity of different MW chitosans to inhibit nine plant pathogenic fungi in solid agar medium. The results indicated that lower MW chitosans exhibited higher mycelial inhibition than higher MW chitosans. Natural polymer chitosans showed 4.9% mycelial inhibition whereas lower MW chitosans ranged between 9.1 and 17.6% mycelial inhibition. The results also suggested that different plant pathogenic fungi showed different responses to different MW chitosans and concentrations. Both investigations concluded that different MW chitosans could exhibit different antimicrobial activity to plant pathogenic fungi when they were tested by different methods *in vitro*. The third experiment studied the efficacy of different MW chitosans to inhibit plant pathogenic fungi *in vivo*. *Colletotrichum gloeosporioides,* the causal organism of mango anthracnose, was used in this antimicrobial activity test *in vivo*. The results revealed that polymer and oligomer chitosans could not inhibit the incidence of the disease; however, oligomer chitosans tended to decrease the disease severity compared to polymer chitosans, which indicated that lower MW chitosans were more effective than higher MW chitosans to inhibit plant pathogenic fungi *in vivo*. The efficacy of chitosans to inhibit the infection of *Helminthosporium turcicum*, the causal agent of northern corn leaf blight, was investigated. The results suggested that lower MW chitosans might be more effective than higher MW chitosans to inhibit the fungus in corn plants.

Keywords: chitosans, polymer and oligomer, antimicrobial activity, *Sphaceloma ampelinum, Colletotrichum gloeosporioides*

INTRODUCTION

Nowadays, organic farming plays a more important role in agricultural production as a means to obtain more food that is considered safe to eat. Both living products and non living natural products are sought and used as substitutes for synthetic chemicals in crop protection. Any natural

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resources such as medicinal plants, microorganisms and animals are being considered for this purpose. Chitosans are an abundant natural biopolymer with strong antimicrobial effects on microorganisms of human, animal and plant pathogenic organisms and they are also safe for humans, animals and the environment. Chitosans have been used previously as antimicrobial products in the food and agricultural industries.

Chitosans, a linear polysaccharide consisting of (1,4) - linked2 – amino – deoxy – β – D - glucan, is a deacetylated derivative of chitin, which is the second most abundant polysaccharide found in nature after cellulose. Chitosans have been found to be nontoxic, biodegradable, biofuntional and biocompatible in addition to having antimicrobial characteristics (Jayakummar *et al*., 2005, 2007). The exact mechanism of the antimicrobial action of chitin, chitosan and their derivatives is still imperfectly known, but different mechanisms have been proposed. One is that the positively charged amino group interacts with the negatively charged microbial cell membrane leading to leakage of proteinaceous and other intracellular constituents of the microorganisms (Helander *et al*., 2002; Kong *et al*., 2008). Chitosans act mainly on the outer surface of bacteria. At low concentration (0.2 mg/mL), the polycationic chitosan probably binds to the negatively charged bacterial surface to cause agglutination, while at high concentration, the large number of positive charges may impart a net positive charge to the bacterial surfaces to keep them in suspension (Papineau *et al*., 1991; Sudarshan *et al*., 1992). There are reports that chitosan causes losses of proteinic materials in *Pythium oaroecandrum* at pH 5.8; it also acts as a chelating agent that selective binds trace metals and inhibits the production of toxins and microbial growth (Cuero, 1991). In addition, it activates several defense processes in the host tissue (El Ghaouth *et al*., 1992), acts as a water binding agent and inhibits various enzymes. Binding of chitosan with DNA and the inhibition of mRNA synthesis occurs through chitosan penetration toward the nuclei of the microorganisms and interference with the synthesis of mRNA and protein (Sudarshan *et al*., 1992).

The effect of the molecular weight (MW) on some antibacterial and antifungal activities has been explored. High MW chitosans are helpful in restraining the growth of bacteria, whereas a lower MW helps in accelerating the growth. There are various factors that affect the antimicrobial activity of chitosan. It has been demonstrated that lower MW chitosan of less than 10 kDa has greater antimicrobial activity than native chitosans (Uchida *et al*., 1989). A degree of polymerization of at least seven is required. Highly deacetylated chitosans are more antimicrobial than those with a higher proportion of acetylated amino groups due to increased solubility and higher charge density. However, solubility can be decreased by using high concentrations of low molecular weight electrolites such as sodium halides, sodium phosphate and organic anions (Roberts, 1992). Moreover, the antimicrobial activity of chitosan is influenced by its concentration in solution and the pH of the medium. Chitosans activated several defense mechanisms including accumulation of chitinases, synthesis of proteinase inhibitor, lignification and callose induction (El Ghaouth *et al*., 2000). When applied on wounded wheat leaves, chitosan induced lignifications and consequently restricted the growth of non pathogenic fungi in wheat. Chitosan inhibited the growth of *A. flavus* and aflatoxin production in liquid medium culture, preharvest maize and groundnut, and it enhanced phytoalexin production in germinating peanut (Cuero *et al*., 1991).

There are several reports on the effects of chitosan to inhibit microorganisms such as *E. coli* and *Staphylococcus aureus* in food, and chitosans at 0.1% have stronger bactericidal effects on Gram-positive bacteria than on Gram-negative ones (Kong *et al*., 2008). Zheng and Zhu (2003)

indicated that antimicrobial activity of chitosans on *S. aureus* was strengthened as the MW increased whereas the antimicrobial effect on *E. coli* increase as the MW was decreased. They explained that chitosans at higher MW formed a film which inhibits nutrient adsorption whereas a lower MW enters the microbial cells more easily which disturbs the metabolism of the cells. They discussed two possible mechanisms for the antimicrobial activity: 1) the chitosans on the surface of the cell can form a polymer membrane which prevents nutrients from entering the cells; or 2) chitosans of low MW enter the cell through pervasion. Since chitosan is able to adsorb the electronegative substances in the cell and flocculate them, it disturbs the physiological activity of the bacteria and kills them. Munoz *et al.* (2009) assessed the ability of chitosan to inhibit the fungus *Colletotrichum* spp. on tomatoes and grapes. They found that chitosan significantly reduced the lesion size on tomato fruits treated with concentrations of 10 and 2.5%. They suggested that the use of a chitosan coating on the fruit is effective in reducing anthracnose in tomatoes and berry fruits.

Because there are only a few reports on the antimicrobial activity of chitosans on plant pathogenic organisms, the objectives of this study were to determine: 1) the effects of different MW chitosans at different concentrations to inhibit the growth of plant pathogenic fungi; and 2) the efficacy of chitosan to control mango anthracnose disease *in vivo* both in the laboratory and the field.

MATERIALS AND METHODS

Chitosans

All chitosans used in the study were prepared from shrimp shells by standard chemical procedures. Chitosans with different molecular weights were used in the study and were divided into two groups. The first group consisted of unknown MW chitosans that included an oligomer type, chitosan I, chitosan II and natural polymer type chitosan III. The oligomer types were lower MW chitosans than the polymer type. They were reduced in MW by a chemical method and an irradiation method. The natural polymer type was assumed to be the highest MW and it was used as the control treatment in the experiment. The second group belonged to known MW chitosans designed by gamma irradiation as follows: chitosan at MW 268,781 Da, irradiated at 50Kgy, chitosan at MW 47,125 Da irradiated at 75Kgy, chitosan at MW 30,790 Da irradiated at 100Kgy. The molecular weight of chitosan was calculated using the Mark-Houwing equation. All chitosans were supplied by Associate Professor Panee Pakong, Department of Irradiation, Faculty of Science, Kasetsart University, Bangkok, Thailand.

Plant pathogens

Nine plant pathogenic fungi of economic crop diseases were used for testing the antimicrobial activity of the chitosans. These were: *Colletotrichum gloeosporioides, Fusarium oxysporum* fsp *cubense, Colletotrichum capsici, Pythium aphanidermatum, Phytophthora parasitica, Curvularia lunata, Rhizoctonia solani, Helminthosporium oryzae,* and *Sphaceloma ampelinum.* All of the organisms were obtained from the laboratory of the Plant Pathology Department, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand.

Chitosan solution preparation

A stock solution of chitosan at a concentration of 1% (w/v) was prepared in 2% (v/v) acetic acid. Different concentrations (0; 10; 100; 1,000; 10,000; and 100,000 ppm) were prepared by diluting each 1% chitosan solution in aseptic distilled water and these were subsequently used for the antimicrobial activity test.

Antimicrobial activity test

A dilution method was used to test the

inhibition activity of the organisms. Solid and liquid media, (potato dextrose agar, PDA; and potato dextrose broth, PDB, respectively) containing chitosans at different concentrations were used as standard media for cultivating the fungi. After cultivation, Petri dishes and flasks were kept at 25–27 °C for incubation. Antimicrobial activity was recorded by measuring the colony diameter and calculating the percentage of mycelial inhibition. The number of colonies was recorded by a serial dilution plate method and the optical density (OD) was measured by a spectrophotometer (Spectronic 20, Bausch & Lomb, Model 2D, Metrology Technical Co. Ltd.)

Experiments

Assays for antimicrobial activity of different MW chitosans to inhibit plant pathogenic fungi in liquid culture

Three different kinds of unknown MW chitosans (the first group) and four different kinds of known MW chitosans (the second group) were used. In the first experiment, the three different kinds of unknown MW chitosans I, II and III were diluted to four concentrations of 0, 1,000, 10,000 and 100,000 ppm in PDB medium. In the second experiment, there were four different kinds of known MW chitosans—namely, chitosan at MW 1,121,543 Da, chitosan at MW 268,781 Da, chitosan at MW 47,125 Da and chitosan at MW 30,790 Da that were each diluted to concentrations of 0, 100, 1,000, and 10,000 ppm. The antimicrobial activity levels of the chitosans were determined by culturing the fungus *Splaceloma ampelinum*, the causal agent of grape scab disease, in liquid medium. Conidia of the fungus were cultured in PDB for 24 h in rotary shakers. Then, 5 mL of conidia culture were inoculated into 45 mL of PDB containing each of the chitosans with a different MW at the different concentrations. Inoculated flasks were incubated on a rotary shaker at 200 rpm and at 25–27 °C for 5 d. For each flask, the number of conidia was measured by the serial dilution plate method and colonies on PDA were counted. The value of the optical density of each serial dilution was also determined by spectrophotometry.

Assays for antimicrobial activity of different MW chitosans to inhibit plant pathogenic fungi in solid culture

Two experiments were used in this assay to determine the antimicrobial activity of the different MW chitosans in solid medium. The first experiment used the three different unknown MW chitosans, chitosan I, II and III that were each diluted to four concentrations (0; 100; 1,000; and 10,000 ppm). The second experiment contained the four different known MW chitosans that had been used in the former experiment. Diluted samples of each chitosan were prepared at each of five concentrations (0; 10; 100; 1,000; and 10,000 ppm). The determination of the inhibiting effect on mycelial growth of the nine plant pathogenic fungi was undertaken using the dilution method. Chitosans at different concentrations were prepared by mixing 1 mL of 1% (w/v) chitosan solution in 9 mL of PDA and pouring into Petri dishes. A small block of agar with mycelia (diameter 0.5 cm) was transferred onto the surface of each medium and incubated at 25–27 °C for 14 d. The diameter of the gross colony was measured and the percentage of mycelial inhibition calculated.

Assays for antimicrobial activity of different MW chitosans to inhibit plant pathogenic fungus *in vivo*:

Assay for efficiency of chitosans to inhibit mango anthracnose disease in the laboratory

The unknown MW chitosan III was diluted to a concentration of 10,000 ppm and benlate fungicide was prepared at a concentration of 500 ppm. Mature mango fruits were sprayed

with a spore suspension of the fungus *Colletotrichum gloeosporioides* and they were incubated to obtain infection for 24 h in a moist chamber. Infected fruits were dipped into the chitosan and benlate fungicide solution for 5 min and air dried at room temperature. Then they were incubated for 10 d at room temperature. Disease incidence, lesion number and lesion size were measured after 10 d of incubation.

Assay for efficiency of chitosans to inhibit mango anthracnose disease in the field

Three different MW chitosans, chitosan I, II and III, were prepared at a concentration of 1% (10,000 ppm). Mature mango fruits were collected from an anthracnose-infected field and used for the chitosan treatments. Infected mango fruits were dipped into chitosan solutions for 5 min, air dried and then incubated for 7–10 d at room temperature until the disease symptoms appeared. A synthetic fungicide (Sportak®) was applied at the recommended concentration of 100 ppm using the same procedure as for the control treatment. Disease incidence was measured by counting the number of fruits that exhibited symptoms, and disease severity was recorded by the number of black spots and the percentage of infected area on the fruit surface. The experiment was conducted in a completely randomized design with four replications with 10 fruits for each treatment

Assay for efficiency of chitosans to inhibit northern corn leaf blight disease in the field

The antimicrobial activity of the three different unknown MW chitosans (chitosan I, II and III) to inhibit infection of plant pathogenic fungi *in vivo* was investigated in the field. A corn cultivar susceptible to *Helminthosporium turcicum,* the causal agent of northern corn leaf blight, was planted in an infected field at a spacing of 75 × 25 cm. Natural infection was also built up in the field by laying corn plants infected with northern corn leaf blight (NCLB) between the rows. Springler irrigation was applied to establish a high relative humidity suitable for disease development. Each chitosan solution was applied by spraying at the rate of 1mL/L, with the first application on seedlings 7 d after germination; additional spraying was conducted every 7 d thereafter until the corn plants had reached the tasselling stage. Disease incidence and severity were recorded by the number of infected plants, number of infected leaves and number of lesions per plant. The experiment was conducted in a randomized block design with four replications with one row for each treatment.

RESULTS

Assays for antimicrobial activity of different MW chitosans to inhibit plant pathogenic fungus in liquid culture

The first experiment was conducted to determine the antimicrobial activity of unknown MW chitosans in liquid medium. Three different kinds of unknown MW chitosans (chitosan I, II and III) in solution were diluted to four concentrations (0; 1,000; 10,000; and 100,000 ppm) in PDB medium. Conidia of *S. ampelinum* were added into the culture medium to determine the antimicrobial activity. The results indicated that the OD values of the three chitosans at a high concentration of 10,000 ppm (OD = 0.03) were lower than the OD values at the lower concentration of 1,000 ppm. (OD = 1.02). The number of colonies at a high concentration from 10,000 ppm upwards was lower than at a low concentration from 1,000 ppm downwards. None of the colonies were observed at the concentration of 100,000 ppm, indicating that all chitosans at this concentration completely inhibited spore multiplication. The results suggested that chitosan at a higher concentration than 10,000 ppm exhibited effective growth inhibition and prevented spore multiplication of *S. ampelinum*.

The data suggested that chitosan III was

more effective in inhibiting the fungus than either chitosan I or chitosan II because the number of colonies (0.15×10^5 spores/mL), was the lowest compared with the other two chitosans. The result suggested that a higher MW chitosan was more effective at inhibiting fungal activity than a lower MW chitosan (Table 1).

The second experiment was conducted to determine the antimicrobial activity of four different chitosans with known MW. Chitosans with molecular weights of 1,121,543 Da, 268,781 Da, 47,125 Da and 30,790 Da were used in this experiment. The spore formation of *Sphaceloma ampelinum* was cultured in a liquid medium containing chitosans at different concentrations. The number of colonies was determined by serial dilution plate counting and the optical density was measured by spectrophotometry. The results (Table 2) revealed that the highest MW chitosan (chitosan III) exhibited the lowest average number of colonies (28.4×10^5 colonies/ mL) while the lower MW chitosans exhibited higher average values for the number of colonies (76.4, 51.5 and 54.6×10^5 spores per mL for MW of 268,781 and 30,790 and 47,125 Da, respectively). On average, the increasing concentration of chitosans decreased the number of colonies.

Assays for antimicrobial activity of different MW chitosans to inhibit plant pathogenic fungi on solid culture

The first experiment was conducted to determine the antimicrobial activity of unknown MW chitosans to inhibit the mycelial growth of nine plant pathogenic fungi *in vitro*. The three unknown MW chitosans were prepared and mixed

Table 1 Optical absorbance value and number of colonies of *S.ampelinum* after culturing in medium containing chitosan solution at four different concentrations.

Parameter	Chitosan	Concentration (ppm)				Average
		0	1,000	10,000	100,000	
OD-value[1]	Chitosan I	1.10	1.10	0.04	0.11	0.59
	Chitosan II	1.10	1.10	0.02	0.10	0.58
	Chitosan III	1.00	0.85	0.02	0.07	0.49
	Average	1.06	1.02	0.03	0.09	0.55
Total Number of Colonies[2]	Chitosan I	2.28	3.40	0.64	0.00	1.58
	Chitosan II	2.57	2.70	0.60	0.00	1.46
	Chitosan III	2.43	2.56	0.02	0.00	1.24
	Average	2.42	2.88	0.41	0.00	1.43

[1] OD = OD value at dilution 10^{-5}.

[2] = Number of colonies $\times 10^7$ per mL.

Table 2 Number of colonies ($\times 10^5$) of *S. ampelinum* after culturing in medium containing chitosans at different concentrations.

Chitosan	Concentration (ppm)				
	0	100	1,000	10,000	Av
Non-irradiated Chitosan III (MW = 1,121,543 Da)	47.0	57.0	9.5	0.5	28.4
Irradiated Chitosan 50 Krad (MW 268,781 Da)	128.5	88.0	40.5	48.5	76.4
Irradiated Chitosan 75 Krad (MW 47,125 Da)	126.0	16.0	21.0	55.5	54.6
Irradiated Chitosan 100 Krad (MW 30,790 Da)	53.0	41.0	60.0	52.0	51.5
Average	88.6	50.5	32.7	39.0	52.7

into PDA to obtain concentrations of 0, 100, 1,000 and 10,000 ppm and then these were used for culturing plant pathogenic fungi to determine the mycelial growth inhibition. The results (Table 3) revealed that chitosan II had a higher average percentage of mycelial inhibition for almost all fungi tested. The mean inhibition was 17.6, 9.1 and 4.9% for chitosan II, I and III, respectively. The results indicated that chitosan at lower MW was more effective at inhibiting plant pathogenic fungi than at higher MW. The different plant pathogenic fungi showed different responses to the chitosans at different concentrations. In addition the most effective concentration was 10,000 ppm indicating that chitosans at higher concentrations were more effective than at lower concentrations.

The second experiment determined the antimicrobial activity of four different known MW chitosans to inhibit four plant pathogenic fungi *in vitro*. A dilution method was used to culture the fungi in PDA containing chitosan solutions at concentrations of 0, 10, 100, 1,000 and 10,000 ppm that were incubated for 14 d and then the diameters of the colonies were measured to calculate the percentage of mycelial inhibition. The results indicated that chitosans at higher MW were less effective than at lower MW as inhibitors of the mycelial growth of the fungi. Chitosan III was a natural polymer having the highest MW of the chitosans used and showed the lowest average percentage of mycelial inhibition (1.9%) compared with the activity of the lower MW chitosans (Table 4).

Assay for antimicrobial activity of different MW chitosans to inhibit plant pathogenic fungi *in vivo*

Assays for efficiency of chitosans to inhibit mango anthracnose disease in the laboratory

The preliminary experiment was conducted to determine the efficacy of chitosans to control mango anthracnose. A natural polymer chitosan (chitosan III) at a concentration 10,000

Table 3 Percent mycelial inhibition[1] of three different MW chitosans on nine plant pathogenic fungi at four different concentrations.

Chitosan	Concentration (ppm)				Average
	0	100	1,000	10,000	
Chitosan I(oligomer)	0.0	5.2	3.4	27.6	9.1
Chitosan II (oligomer)	0.0	16.2	14.6	39.6	17.6
Chitosan III (polymer)	0.0	5.3	1.3	13.0	4.9
Average	0.0	8.9	6.4	26.7	10.5

[1] = Percentage mycelial inhibition were averaged from the nine tested plant pathogenic fungi.

Table 4 Percentage mycelial inhibition[1] of different MW chitosans on four plant pathogenic fungi at different concentrations.

Chitosan	Concentration (ppm)					Average
	0	10	100	1,000	10,000	
Chitosan III (MW = 1,121,543 Da)	0.0	2.8	5.0	2.5	-0.8	1.9
Chitosan 50 Krad[2](MW = 268,781 Da)	0.0	2.1	3.3	7.5	14.1	5.4
Chitosan 75 Krad[2] (MW = 47,125 Da)	0.0	2.3	6.5	6.2	11.9	5.4
Chitosan 100 Krad[2](MW = 30,790 Da)	0.0	10.5	1.3	13.7	5.4	6.2
Average	0.0	4.4	4.0	7.5	7.6	4.7

[1] = Percentage mycelial inhibition values were averaged from the four tested plant pathogenic fungi.

[2] = Irradiated chitosan at 50, 75 and 100 Krad to obtain different MW chitosans.

ppm was compared with benlate fungicide at the recommended dosage (500 ppm). Disease incidence and severity were recorded at 10 d after incubation. The results revealed that chitosan produced a lower number of lesions and smaller lesion size compared to the fungicide treatment and the control with the control exhibiting the highest level of disease incidence and severity. The results indicated the possibility that chitosan might be used for the control of anthracnose disease of mango postharvest (Table 5).

Assay for efficiency of chitosans to inhibit mango anthracnose disease in the field

The mature mango fruits were harvested from natural anthracnose-infected fields and were dipped in chitosan solutions at concentrations of 10 mL/L for 5 min to determine the efficacy of controlling the disease post harvest. The incidence and severity of disease were recorded 7–10 d after incubation. The results showed that anthracnose incidence was exhibited as high as in the control treatment whereas the fungicide treatment showed a lower disease incidence (Table 6). Chitosan I and chitosan II exhibited lower percent disease incidence, (94.7% for both) whereas chitosan II showed 100% disease incidence compared to the fungicide and control treatments that exhibited 50 and 100% disease incidence, respectively. The results revealed that chitosan could not effectively control the incidence of anthracnose disease caused by *C. gloeosporioides* compared to synthetic fungicide (Table 6). However the efficacy of chitosan to reduce the severity of mango anthracnose was evaluated by dividing the severity into five levels and the percentage of each severity level was observed. It was found that chitosans I, II and III all exhibited lower disease severity than the control treatment. The level of severity of disease after chitosan treatments ranged from level 1 to level 4, and the level of severity of the control ranged from level 3 to level 5, whereas the level of severity of the fungicide treatment ranged from level 0 to level 1 (Table 7). The results suggested that chitosan might be effective in decreasing the severity of anthracnose disease.

Assay for efficiency of chitosans to inhibit northern corn leaf blight disease in the field

The field experiment was conducted to determine the antimicrobial activity of chitosan to inhibit infection of northern corn leaf blight (NCLB) in corn plants. A susceptible inbred line

Table 5 Disease incidence and severity of disease developed on mango fruit after dipping into chitosan or fungicide solution at 10 d of incubation.

Treatment	pH of solution	Number of lesions	Diseased area (%)	Lesion size (cm)
Chitosan III (10,000 ppm)	7.0	112.2	83.7	0.39
Benlate (500 ppm)	7.0	150.7	81.2	0.52
Control	-	150.0	91.2	0.41

Values are averages from four replications.

Table 6 Efficiency of different MW chitosans to control mango anthracnose and fruit rot post harvest.

Treatment	Rate of application(mL/L)	Anthracnose incidence (%)
Chitosan I (Oligomer)	10.0	94.7
Chitosan II (Oligomer)	10.0	100.0
Chitosan III (Polymer)	10.0	94.7
Fungicide	0.2	50.0
Control	0.0	100.0

was planted in the field and *H. turcicum* infection was built up in the field by natural infection. Chitosan solutions at a concentration of 1 mL/L were sprayed onto the plants at intervals of 7 d. Disease incidence was recorded by counting number of infected plants at the teaseling stage. The results revealed that chitosan I and chitosan II whose MWs were designed to be lower than the natural polymer chitosan exhibited lower percentages of disease incidence (50.0 and 54.8%, respectively) compared to the higher MW chitosan III and the control treatment (73.6 and 75.5% disease incidence, respectively) as shown in Table 8. The results suggested that lower MW chitosan might be more effective than higher MW chitosan to inhibit fungal growth *in vivo*.

DISCUSSION

Due to the poor water solubility of chitosans, they are usually dissolved in acetic acid, which has widely known antimicrobial activity. No *et al*. (2007) observed that the antimicrobial activity of chitosan was enhanced by acetic, formic or lactic acid in the medium. Lin *et al*. (2006) showed that acetic acid solutions with more than 200 ppm have a marked biocide response to *E. coli*. However, it was also demonstrated that chitosan samples exceeding 50 ppm were more effective than the action of acetic acid. To identify the effect of 2% acetic acid that was not confounded by the product of the antimicrobial activity of chitosans in the experiments, the preliminary experiment was conducted by culturing nine plant pathogenic fungi in a medium containing 2% acetic acid at different concentrations of 0, 1, 10, 100, 1,000, 10,000 and 100,000 ppm. The results indicated that acetic acid at a concentration lower than 10,000 ppm did not inhibit plant pathogenic fungi *in vitro*. Thus, this means that the antimicrobial activity exhibited in this experiment resulted from the effect of the chitosans.

The *in vitro* antimicrobial activity test of the different MW chitosans to plant pathogenic fungi indicated that chitosans with a higher molecular weight were more effective at inhibiting spore multiplication of the fungus *S. ampelinum* when tested in liquid culture (Tables 1 and 2) whereas they inhibited lower levels of mycelial growth of other plant pathogenic fungi when tested in solid culture (Tables 3 and 4).This could

Table 7 Percentage disease severity after treating mango fruit with different MW chitosans.

Treatment	Rate	incidence at each severity level (%)					
	(mL/L)	0	1	2	3	4	5
Chitosan I (Oligomer)	10.0	0.0	5.3	5.3	42.1	47.0	0.0
Chitosan II (Oligomer)	10.0	0.0	0.0	5.9	64.7	29.4	0.0
Chitosan III (Polymer)	10.0	0.0	5.3	26.3	10.5	57.8	0.0
Fungicide	0.2	50.0	5.0	0.0	0.0	0.0	0.0
Control	0.0	0.0	0.0	0.0	23.6	47.7	23.5

Table 8 Northern corn leaf blight incidence after spraying with different MW chitosans.

Chitosan	Disease incidence (%)
Chitosan I	50.8
Chitosan II	54.8
Chitosan III	73.6
Control	75.5

be explained by the high MW chitosans in liquid culture forming a layer around the spores and disturbing the absorption of nutrients and the process of spore budding by the fungus. It may also result from the pH imbalance between the culture solution and the spores of the fungus resulting in cell death. In contrast, in solid culture, the lower MW chitosans are more insoluble and are dispersed through the agar medium and the fungal mycelia and so can be absorbed into the mycelia and interfere with the process of mycelial growth.

Qin *et al*. (2006) correlated the water solubility of chitosans and antimicrobial activity. Water soluble chitosans did not show any antimicrobial activity. In contrast, water insoluble chitosans that were dissolved in an acid medium were shown to be microbiocidal. Due to the physiological pH in the cell that is approximately neutral, the water insoluble samples can precipitate, forming an impervious layer around the cell, blocking channels which are crucial for living cells. Water soluble samples could not form these layers. Another important concept was highlighted by Qin *et al*. (2006) that when the chitosan molecules are too large, the chitosan layer may be not very compact, which may help to understand the high amount of antimicrobial activity with low MW chitosans in several studies (Lin *et al*., 2006).

Chitosans are known to inhibit several plant pathogens; for example, it has been reported that mycelial growth of *Pythium irregulare* was inhibited by 58% at a concentration of 0.125 g chitosan/L (Park *et al*., 2002). Growth disturbance of *Botrytis cinerea* in combination with structural plant defense responses, such as the formation of wall appositions and plugging of intercellular space with fibrillar material in bell pepper fruit were observed by El Ghaouth *et al*. (1994).

The effect of MW and the concentration of chitosan on antibacterial activity of *E. coli* has been investigated by Lin *et al*. (2006). Different molecular weight chitosans (5.5×10^4 to 15.5×10^4 Da) with the same degree of deacetylation (80%) were obtained by the method of acetic acid hydrolysis and used for investigation. All of the chitosans had antimicrobial activity at a concentration higher than 200 ppm; the growth of *E. coli* was promoted at concentrations lower than 200 ppm. The antimicrobial activity of low MW chitosans was higher than that of the high MW samples. However, a chitosan with a middle MW (9.0×10^4 Da) could promote the growth of bacteria.

The main factors affecting the antibacterial activity of chitosans are molecular weight (MW) and concentration. There are some reports that natural polymer chitosans were more effective in inhibiting growth of bacteria than chitosan oligomers (No *et al*., 2007). The minimum inhibitory concentration (MIC) of chitosans ranged from 0.005 to 0.1% depending on the species of bacteria and the MW of the chitosan (No *et al*., 2007) and varied depending upon the pH of the chitosan preparation (Lin *et al*., 2006).

The effects of different MW chitosans were evaluated in several plant pathogens. The results indicated that lower MW chitosans were more effective than higher MW chitosans at inhibiting mycelial growth *in vitro*. This result was the same as the previously reported where a low MW chitosan (1.74×10^4 g/mol) was more effective at inhibiting the mycelial growth of *R. stolonifer* while a higher MW (3.07×10^4 g/mol) affected spore shape, sporulation and germination (Hernandez-Lauzardo *et al*., 2008). Oligochitosans showed higher activity at inhibiting the mycelial growth of several fungi than the high MW chitosans (Kim and Rajapakse, 2005). They also elicited maximal pisatin formation and exhibited higher antifungal activity against *F. solani* (Kendra and Hadwiger, 1984). However, the present experiment was in disagreement with Badawy and Rabea (2008) who reported that the inhibitory

effect on the mycelial growth of plant pathogens occurred when the fungi grew on media with high MW chitosans.

The potential of chitosans with different MW values to control mango anthracnose disease and northern corn leaf blight (NCLB) was investigated. The results revealed that the incidence of anthracnose was the same after treating mango-infected fruit with three different kinds of different MW chitosans (Table 6). However, the incidence of NCLB after spraying with the lower MW chitosan was reduced compared to spraying with higher MW chitosans (Table 8). These results corresponded to the former study by Badawy and Rabea (2008) on gray mold of tomato fruit where antimicrobial activity was increased when the chitosan MW was decreased *in vitro* and chitosan treatment significantly reduced fungal decay *in vivo*. Their results revealed that high chitosan concentration correlated with low disease incidence regardless of stress conditions. In the present study, it could be suggested that the effects of chitosans with different MW on NCLB may be associated with direct fungal toxic properties against the pathogen and the elicitation of biochemical defense responses in the corn leaves as was reported in El Ghaouth *et al*.(1992, 2000) Sudarshan *et al*. (1992) and Cuero *et al*.(1992).

CONCLUSION

The investigation suggested that both low and high MW chitosans have potential to inhibit the plant pathogenic fungi *in vitro* whereas the lower MW chitosans were more effective at inhibiting the fungi *in vivo*. The antimicrobial activity of chitosans was strengthened as the MW increased or decreased depending on the conditions of application, as high MW chitosans form a film which inhibits nutrient adsorption but lower MW chitosans enter the microbial cells more easily which disturbs the metabolism of the cell. The antimicrobial efficiency of chitosan is known to depend on its physical properties, such as solubility, degree of deacetylation (DA) and molecular weight (MW). In addition, chitosan is also known to be a potential elicitator of many plant defense mechanisms including accumulation of chitinase, chitosanase, synthesis of proteinase inhibitor, lignification, induction of callose synthesis and phytoalexin. Thus chitosan appears to play important to activate several biological processes in plant tissue. In the case of plant diseases, if a smaller molecule is applied to plants to prevent infection by plant pathogens, it may increase the efficiency with which the chitosan molecules can penetrate through the epidermal cell and increase the effectiveness of preventing the fungal infection and elicit defense mechanisms in the plant. In the future, more attention should be paid to the investigation of areas that relate to increasing the possibility and efficiency of chitosan for penetrating though host tissue. Nano-particle chitosan has a high potential for use in the enhancement and activation of plant defense mechanisms to pathogens.

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