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

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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

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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