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Variation of Antifungal Activities of Chitosans on Plant Pathogens

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Abstract The effect of chitosan on the growth of plant pathogenic fungi was investigated. Chitosan solubilized in acetic acid showed much higher and more consistent antifungal activity than that solubilized in HCl. The antifungal activity was not significantly affected within a DA (degree of deacetylation) range of 57.3–99.2% tested. Water-soluble and low molecular weight chitosan (57.3% DA) against 6 plant pathogens showed that *Monosporascus canonballus* and *Pythium irregulare* were the most susceptible to the chitosan, while *Fusarium oxysporum* and *F. graminearum* were the most resistant. At a concentration of 2.5 mg/ml, the growth of pathogens was completely inhibited except for *F. oxysporum*. The MIC_{50} values varied depending on both the DA of the chitosan and the plant pathogens. A chitosan with 57.3% DA exhibited the lowest MIC_{50} (ranging <0.1–1.8 mg/ml) and that with 84.7% DA the highest MIC_{50} (ranging <0.4–4.0 mg/ml) depending on the pathogen.

Key words: Chitosan, antifungal activity, degree of deacetylation, MIC_{50}

Chitosan, a high molecular weight cationic polysaccharide, has already been shown to be fungicidal against several fungi [1, 7, 11, 14]. The inhibitory effect of chitosan has also been demonstrated with soilborne phytopathogenic fungi [23]. Stossel and Leuba [23] showed that the inhibitory activity of chitosan is higher at pH 6.0 (*pKa* value of chitosan = 6.2) than at pH 7.5, when most amino groups are in free-base form.

El Ghaouth *et al.* [5] found that the chitosan coating of strawberries is effective in reducing the decay of the fruit caused by *Botrytis cinerea* and *Rhizopus stolonifer*, since chitosan inhibits spore germination and radial growth of both fungi. However, since complete inhibition is not achieved even at a high concentration (6 mg/ml), this indicates that chitosan is fungistatic rather than fungicidal. Benhamou *et al.* [3] demonstrated by light and electron microscopies that chitosan can induce gross morphological alterations, including hypal swelling, increased vacuolation, retraction and alterations of plasma membrane, cytoplasm aggregation, and abnormal cell wall deposition. However, a question of whether such disturbances in the overall fungal cell organization are a direct effect of chitosan remains to be a matter of speculation.

Chitosan is also known to be a potential elicitor of many plant defenses, including accumulation of chitinase [4, 18, 20] and chitosanase [8], synthesis of proteinase inhibitors in tomato leaves [25], lignification in wheat leaves [21], and induction of callose synthesis [13] and phytoalexin [10, 25]. Thus, chitosan appears to play a dual function, by directly interfering with fungal growth and also by activating several biological processes in plant tissues.

The antifungal efficacy of chitosan is known to depend on its physical properties, such as solubility, degree of deacetylation (DA), and molecular weight [13, 24]. Uchida [24] reported that the higher the degree of deacetylation of chitosan, the stronger the antifungal activity against *Fusarium solani*. Kendra *et al.* [14] also demonstrated that the maximal antifungal and pisatin-inducing activities of chitosan were exhibited by chitosan oligomers of seven or more residues.

Recently, alternative approaches have been developed to reduce the incidence of plant disease without negative aspects of hazardous pesticides. One such approach involves the use of bioactive substances such as chitosan. Previous research using chitosan as a fungistatic and antifungal has been conducted against apple white rot (*Botryosphaeria dothidea*) [16], tomato crown and root rot (*Fusarium oxysporum* f. sp. *radicis-lycopersici*) [2, 3], cucumber rot (*Pythium aphanidermatum*), and strawberry gray mold rot (*Botrytis cinerea* Pers: Fr) [6]. However, these studies focused only on the antifungal activity of chitosan against very few fungi, i.e., mainly against *Fusarium*

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spp., and did not carefully define the physical properties of chitosan [2, 3, 5, 6, 22]. Moreover, to apply chitosan as an alternative or additive for the control of fungal diseases, more defined information is needed on its antifungal effects against various fungi causing plant diseases.

Accordingly, the objectives of the current study were to demonstrate the antifungal effect of chitosan with various degrees of deacetylation and determine the MIC_{50} of chitosan against various pathogenic fungi.

MATERIALS AND METHODS

Pathogenic Fungi

The plant pathogens *Didymella bryoniae* DO 96-26, *Fusarium gramineanum* FG 93-36, *Fusarium oxysporum*, *Monosporascus canonballus*, *Pythium irregulare*, and *Rhizoctonia solani* 40139 were all obtained from the National Institute of Agricultural Science and Technology (NIAST) (Suwon, Korea), and maintained on a potato-dextrose agar (PDA).

Chitosans and Solubilizations

The chitosans (84.7% DA, MW 150 kD and 99.2% DA, MW 120 kD) and chitin (2.5% DA, MW 200 kD) were obtained from Taehoon Bio (Seoul, Korea) and the water-soluble chitosan (DA 57.3%, MW 3–20 kD) from Wako Pure Chemical Industries Co. Ltd. (Osaka, Japan), and used as received. The swollen chitin was prepared according to Monreal and Reese [19]. The chitosan was dissolved in 0.25 N HCl and neutralized to pH 5.6 with 2 N NaOH according to Benhamou *et al.* [3]. The chitosan was also dissolved in acetate buffer (pH 5.5) according to Izume and Ohtakara [12].

Antifungal Assays

The antifungal assays were carried out on PDA plates amended with chitosan at different concentrations. The PDA plates amended with chitosan or chitin were prepared as follows [5]. The chitosan solution was autoclaved and subsquently added to sterile molten PDA to obtain chitosan concentrations of 0, 0.125, 0.25, 0.5, 1.0, and 2.5 mg/ml. Aliqouts of 20 ml of these solutions were immediately dispensed into 87 mm-diameter polystyrene petri plates. The plates were then seeded with 5 mm-diameter mycelial plugs taken from the margin of 3–7 day-old cultures, which were dependent on the growth rate of the fungus. Three replicates of 5 plates were used for each fungus at each concentration of chitosan, and the plates were incubated in dark at 27±1°C. The fungal growth was recorded at 1-day intervals until the control (0 mg/ml of chitosan) reached the edge of the plate. The test was repeated twice. The growth inhibition was expressed as the percentage of inhibition of radial growth relative to the control [2]. That is, 1-ds/dc×100, where ds stands for the radial growth diameter in the chitosan-amended plate and dc for the diameter in the control plate.

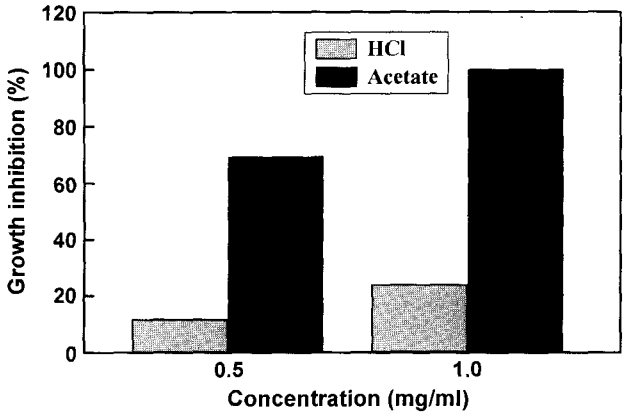

Fig. 1. Effect on antifungal activity of chitosan (84.7% DA) by solubilization media.
Chitosan was solubilized in acetic acid or HCl and amended to PDA at the final concentrations of 0.5 or 1.0 mg/ml. *Monosporascus canonballus* was inoculated, and the radial growth was recorded, as in Materials and Methods.

RESULTS

Effect of Chitosan Solubilization Solvents

Chitosan has previously been solubilized in acidic solvents, most often in acetic acid and HCl, and we also investigated the antifungal activity of chitosan solubilized in these two solvents. As shown in Fig. 1, the chitosan solubilized in acetic acid exhibited much higher and more consistent antifungal activity against *M. canonballus* than the chitosan in HCl. At 0.5 mg/ml concentration, 70% growth inhibition was observed in the plates amended with chitosan-acetic

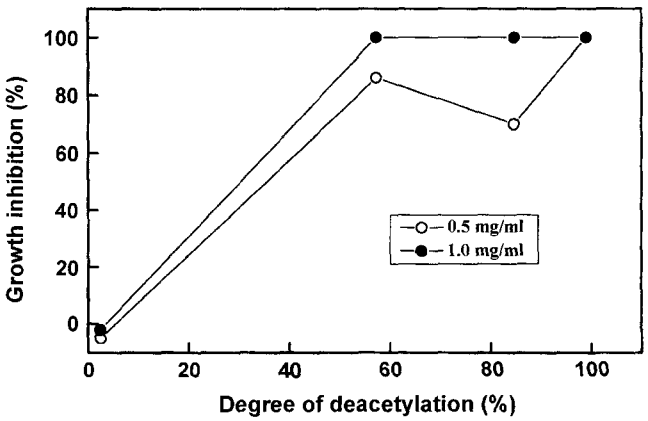

Fig. 2. Effect of degree of deacetylation on antifungal activity of chitosan.
Each chitosan varying in DA was amended to PDA at the final concentrations of 0.5 or 1.0 mg/ml. *Monosporascus canonballus* was inoculated, and the radial growth recorded, as in Materials and Methods.

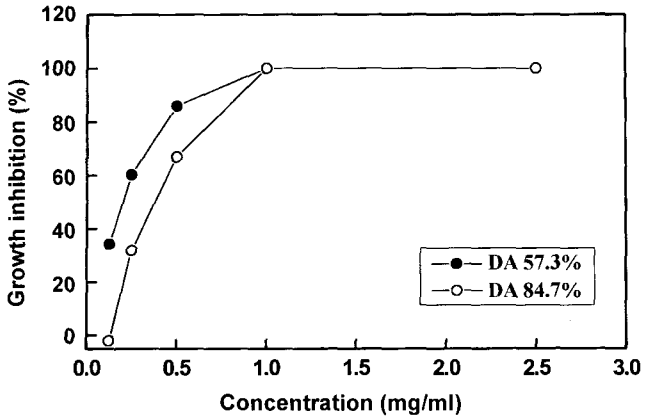

Fig. 3. Dependence of antifungal activity on concentration of chitosan.
Chitosan with 57.3% and 84.7% DA was amended to PDA at specified concentrations. *Monosporascus canonballus* was inoculated and the radial growth was recorded, as in Materials and Methods.

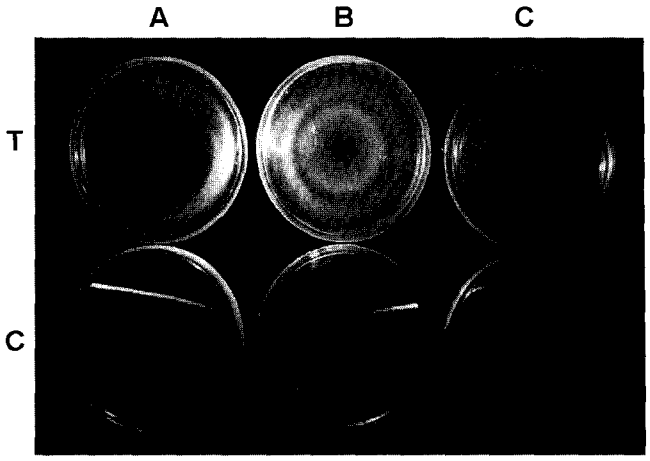

Fig. 4. Effect of chitosan (57.3% DA) on growth of *Didymella bryoniae* (A), *Fusarium oxysporum* (B), and *Rhizoctonia solani* (C) at the concentration of 1.0 mg/ml (upper row T) and without chitosan (lower row C).

acid, while only 10% inhibition was observed in the chitosan-HCl. The chitosan-HCl produced less consistent results due to aggregation of the chitosan in the culture medium, and less efficient antifungal activity possibly due to the lower accessibility of the chitosan to the cell wall components [23]. Therefore, acetic acid was chosen as the chitosan solubilization solvent in all the following experiments.

Effect of Degree of Deacetylation

Figure 2 shows the effect of DA of chitosan on the antifungal activity against *M. canonballus*. Four chitosans (2.5, 57.3, 84.7, and 99.2% DA) were tested as antifungals. The antifungal activity was not significantly affected within the range of 57.3–99.2% DA tested. However, the chitosan with 2.5% DA did not exhibit the antifungal activity. Chitosan contains primary amino groups in its structure and the number of these amino groups is related to its antifungal activity [1].

Effect of Chitosan Concentration

The effect of chitosan concentration on the antifungal activity against *M. canonballus* is shown in Fig. 3. The growth of the fungus was completely inhibited at 1.0 mg/ml concentration with either 57.3% or 84.7% DA. In the case of 99.2% DA, over 80% radial growth inhibition was observed even in the plates amended with only 0.125 mg/ml chitosan (data not shown).

Inhibition of Radial Growth of Various Pathogenic Fungi by 57.3% DA Chitosan

Table 1 shows the radial growth inhibition of 57.3% DA water-soluble and low molecular chitosan against 6 plant pathogens. *M. canonballus* and *P. irregulare* were the most susceptible to chitosan, and *F. oxysporum* and *F. gramineanum* were the most resistant. At a concentration of 2.5 mg/ml, the growth of most pathogens, except *F. oxysporum* and *P. irregulare*, was completely inhibited. At the same concentration, the degree of inhibition rate against *F. oxysporum* was 71.7%. At 1.0 mg/ml concentration, over 80% growth inhibition was observed with *M. canonballus* and *P. irregulare*, yet little inhibition with any of the other pathogens. Figure 4 shows the growth of *D.*

Table 1. Inhibition rate of radial growth of various pathogenic fungi by 57.3% DA chitosan.

Pathogen	Concentration (mg/ml)				
	0.125	0.25	0.5	1.0	2.5
	(%)				
Didymella bryoniae DO96-26	11.7	14.0	15.2	33.6	100.0
Fusarium graminearum FG93-36	−7.4	1.9	11.8	25.5	100.0
Fusarium oxysporum	3.9	2.2	11.0	21.8	71.7
Monosporascus canonballus	34.3	60.4	86.0	100.0	100.0
Pythium irregulare	58.3	68.3	77.9	83.1	91.8
Rhizoctonia solani 40139	11.9	11.0	19.7	42.4	100.0

Table 2. MIC_{50} values of three chitosans against various plant pathogens.

Pathogen	Chitosan		
	DA 57.3%	DA 84.7%	DA 99.2%
	(mg/ml)		
Didymella bryoniae DO96-26	1.4	3.2	1.6
Fusarium graminearum FG93-36	1.5	4.0	1.7
Fusarium oxysporum	1.8	3.2	2.5
Monosporascus canonballus	0.2	0.4	<0.1
Pythium irregulare	<0.1	1.0	0.3
Rhizoctonia solani 40139	1.0	3.4	2.2

bryoniae, *F. oxysporum*, and *R. solani* in the presence of 1.0 mg/ml chitosan with 57.3% DA.

MIC_{50} of Three Chitosans Against Various Plant Pathogens

The MIC_{50} of three chitosans was measured for each pathogen. As shown in Table 2, the MIC_{50} values were dependent on DA of the chitosan and the plant pathogens. The chitosan with 57.3% DA exhibited the lowest MIC_{50} (ranging <0.1–1.8 mg/ml), while 84.7% DA exhibited the highest MIC_{50} (ranging <0.4–4.0 mg/ml), depending on the pathogens. Among the six pathogens, *M. canonballus* and *P. irregulare* were highly susceptible to the chitosans (MIC_{50} of <0.1–0.4 mg/ml and <0.1–1.0 mg/ml, respectively).

DISCUSSION

The currect study demonstrated that the antifungal activity of chitosan was related to the solubilization method, degree of deacetylation, and fungal species. Although chitosan is generally dissolved in acetic acid, El Ghaouth *et al.* [5] dissolved chitosan in 0.25 N HCl and then neutralized it with 2 N NaOH to test its antifungal activity on the postharvest pathogens of strawberry fruits. Walker-Simmons *et al.* [25] and Lee *et al.* [16] dissolved chitosan in 0.01 M phosphate buffer (pH 6.5) with a small amount of glacial acetic acid. In the current study, the antifungal activity of chitosan exhibited a great variation depending on whether the chitosan was dissolved in acetic acid or HCl, therefore, the result on the antifungal activity should be carefully compared.

Allan and Hadwiger [1] reported that the positively charged amino groups in chitosan inhibit the growth of fungi or microbacteria through polyelectrolyte complexes with negatively charged carboxyl groups present in their cell walls. This suggests that the higher the number of amino groups, the higher the antimicrobial activity. In the current study, chitin with 2.5% DA exhibited little antifungal activity, while chitosan with 57.3% DA or more showed a strong activity, as shown in Fig. 2, thereby indicating that the free amino groups of chitosan were involved in the physical interaction of the chitosan molecules with the fungus.

The MIC_{50} values were found to be dependent on the DA of the chitosan and the plant pathogens. The values ranged from <0.1 up to 4.0 mg/ml, depending on the pathogen and the DA% of the chitosan. *M. canonballus* and *Pythium irregulare* were the most susceptible to the chitosans. Uchida [24] observed that chitosan at 1.0 mg/ml concentration completely inhibits the growth of *F. solani*, while *Botrytis* sp., *Pestalotia* sp., *Rhizopus* sp., *Mucor* sp., *Penicillium* sp., and *Aspergillus* sp. are less inhibited at the same concentration. Allan and Hadwiger [1] also reported that chitosan (1.0 mg/ml) is effective in reducing the radial growth of most fungi, except those containing chitosan as a major cell wall component (i.e., *Rhizopus* sp. and *Mucor* sp.). Thus, the activity of chitosan would seem to be highly related to the cell wall structure and composition.

Although chitosan is known to affect the growth of most fungi, the mechanism involved in its antifungal action has not yet been fully elucidated. Two models have been proposed to explain the antifungal activity of chitosan. In the model of Hadwiger and Loschke [9], the interaction of chitosan with the fungal DNA and mRNA is the basis of its antifungal effect. On the other hand, Stossel and Leuba [23] suggested that the activity of chitosan is related to its ability to interfere with the plasma membrane function. El Ghaouth *et al.* [5, 6] found that chitosan is very effective in inhibiting the spore germination, germ tube elongation, and radial growth of *Botrytis cinerea* and *Rhizopus stolonifer* in a culture, and also that *B. cinerea* is more susceptible to chitosan than *R. stolonifer*. This indicates that the growth inhibition by chitosan is dependent on the fungal species, which was also demonstrated in the current study. To date, this is the first report on the growth inhibition of *Didymella* sp., *Monosporascus* sp., and *Rhizoctonia* sp. by chitosan. To elucidate the interaction of chitosan with the cell wall structure, further studies on the effect of structurally modified chitosan on the growth of microorganisms are currently in progress.

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