Available online at www.sciencedirect.com
ScienceDirect
Pesticide Biochemistry and Physiology 87 (2007) 220–228
PESTICIDE
Biochemistry & Physiology
www.elsevier.com/locate/ypest

Antifungal activity of oligochitosan against *Phytophthora capsici* and other plant pathogenic fungi *in vitro*

Junguang Xu [a,b], Xiaoming Zhao [a], Xiuwen Han [a], Yuguang Du [a,*]

[a] *Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, PR China*
[b] *Graduate School of the Chinese Academy of Sciences, Beijing 100049, PR China*

Received 10 July 2006; accepted 31 July 2006
Available online 4 August 2006

Abstract

Antifungal activity of oligochitosan against nine phytopathogens was investigated *in vitro*. Oligochitosan was more effective than chitosan in inhibiting mycelial growth of *Phytophthora capsici* and its inhibition on different stages in life cycle of *P. capsici* was observed. Rupture of released zoospores induced by oligochitosan was reduced by addition of 100 mM glucose. The effects of oligochitosan on mycelial growth and zoospore release, but not zoospore rupture, were reduced largely when pH value was above 7. The ultrastructural study showed that oligochitosan caused distortion and disruption of most vacuoles, thickening of plasmalemma, and appearance of unique tubular materials. Plasmalemmasomes in hyphal tip cells were not found in the presence of oligochitosan. These results suggest polycationic nature of oligochitosan contributes only partly to its antifungal activity and multiple modes of action of oligochitosan exist including the disruption of endomembrane system.

Keywords: Oligochitosan; Antifungal; *Phytophthora capsici*; Ultrastructure

1. Introduction

Chitosan, the deacetylated derivative of chitin, is a polycationic copolymer consisting of β-1,4-linked 2-acetamido-D-glucose and β-1,4-linked 2-amino-D-glucose units with the latter usually exceeding 80% [1]. It is currently obtained from shrimp or crabshell chitin by alkaline deacetylation. Chitosan is biodegradable, non-toxic and biocompatible and has shown to be particularly useful in plant protection due to its dual function: antifungal effects and elicitation of defense mechanisms in plant [2,3]. However, its high viscosity and insolubility in neutral aqueous solution restrict chitosan uses in practice.

Numerous studies on antifungal activity of chitosan against plant pathogens have been carried out [4–11] and reviewed [2,3]. Chitosan's inhibition was observed on different development stages such as mycelial growth, sporulation, spore viability and germination, and the production of fungal virulence factors. It has been commonly recognized that antifungal activity of chitosan depends on its molecular weight, deacetylation degree, pH of chitosan solution and, of course, the target organism. Mechanisms proposed for the antifungal activity of chitosan focused mainly on its effect on fungal cell wall [4–6,12] and cell membrane [13,14]. Recently, many chitosan derivatives prepared by chemical modifications have been reported with increased solubility in water as well as improved fungicidal and insecticidal activity [15–18]. So far, little is known about their ability to induce resistance of plant.

Recent studies on chitosan have attracted interest for converting chitosan to oligosaccharides [19]. Oligochitosan, obtained by hydrolysis or degradation of chitosan, is not only water-soluble but also has shown to be more effective than chitosan to elicit multiple plant defense responses. These responses include production of hydrogen peroxide (H_2O_2) [20], increases in the activities of phenylalanine

* Corresponding author. Fax: +86 411 84379060.
E-mail address: duyg@dicp.ac.cn (Y. Du).

0048-3575/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.pestbp.2006.07.013

ammonialyase (PAL) and peroxidase (POD) [21], up-regulation of genes transcription of β-1,3-glucanase and chitinase [20], formation of pisatin [22–24], and lignin deposition [21]. The mechanisms by which oligochitosan elicits the defense response of plant have not been fully elucidated and octadecanoid pathway was suggested to play a role in signaling by oligochitosan [25].

Moreover, antifungal activity of oligochitosan has also been observed against several fungi [11]. Interestingly, oligochitosan (hexamer unit) that elicited maximal pisatin formation also exhibited higher antifungal activity against *Fusarium solani* than the lower DP (degree of polymerization) oligomers and chitosan [22]. Recently, penetration of fluorescent labeled chitosan oligomers with molecular weight under 8000 into living cells of *Escherichia coli* were observed and oligochitosan was suggested to inhibit bacteria from inside the cell [26,27]. This is clearly different from chitosan that adsorbed to bacterial walls leading to walls covering, membrane disruption and cell leakage [28–31]. At present, relative little attempt has been made to elucidate the mechanisms of antifungal activity of oligochitosan.

We have previously reported the preparation of oligochitosan by enzymatic depolymerization [32] and its elicitor activity [33–35]. This paper describes the antifungal activity of oligochitosan against phytopathogens and more specifically to *Phytophthora capsici in vitro*. The effects of oligochitosan on different stages in the life cycle of *P. capsici* and on hyphal ultrastructure were examined to gain more information on its mode of action.

2. Materials and methods

2.1. Preparation of oligochitosan

Chitosan (minimum 95% deacetylated, molecular weight (MW): 300,000–500,000 Da) was purchased from Jinan Haidebei Marine Bioengineering Co., Ltd. (Shangdong, China). Oligochitosan with a degree of polymerization (DP) of 3–9 was prepared by enzymatic hydrolysis of chitosan according to our previous method [32,34]. Oligochitosan was prepared as a stock solution at 40 mg ml^{-1} in sterile distilled water and stored in the dark at −20 °C.

2.2. Pathogens and cultures

Fusarium graminearum, *Phytophthora capsici*, *Verticillium dahliae* were kindly provided by Dr. Xiaoming Zhao (Department of Plant Pathology, Northwest Agricultural University, Yangling, China); *Alternaria solani*, *Botrytis cinerea*, *Colletotrichum orbiculare*, *Exserohilum turcicum*, *Fusarium oxysporum*, *Pyricularia oryzae* by Dr. Xuerui Yan (Department of Plant Pathology, Shenyang Agricultural University, Shenyang, China). All nine isolates were maintained on potato dextrose agar (PDA) (potato infusion from 200 g l^{-1}, 20 g l^{-1} dextrose, and 15 g l^{-1} agar) in the dark at 25 °C.

2.3. Effect of oligochitosan on mycelial radial growth

Mycelial discs (6 mm in diameter) of test fungi grown on PDA plates were cut from the margins of the colony and placed on PDA plates containing different concentrations of oligochitosan (0, 250, 500, 1000, 2000 μg ml^{-1}). Stock solution of oligochitosan was diluted with sterile distilled water and added to sterile molten PDA to obtain the desired oligochitosan concentrations. After incubation at 25 °C for 3–10 days, mycelial radial growth was measured and activity was expressed as EC_{50} (the concentration inhibiting growth by 50%) and MIC (the minimum concentration showing over 90% inhibition of mycelial growth) estimated by probit analysis. Effect of chitosan (in 1% v/v aqueous acetic acid) on mycelial radial growth of four strains (*F. graminearum*, *B. cinerea*, *P. capsici*, *V. dahliae*) was tested under the same condition as described above. Results were analyzed statistically using the two-tailed unpaired student's *t*-test to determine differences with oligochitosan ($P < 0.05$).

Effect of pH on efficacy of oligochitosan (1 mg ml^{-1}) was tested on *B. cinerea* and *P. capsici* under the same conditions described above except medium pH values, from 4 to 7 with one pH unit interval, were adjusted with 1 M NaOH or HCl. All experiments were repeated two times with three replicates.

2.4. Effect of oligochitosan on zoosporangia production, zoospore release, zoospore motility, cystospore germination of P. capsici

For zoosporangia production test, five mycelial disks (6 mm in diameter) were cut from the edge of the actively growing culture and immersed in 15 ml sterile distilled water containing 0.3, 1, 3, and 10 μg ml^{-1} oligochitosan in plates ($\phi = 9$ cm). Three replicate plates of each concentration, in addition to control plates containing sterile distilled water, were prepared. Following 36-h incubation under a 25 W daylight lamp at 25 °C, zoosporangia along the margins of each mycelial disk were observed with a light inverted microscope (COIC XSZ-D, Chongqing Optical Instrument Factory, China).

Effect of oligochitosan (in sterile distilled water) on zoospore release, zoospore motility, cystospore germination was tested in 96 well microtiter plates (MaxiSorp Nunc). Zoosporangia suspension (2×10^4 zoosporangia ml^{-1}) and zoospore suspension (1×10^5 zoospore ml^{-1}) of *P. capsici* were obtained by the method of Young, D.H. [36]. Oligochitosan solution (50 μl) was added to the well containing 50 μl zoosporangia suspension for zoospore release test. For zoospore motility and cystospore germination tests, 50 μl oligochitosan solution was added to the well containing 50 μl zoospore suspension at the beginning of and 120 min after the incubation, respectively. Fifty microlitres of sterile distilled water was added to the control well. After incubation at 25 °C for 240 min, approximately 150 zoosporangia were observed with the light microscope and

inhibition percentages of zoospore release of *P. capsici* were calculated. Zoospore motility and cystospore germination were assessed after 60 min and 22 h incubation at 25 °C, respectively.

In some tests, ATP (an energy supplier) or glucose (an osmotic stabilizer) was added to the zoosporangia or zoospore suspension to gain more information on mode of action of oligochitosan by the method described by Mitani S. [37]. Briefly, ATP in Tris–HCl buffer (pH 7.3), or glucose in distilled water, was added to the zoosporangia or zoospore suspension at 10 min before addition of oligochitosan to give final concentrations of 20 μM and 100 mM, respectively. All experiments were repeated two times with three replicates. EC_{50} and MIC values of oligochitosan on zoosporangia production, zoospore release, zoospore motility, and cystospore germination of *P. capsici* were estimated by probit analysis.

2.5. Transmission electron microscopy

P. capsici mycelial tips (5 mm) from the margins of activity growing colony on Erwin medium [38] amended with 0, 10, and 100 μg ml^{-1} oligochitosan were cut down and fixed with 1% w/v OsO_4 solution. After fixation, samples were dehydrated with ethanol and embedded in Epon 112. Thin sections were cut and double-stained with uranyl acetate and lead citrate. The grids were examined with a JEM-2000EX (JEOL, Japan) transmission electron microscope.

3. Results

3.1. Effect of oligochitosan and chitosan on mycelial growth

EC_{50} and MIC values of oligochitosan on mycelial radial growth were examined to determine its fungicidal spectrum (Table 1). Of nine strains of phytopathogens tested, mycelial growth of *P. capsici* was most sensitive to oligochitosan with EC_{50} and MIC values of 100 and 580 μg ml^{-1}, respectively. Mycelial growth of *B. cinerea* was less inhibited by oligochitosan with MIC value of 1640 μg ml^{-1}. Five strains (*C. orbiculare*, *E. turcicum*, *F. graminearum*, *F. oxysporum*, *V. dahliae*) showed weak sensitivity when exposed to oligochitosan supplemented PDA (EC_{50} > 2 mg ml^{-1}). However, 6 mg ml^{-1} oligochitosan could not completely inhibit the growth of all nine strains (data not shown).

Table 1
Inhibition of mycelial radial growth by oligochitosan against different phytopathogens

Fungi[a]	Oligochitosan	
	EC_{50} (μg ml^{-1})[b]	MIC (μg ml^{-1})[c]
Alternaria solani	650	>2000
Botrytis cinerea	580	1640
Colletotrichum orbiculare	>2000	>2000
Exserohilum turcicum	>2000	>2000
Fusarium graminearum	1086	>2000
Fusarium oxysporum	>2000	>2000
Phytophthora capsici	100	580
Pyricularia oryzae	>2000	>2000
Verticillium dahliae	>2000	>2000

[a] All fungi were tested on PDA in the dark at 25 °C.
[b] The concentration inhibiting by 50%.
[c] The minimum inhibitory concentration showing over 90% inhibition.

Four strains with different sensitivity to oligochitosan were tested for comparison of the inhibition efficacy of chitosan and oligochitosan. As shown in Fig. 1, only *P. capsici* was more effectively inhibited by oligochitosan at concentrations below 2 mg ml^{-1}. Mycelial growth of *B. cinerea* was more sensitive to oligochitosan than to chitosan at 2 mg ml^{-1}. There was no significant difference between oligochitosan and chitosan on growth inhibition of *F. graminearum*. Although *V. dahliae* was most resistant to both chitosan and oligochitosan among the test fungi, it was more sensitive to chitosan at tested concentrations.

On PDA medium with pH values from 4 to 6, inhibition of oligochitosan on mycelial growth of *P. capsici* and *B. cinerea* was not significantly affected (Fig. 2). When pH value was adjusted to 7, effect of oligochitosan on *P. capsici* dropped significantly from 86% to 28%. While on the contrary, effect of oligochitosan on *B. cinerea* was not changed. Mycelial growth of both two strains in the absence of oligochitosan was not significantly affected at all pH values tested (data not shown).

3.2. Effect of oligochitosan on different stages in the life cycle of *P. capsici*

Strong inhibition of oligochitosan on different stages in the life cycle of *P. capsici* were observed. The EC_{50} and MIC values of oligochitosan for sporangium formation, zoospore release, and cystospore germination are shown in Table 2. Zoosporangia production was the most sensitive stage to oligochitosan. The EC_{50} and MIC values of oligochitosan on zoosporangia formation were 0.6 and 5.3 μg ml^{-1}, respectively. After incubation for 36 h, there was ca. 123 (±10) zoosporangia formed around margin of one mycelial disk in sterile distilled water. In the presence of 10 μg ml^{-1} oligochitosan, there was nearly no new mycelium around original mycelial disks and formation of sporangium was completely inhibited.

Oligochitosan also inhibited zoospore release from zoosporangia with EC_{50} and MIC values of 41.7 and 218.7 μg ml^{-1}, respectively. After incubation for 4 h, zoospore release in sterile distilled water was ca. 90%. Oligochitosan at 50 μg ml^{-1} exhibited 78% inhibition on zoospore release and this effect reduced markedly to 31% when 20 μM ATP in pH 7.3 Tris–HCl buffer was applied 10 min before oligochitosan (Fig. 3). However, Tris–HCl buffer applied alone was able to reduce this effect to 20%. At a higher concentration of 150 μg ml^{-1}, inhibition of oligochitosan was less reduced by Tris–HCl buffer with and without ATP.

Zoospore motility of *P. capsici* used in this study was not notable in control (sterile distilled water) from the beginning of incubation, so no data was obtained about the effect of oligochitosan on zoospore motility.

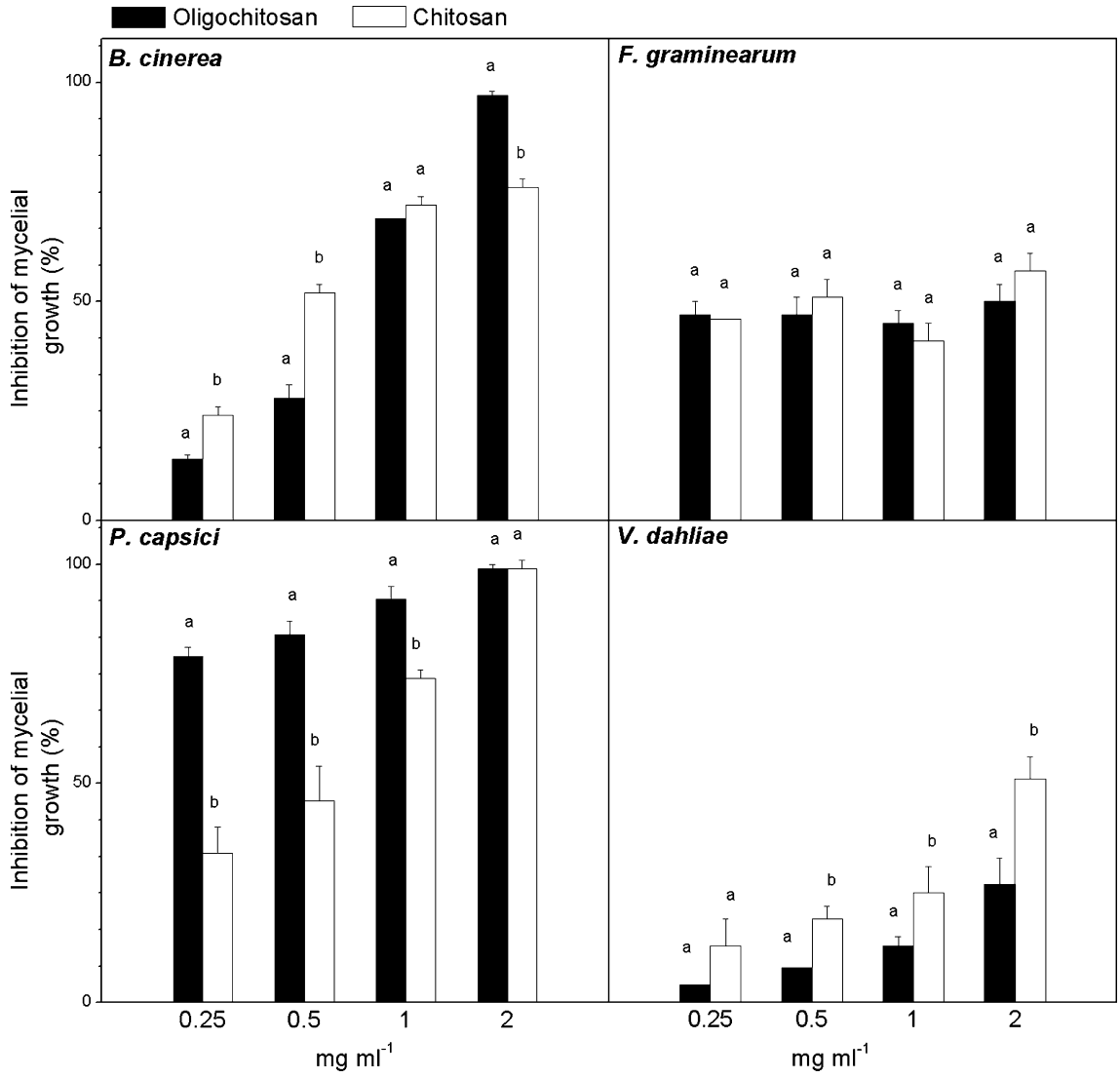

Fig. 1. Effect of chitosan and oligochitosan on mycelial growth of *P. capsici*, *B. cinere*, *F. graminearum* and *V. dahliae*. Measurement of mycelial growth was performed three days after inoculation with *P. capsici* and *B. cinerea* and six days after inoculation with *F. graminearum* and *V. dahliae*. Vertical bars within the same concentration with different letters are significantly different according to two-tailed unpaired student's *t*-test ($P = 0.05$).

Incubation with oligochitosan could lead to the rupture of zoospores. After 60-min incubation with 50 μg ml^{-1} oligochitosan, 98% zoospores were ruptured. Glucose with the final concentration of 100 mM protected zoospore from rupture when concentrations of oligochitosan were 50 μg ml^{-1} or below (Fig. 4). While on the contrary, presence of 20 μM ATP at pH 7.3 in Tris–HCl buffer showed no effect on oligochitosan induced zoospores rupture.

After 22-h incubation, cystospore germination in control was ca. 89% while oligochitosan at 150 μg ml^{-1} completely inhibited cystospore germination. The EC_{50} and MIC values of oligochitosan on cystospore germination were 36.5 and 64.3 μg ml^{-1}, respectively.

3.3. Effect of oligochitosan on ultrastructure of *P. capsici*

Oligochitosan was more effective on inhibiting mycelial radial growth on a synthesis Erwin medium (Table 2). 10 and 100 μg ml^{-1} oligochitosan inhibited growth of *P. capsici* mycelium by 39% and 86%, respectively. Mycelial tips from the actively growing culture were examined by electron microscopy. Hyphae of *P. capsici* grown in the absence of oligochitosan showed typical ultrastructural features of the genus (Figs. 5A and D). The hyphal cell was enclosed by a distinct electron-opaque cell wall (CW) and an undulated plasmalemma. Many organelles were observed such as vacuole (V), lipid body (L), mitochondria (M) and dense body (DB). Vigorous hyphal tip growth was marked by plasmalemmasomes (Pl) between cell wall and plasmalemma (Figs. 6A and C). In the presence of 10 μg ml^{-1} oligochitosan, vacuole distortion was the most pronounced ultrastructural feature in the hyphal tip (Figs. 5B and E). Besides, plasmalemmasomes were no longer observed. Distortion of cell wall, although less frequently, was also observed in hyphal tip (Fig. 5B). At a higher concentration of 100 μg ml^{-1}, oligochitosan led to more conspicuous cytological changes. The

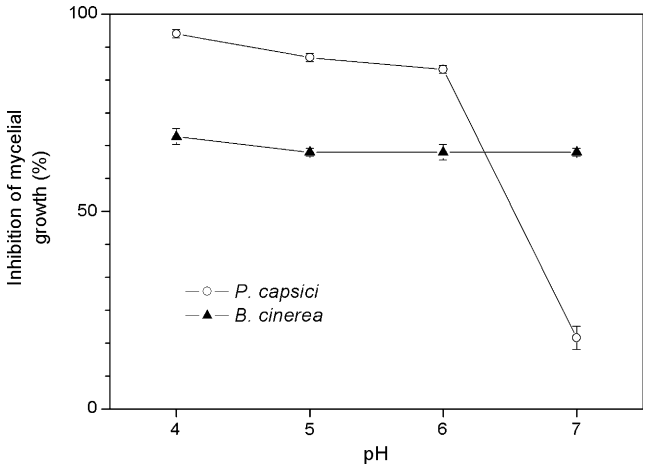

Fig. 2. Effect of pH on antifungal activity of oligochitosan against *P. capsici* and *B. cinerea*. Measurement of mycelial growth on pH-adjusted PDA amended with 1 mg ml^{-1} oligochitosan was performed three days after inoculation with *P. capsici* and *B. cinerea*.

Table 2
Effect of oligochitosan on different stages in the life cycle of *P. capsici*

Stages in the life cycle of *P. capsici*	Oligochitosan	
	EC_{50} (μg ml^{-1})[a]	MIC (μg ml^{-1})[b]
Zoosporangia production	0.6	5.3
Zoospore release	41.7	218.7
Zoospore rupture[c]	12.0[c]	100.1[d]
Cystospore germination	36.5	64.3

[a] The concentration inhibiting by 50%.
[b] The minimum inhibitory concentration showing over 90% inhibition.
[c] The concentration causing 50% zoospore rupture.
[d] The concentration causing 90% zoospore rupture.

hyphal cell was disordered and almost no vacuole could be observed (Figs. 5C and F). Thickening of plasmalemma (Figs. 6B and D) and appearance of tubular materials (Figs. 6B, E and F) linked with plasmalemma or vesicles in apical

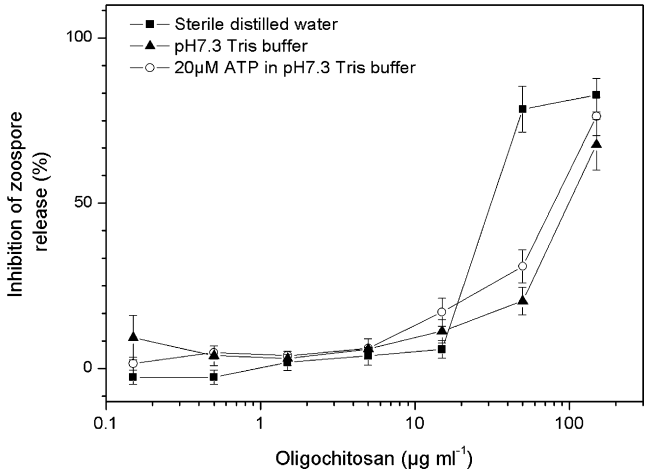

Fig. 3. Effect of ATP on zoospore release inhibition by oligochitosan. pH 7.3 Tris–HCl buffer with or without 20 μM ATP was added 10 min before addition of oligochitosan. Inhibition of oligochitosan on zoospore release was measured after 4 h.

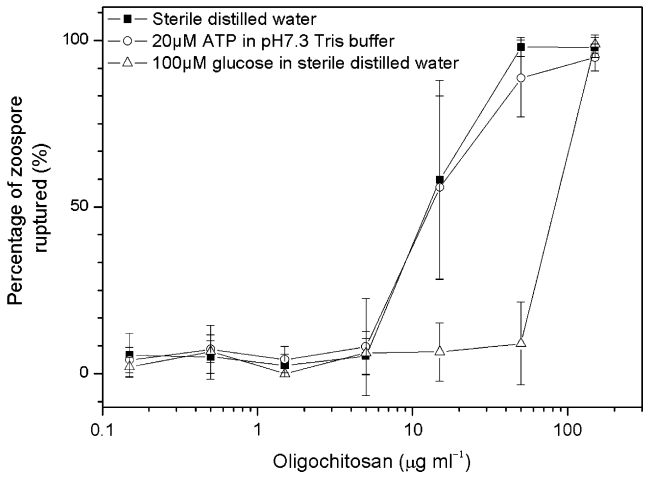

Fig. 4. Effect of glucose on zoospore rupture caused by oligochitosan. 100 mM glucose in sterile distill water was added 10 min before addition of oligochitosan. Zoospore rupture was measured after 60 min incubation.

were the most unique changes. Some swollen mitochondria were also observed (Fig. 5F) and plasmalemmasomes were also not found.

4. Discussion

Oligochitosan, with higher elicitor activity and water solubility than chitosan, has attracted much attention as an environmentally safe means of plant disease control [20,21,24,34]. However, little attention has been paid to its antifungal activity and related mode of action. We have successfully obtained oligochitosan with a DP of 3–9 by enzymatic hydrolysis and investigated its elicitor activity [32–35]. In the present study, we demonstrated that oligochitosan was more effective than chitosan in inhibiting mycelial growth of *P. capsici*. More importantly, investigation of oligochitosan on different stages in life cycle of *P. capsici* as well as study on ultrastructural alteration also indicated that different modes of action may exist in the antifungal mechanism of oligochitosan.

Of nine strains of phytopathogens tested in this study, mycelial growth of *P. capsici*, an rapidly growing oomycete with no chitosan in cell wall [38], was most sensitive to oligochitosan. Some conflict evidences for a correlation between antifungal activity of chitosan and the cell wall composition of fungi tested were found. It was suggested that presence of chitosan within the cell walls rendered some strains such as *Rhizopus nigricans* and *Mucor* sp. more resistant to 1 mg ml^{-1} chitosan [4]. While in other studies, growth inhibition has been observed on *Rhizopus stolonifer* [5,39] and *Mucor racemosus* [40] at similar concentrations. Thus, antifungal activity of chitosan seemed to vary even within fungal species. Different sensitivity to oligochitosan was also observed with two *Fusarium* species in our study. A large variety of fungal strains should be tested with oligochitosan to validate the proposition above.

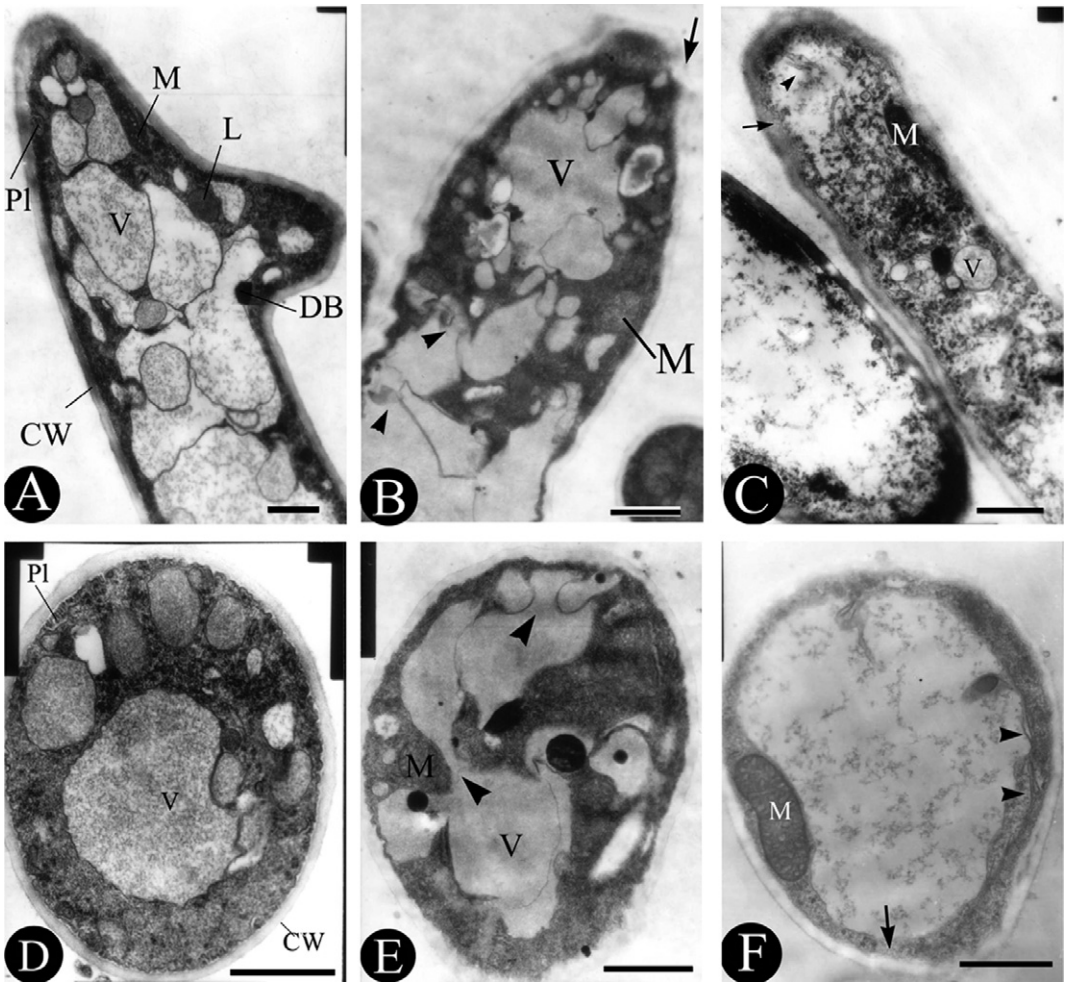

Fig. 5. Transmission electron micrographs of *P. capsici* hyphae. (A and D) – Longitudinal (A) and transverse (D) sections of *P. capsici* hyphae grown on Erwin solid medium in the absence of oligochitosan (control), showing normal cell wall (CW), vacuoles (V), lipid bodies (L), mitochondria (M), dense body (DB) and plasmalemmasome (Pl). (B and E) Longitudinal (B) and transverse (E) sections of *P. capsici* hyphae grown on Erwin solid medium containing 10 μg ml^{-1} oligochitosan. Note distorted and disorganized vacuoles (arrowheads) and the distortion of cell wall (arrow). Plasmalemmasome is no longer found. (C and F) Longitudinal (C) and transverse (F) sections of *P. capsici* hyphae grown on Erwin solid medium containing 100 μg ml^{-1} oligochitosan. Note disruption of most vacuoles, thickening of plasmalemma (arrow) and appearance of tubular materials (arrowheads) in apical hyphae. Plasmalemmasome is also not found. Scale bar = 1 μm.

Four strains with different sensitivity to oligochitosan were also tested for their sensitivity to chitosan in our study and *P. capsici* was the only strain that showed more sensitivity to oligochitosan at low concentrations (Fig. 1). Difference in sensitivity of *B. cinerea* to oligochitosan and chitosan depended on the concentration. Oligochitosan (purified hexamer or synthesized octamer) has exhibited higher antifungal activity against *Fusarium solani* than the lower DP oligomers and chitosan [22,24]. While our studies showed no difference between oligochitosan and chitosan on mycelial growth of *F. graminearum*. These diverging results may originate from the difference in degree of polymerization of oligochitosan used in the research. Because oligochitosan used in our research was a mixture of chitosan oligosaccharides with DP of 3–9.

Oligochitosan at low concentrations inhibited different stages in the life cycle of *P. capsici* including zoosporangia production, zoospore release, cystospore germination, and induced the rupture of released zoospore. It is well known that both zoospore release and zoospore rupture of *Phytophthora* are connected to impairment of the energy generation system [37,41] and osmotic pressure [42,43]. Addition of ATP (an energy supplier) or glucose (an osmotic stabilizer) prior to treatment with respiration inhibitor was able to reduce the inhibition on zoospore release and rupture of zoospore, respectively [37]. It is notable that addition of ATP was not able to reduce the inhibition of oligochitosan on zoospore release (Fig. 3) and zoospore rupture (Fig. 4). On the contrary, zoospore rupture was greatly reduced by addition of 100 mM glucose prior to treatment with 15–50 μg ml^{-1} oligochitosan (Fig. 4). Oligochitosan also induced leakage of electrolytes from mycelium of *P. capsici* (Junguang Xu, unpublished data) which has been found on different fungi treated with chitosan [5,8]. These observations indicate that, similar to chitosan, oligochitosan may act on the cell membrane by upsetting osmotic pressure.

Polycationic nature of chitosan is believed to be the key to its antimicrobial properties as has been shown in tests

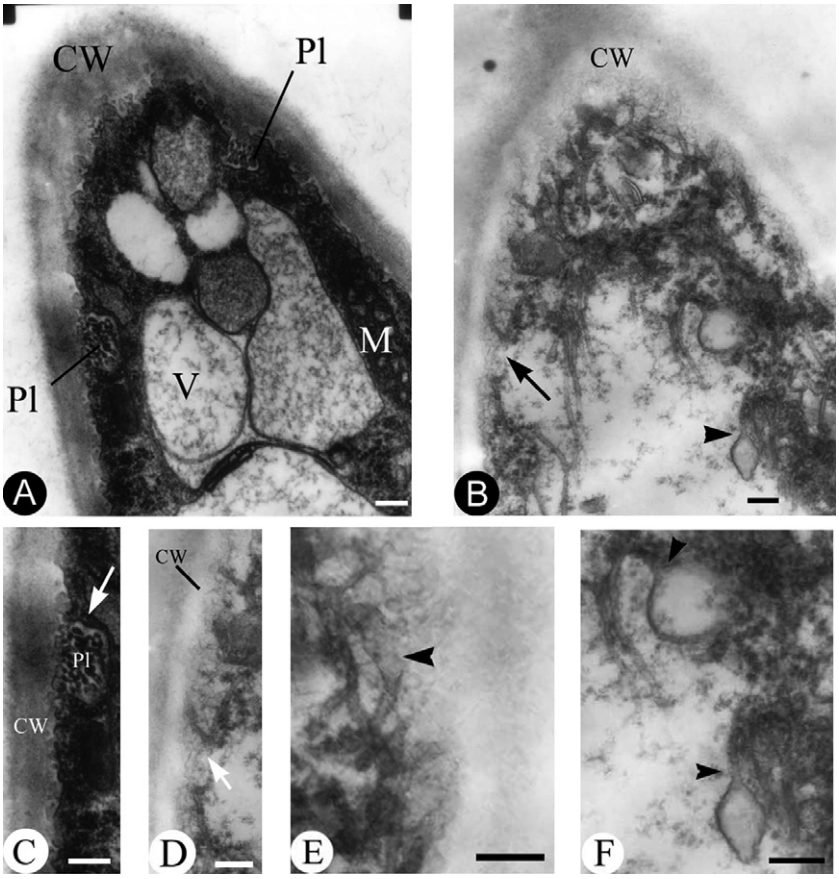

Fig. 6. Transmission electron micrographs of *P. capsici* hyphae tip. (A and C) Hyphal tip of *P. capsici* grown on Erwin solid medium in the absence of oligochitosan (control). Plasmalemmasomes (Pl) between cell wall (CW) and plasmalemma, shown in detail in (C), are clearly visible. (B, D, E and F) Hyphal tip of *P. capsici* grown on Erwin solid medium containing 100 μg ml^{-1} oligochitosan. (B) Vacuoles are not visible. Thickening of plasmalemma (arrow) and appearance of tubular materials (arrowheads) in apical hyphae are notable. (D) Thickening of plasmalemma (arrow) compared with normal thin layer plasmalemma (arrow in (C)). Tubular materials linked with plasmalemma (arrowhead in (E)) or vesicles (arrowheads in (F)) are clearly visible. Scale bar = 0.2 μm.

Table 3
Effect of oligochitosan on mycelial radial growth of *P. capsici* on Erwin medium

Oligochitosan (μg ml^{-1})	Inhibition (%) of mycelial growth ± std.[a]
1	1 ± 1
10	39 ± 1
100	86 ± 1

[a] Mean and SD of inhibition of mycelium radial growth ($n = 3$).

focusing on the influence of pH values [40,44,45]. The reason for low activity of chitosan at pH values above 7 was suggested to be reduction of both proportion of charged amino groups and solubility of chitosan. Because solubility of oligochitosan used here was not reduced at tested pH values (data not shown), reduction proportion of charged amino groups at high pH values would be the reason for activity reduction. In this study, effect of pH on antifungal activity of oligochitosan was quite confusing. At pH 7, inhibition of oligochitosan on mycelial growth of *P. capsici* reduced significantly from 86% to 28% while no reduction was found on *B. cinerea* (Fig. 2). Moreover, effect of oligochitosan on zoospore release, but not zoospore rupture, was reduced in Tris–HCl buffer (pH 7.3) (Figs. 3 and 4). These results clearly indicate that polycationic nature of oligochitosan contributes only partly to its antifungal activity and multiple modes of action of oligochitosan exist.

Electron microscopic studies have been carried out for understanding of the action of chitosan and its derivatives on different fungi [5,6,12,46–50]. Chitosan and its derivatives induced marked structural alterations including cell wall loosening, vacuolation, and protoplasm degradation. Moreover, a histochemical analysis on *Rhizopus stolonifer* with chitin specified wheat germ agglutinin/ovomucoid–gold complex has suggested that cell wall loosening was the result of upset balance between biosynthesis and turnover of chitin at the hyphal apex [5]. In this study, only slight distortion on some hyphal cell wall of *P. capsici* was observed in presence of 10 μg ml^{-1} oligochitosan. Considering the fact that no chitosan presences in the cell wall of *P. capsici*, it is not strange to see this result. The most profound structural alteration of *P. capsici* treated with oligochitosan was the disruption of the endomembrane system, especially vacuole and secretory vesicles such as plasmalemmasomes. The vacuoles in the hyphal tips of *P. capsici* treated with 10 and 100 μg ml^{-1} oligochitosan were observed to be distorted and disrupted totally, respectively. Alteration of vacuoles

also accorded well with inhibition extent of oligochitosan on mycelial growth (Table 3). Vacuole plays an important role in maintaining the fungal turgor pressure and it is more important for mycelial growth of aseptate oomycetes [38]. Thus, it may well explain the higher sensitivity of mycelial growth of *P. capsici* to oligochitosan than other pathogens. Plasmalemmasome, a membranous structure exists between fungal cell wall and plasmalemma [51,52], has been found abundantly during the period of active cell growth and was supposed to be the secretory vesicle related with cell wall synthesis [53]. In this study, plasmalemmasomes observed in control cells of *P. capsici* were not detected in oligochitosan treated cells. Hence, oligochitosan may also retard fungal growth by indirectly hindering cell wall synthesis. Changes including thickening of plasmalemma and appearance of tubular materials linked with plasmalemma or vesicles in apical hyphae may be the result of disruption of endomembrane. Further investigation is undergoing to elucidate the composition of the unique tubular materials appeared in the hyphal tip cells. We are not, however, able to address whether oligochitosan disrupts endomembrane system indirectly by binding to the cell surface or directly from inside the cell as has been found in bacteria.

In conclusion, our results showed that oligochitosan exhibited higher antifungal activity than chitosan against *P. capsici*. Oligochitosan also inhibited different stages in the life cycle of *P. capsici*. Polycationic nature of oligochitosan contributed only partly to its antifungal activity and multiple modes of action of oligochitosan existed including the disruption of endomembrane system. An investigation with fluorescent labeled oligochitosan is under progress in our laboratory to find the exact action site of oligochitosan.

Acknowledgments

We thank Prof. Zhouchuan Gao for help in preparing and examining the ultrathin sections. We thank Prof. Keyi Wang and Fanzuo Kong for revising this paper. This research was supported by Hi-Tech Research and Development program of China (Nos. 2003AA625010 and 2002AA245131).

References

[1] I.S. Arvanitoyannis, A. Nakayama, S.i. Aiba, Chitosan and gelatin based edible films: state diagrams, mechanical and permeation properties, Carbohydr. Polym. 37 (1998) 371–382.

[2] E.I. Rabea, M.E.T. Badawy, C.V. Stevens, G. Smagghe, W. Steurbaut, Chitosan as antimicrobial agent: Applications and mode of action, Biomacromolecules 4 (2003) 1457–1465.

[3] S. Bautista-Banos, A.N. Hernandez-Lauzardo, M.G. Velazquez-del Valle, M. Hernandez-Lopez, E. it Barka, E. Bosquez-Molina, C.L. Wilson, Chitosan as a potential natural compound to control pre and postharvest diseases of horticultural commodities, Crop Prot. 25 (2006) 108–118.

[4] C.R. Allan, L.A. Hadwiger, The fungicidal effect of chitosan on fungi of varying cell wall composition, Exp. Mycol. 3 (1979) 285–287.

[5] A. Elghaouth, J. Arul, A. Asselin, N. Benhamou, Antifungal activity of chitosan on postharvest pathogens – induction of morphological and cytological alterations in *Rhizopus stolonifer*, Mycol. Res. 96 (1992) 769–779.

[6] A. Elghaouth, J. Arul, J. Grenier, N. Benhamou, A. Asselin, R. Belanger, Effect of chitosan on cucumber olants – suppression of *Phythium aphanidermatum* and induction of defense reactions, Phytopathology 84 (1994) 313–320.

[7] Y. Baba, T. Yamashita, Y. Kawano, Y. Uchida, Antifungal activity of aqueous soluble chitosan derivatives on *Fusarium* and *Verticillium*, Nippon Kagaku Kaishi (1996) 48–53.

[8] M.V.B. Reddy, J. Arul, E. Ait-Barka, P. Angers, C. Richard, F. Castaigne, Effect of chitosan on growth and toxin production by *Alternaria alternata* f. sp. *lycopersici*, Biocontrol Sci. Technol. 8 (1998) 33–43.

[9] M.V.B. Reddy, P. Angers, F. Castaigne, J. Arul, Chitosan effects on blackmold rot and pathogenic factors produced by *Alternaria alternata* in postharvest tomatoes, J. Am. Soc. Hortic. Sci. 125 (2000) 742–747.

[10] N. BenShalom, R. Ardi, R. Pinto, C. Aki, E. Fallik, Controlling gray mould caused by *Botrytis cinerea* in cucumber plants by means of chitosan, Crop Prot. 22 (2003) 285–290.

[11] M. Zhang, T.W. Tan, Insecticidal and fungicidal activities of chitosan and oligo-chitosan, J. Bioact. Compat. Pol. 18 (2003) 391–400.

[12] A. Elghaouth, J. Arul, C. Wilson, N. Benhamou, Ultrastructural and cytochemical aspects of the effect of chitosan on decay of bell pepper fruit, Physiol. Mol. Plant Pathol. 44 (1994) 417–432.

[13] A. Zakrzewska, A. Boorsma, S. Brul, K.J. Hellingwerf, F.M. Klis, Transcriptional response of *Saccharomyces cerevisiae* to the plasma membrane-perturbing compound chitosan, Eukaryot. Cell 4 (2005) 703–715.

[14] J.L. Leuba, P. Stossel, Chitosan and other polyamines: antifungal activity and interaction with biological membranes, in: R. Muzzarelli, C. Jeuniaux, G.W. Gooday (Eds.), Chitin in Nature and Technology, Plenum Press, New York, 1986, pp. 215–222.

[15] E.I. Rabea, M.E. Badawy, W. Steurbaut, T.M. Rogge, C.V. Stevens, G. Smagghe, M. Hofte, Fungicidal effect of chitosan derivatives containing an *N*-alkyl group on grey mould *Botrytis cinerea* and rice leaf blast *Pyricularia grisea*, Commun. Agric. Appl. Biol. Sci. 70 (2005) 219–223.

[16] M.E. Badawy, E.I. Rabea, W. Steurbaut, T.M. Rogge, C.V. Stevens, G. Smagghe, M. Hofte, Fungicidal activity of some *O*-acyl chitosan derivatives against grey mould *Botrytis cinerea* and rice leaf blast *Pyricularia grisea*, Commun. Agric. Appl. Biol. Sci. 70 (2005) 215–218.

[17] E.I. Rabea, M.E. Badawy, T.M. Rogge, C.V. Stevens, M. Hofte, W. Steurbaut, G. Smagghe, Insecticidal and fungicidal activity of new synthesized chitosan derivatives, Pest. Manag. Sci. 61 (2005) 951–960.

[18] M.E. Badawy, E.I. Rabea, T.M. Rogge, C.V. Stevens, G. Smagghe, W. Steurbaut, M. Hofte, Synthesis and fungicidal activity of new *N*,*O*-acyl chitosan derivatives, Biomacromolecules 5 (2004) 589–595.

[19] S.K. Kim, N. Rajapakse, Enzymatic production and biological activities of chitosan oligosaccharides (COS): A review, Carbohy. Polym. 62 (2005) 357–368.

[20] W. Lin, X. Hu, W. Zhang, W.J. Rogers, W. Cai, Hydrogen peroxide mediates defence responses induced by chitosans of different molecular weights in rice, J. Plant Physiol. 162 (2005) 937–944.

[21] P. Vander, K.M. Varum, A. Domard, G.N. Eddine El, B.M. Moerschbacher, Comparison of the ability of partially N-acetylated chitosans and chitooligosaccharides to elicit resistance reactions in wheat leaves, Plant Physiol. 118 (1998) 1353–1359.

[22] D.F. Kendra, L.A. Hadwiger, Characterization of the smallest chitosan oligomer that is maximally antifungal to *Fusarium solani* and elicits pisatin formation in *Pisum sativum*, Exp. Mycol 8 (1984) 276–281.

[23] A. Kobayashi, K. Akiyama, K. Kawazu, Partially N-deacetylated chitin fragments are strong elicitors for (+)-pisatin induction in epicotyls of pea, Z. Naturforsch. C. 49 (1994) 302–308.

[24] L.A. Hadwiger, T. Ogawa, H. Kuyama, Chitosan polymer sizes effective in inducing phytoalexin accumulation and fungal suppression are

verified with synthesized oligomers, Mol. Plant Microbe Interact. 7 (1994) 531–533.
[25] S.H. Doares, T. Syrovets, E.W. Weiler, C.A. Ryan, Oligogalacturonides and chitosan activate plant defensive genes through the octadecanoid pathway, Proc. Natl. Acad. Sci. USA 92 (1995) 4095–4098.
[26] X.F. Liu, Y.L. Guan, D.Z. Yang, Z. Li, K. De Yao, Antibacterial action of chitosan and carboxymethylated chitosan, J. Appl. Polym. Sci. 79 (2001) 1324–1335.
[27] S. Tokura, K. Ueno, S. Miyazaki, N. Nishi, Molecular weight dependent antimicrobial activity by chitosan, Macromol. Symp. 120 (1997) 1–9.
[28] Y.C. Chung, Y.P. Su, C.C. Chen, G. Jia, H.I. Wang, J.C.G. Wu, J.G. Lin, Relationship between antibacterial activity of chitosan and surface characteristics of cell wall, Acta Pharm. Sin. 25 (2004) 932–936.
[29] F. Yang, X. Cui, X. Yang, Interaction of low-molecular-weight chitosan with mimic membrane studied by electrochemical methods and surface plasmon resonance, Biophys. Chem. 99 (2002) 99–106.
[30] H. Liu, Y. Du, X. Wang, L. Sun, Chitosan kills bacteria through cell membrane damage, Int. J. Food Microbiol. 95 (2004) 147–155.
[31] I.M. Helander, E.L. Nurmiaho-Lassila, R. Ahvenainen, J. Rhoades, S. Roller, Chitosan disrupts the barrier properties of the outer membrane of Gram-negative bacteria, Int. J. Food Microbiol. 71 (2001) 235–244.
[32] H. Zhang, Y. Du, X. Yu, M. Mitsutomi, S. Aiba, Preparation of chitooligosaccharides from chitosan by a complex enzyme, Carbohydr. Res. 320 (1999) 257–260.
[33] Y. Du, X. Bai, X. Zhao, H. Jiang, The effect of oligochitosan on the activity of defensive enzyme and zymogram of isoenzyme in tobacco leaves, Chin. J. Biol. Control 18 (2002) 83–86.
[34] X. Zhao, X. She, Y. Du, X. Liang. Induction of antiviral resistance and stimulatory effect by oligochitosan in tobacco. Pest. Biochem. Physiol, 2006, in press, doi:10.1016/j.pestbp.2006.06.006.
[35] B. Feng, C. Wang, Y. Chen, X. Bai, Y. Du, mRNA different display analysis of plant resistance-related gene induced by oligochitosan, Biotechnology 14 (2004) 5–7.
[36] D.H. Young, Effects of zarilamide on microtubules and nuclear division in *Phytophthora capsici* and tobacco suspension cultured cells, Pest. Biochem. Physiol. 40 (1991) 149–161.
[37] S. Mitani, S. Araki, T. Yamaguchi, Y. Takii, T. Ohshima, N. Matsuo, Antifungal activity of the novel fungicide cyazofamid against *Phytophthora infestans* and other plant pathogenic fungi *in vitro*, Pest. Biochem. Physiol. 70 (2001) 92–99.
[38] D.C. Erwin, O.K. Ribeiro, *Phytophthora* Disease Worldwide, APS Press, St. Paul, MN, 1996, p. 86.
[39] A. Elghaouth, J. Arul, J. Grenier, A. Asselin, Antifungal activity of chitosan on 2 postharvest pathogens of strawberry fruits, Phytopathology 82 (1992) 398–402.
[40] S. Roller, N. Covill, The antifungal properties of chitosan in laboratory media and apple juice, Int. J. Food Microb. 47 (1999) 67–77.
[41] N. Andrieu, G. Jaworska, J.L. Genet, G. Bompeix, Biological mode of action of Famoxadone on *Plasmopara viticola* and *Phytophthora infestans*, Crop Prot. 20 (2001) 253–260.
[42] U. Gisi, G.A. Zentmyer, Mechanism of zoospore release in *Phytophthora* and *Pythium*, Exp. Mycol. 4 (1980) 362–377.
[43] J. Ambikapathy, J.S. Marshall, C.H. Hocart, A.R. Hardham, The role of proline in osmoregulation in *Phytophthora nicotianae*, Fungal. Genet. Biol. 35 (2002) 287–299.
[44] M. Jumaa, F.H. Furkert, B.W. Muller, A new lipid emulsion formulation with high antimicrobial efficacy using chitosan, Eur. J. Pharm. Biopharm. 53 (2002) 115–123.
[45] N.R. Sudarshan, D.G. Hoover, D. Knorr, Antibacterial action of chitosan, Food Biotechnol. 6 (1992) 257–272.
[46] L.A. Hadwiger, J.M. Beckman, M.J. Adams, Localization of fungal components in the Pea–*Fusarium* interaction detected immunochemically with anti-chitosan and anti-fungal cell wall antisera, Plant Physiol. 67 (1981) 170–175.
[47] R.A.A. Muzzarelli, C. Muzzarelli, R. Tarsi, M. Miliani, F. Gabbanelli, M. Cartolari, Fungistatic activity of modified chitosans against *Saprolegnia parasitica*, Biomacromolecules 2 (2001) 165–169.
[48] M. Plascencia-Jatomea, G. Viniegra, R. Olayo, M.M. Castillo-Ortega, K. Shirai, Effect of chitosan and temperature on spore germination of *Aspergillus niger*, Macromol. Biosci. 3 (2003) 582–586.
[49] R. Muzzarelli, R. Tarsi, O. Filippini, E. Giovanetti, G. Biagini, P.E. Varaldo, Antimicrobial properties of *N*-carboxybutyl chitosan, Antimicrob. Agents Chemother. 34 (1990) 2019–2023.
[50] P.J. LaFontaine, N. Benhamou, Chitosan treatment: An emerging strategy for enhancing resistance of greenhouse tomato plants to infection by *Fusarium oxysporum* f sp *radicis-lycopersici*, Biocontr. Sci. Technol. 6 (1996) 111–124.
[51] R.S. Mehrotra, K.R. Aneja, An Introduction to Mycology, Wiley Eastern Ltd., New Delhi, 1990, p. 19.
[52] R. Marchant, R.T. Moore, Lomasomes and plasmalemmasomes in fungi, Protoplasma 76 (1973) 235–247.
[53] K.C. Rajasingham, R.A. Cawson, Plasmalemmasomes and lomasomes in *Candida albicans*, Cytobios 40 (1984) 21–25.