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Antifungal effect of chito-oligosaccharides with different degrees of polymerization

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Abstract:	Chitosan, obtained from chitin by partial N-deacetylation, shows little or no toxicity towards mammalian cells, is biodegradable, and non-allergenic. It is known that chitosan may have antifungal properties, but the effect of defined chitosan or chito-oligosaccharides (CHOS) with different degree of polymerization is not well known. The objective of this study was to produce CHOS with different DPn (average degree of polymerization) and determine the most effective DPn of chitosan and CHOS against Botrytis cinerea Pers. Ex Fr. and Mucor piriformis Fischer. In vitro testing showed that CHOS of DPn 23 and 40 had the highest germination inhibition against the tested pathogens. The original chitosan (DPn 206) and a collection of short CHOS (degree of polymerization of 3-10) were significantly ($P<0.01$) less effective than CHOS of DPn 23 and 40. M. piriformis M119J showed the most abnormal swelling in presence of CHOS DPn 40, but all abnormally swollen conidia showed further germ tube elongation. In vivo testing showed that CHOS DPn 23 was the most effective in reducing flower infection by two isolates of B. cinerea. Our results show that CHOS inhibit fungal germination and growth and that the effect depends highly on the level of polymerization of the oligomers.
Response to Reviewers:	Dear Editor, We are very grateful for the positive and helpful review of our paper entitled “Antifungal effect of chito-oligosaccharides with different degrees of polymerization”. We have addressed all of your suggested changes for revision as outline below and hope that our manuscript is acceptable for publication in European Journal of Plant Pathology. Sincerely, Morten Sørlie

	Dear Professor Morten Sørlie, I have evaluated the revised version of your manuscript which is very much improved compared to the previous version. However, the manuscript will need another round of revision and editing before it will be acceptable for publication. The first part of the introduction (line 4 to 37) is not well structured and some of it is too general. I suggest that you remove the sentence: "B. cinerea is also the main production constraint..." (line 15-20) as well as "Chemical fungicides are the primary means to control fungal diseases6, and" (line 22 to 24). Response: We appreciate this input and have removed the sentences as suggested. In addition, the manuscript has to be prepared according to the Instructions for Authors. Cite references in the text by name and year in parentheses and, reference list entries should be alphabetized by the last names of the first author of each work. Response: The references are now cited using the standards of “European Journal of Plant Pathology”. With kind regards, Christine Struck Associate Editor

Antifungal effect of chito-oligosaccharides with different degrees of polymerization

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Abstract

Chitosan, obtained from chitin by partial *N*-deacetylation, shows little or no toxicity towards mammalian cells, is biodegradable, and non-allergenic. It is known that chitosan may have antifungal properties, but the effect of defined chitosan or chito-oligosaccharides (CHOS) with different degree of polymerization is not well known. The objective of this study was to produce CHOS with different DP_n (average degree of polymerization) and determine the most effective DP_n of chitosan and CHOS against *Botrytris cinerea* Pers. Ex Fr. and *Mucor piriformis* Fischer. *In vitro* testing showed that CHOS of DP_n 23 and 40 had the highest germination inhibition against the tested pathogens. The original chitosan (DP_n 206) and a collection of short CHOS (degree of polymerization of 3-10) were significantly ($P<0.01$) less effective than CHOS of DP_n 23 and 40. *M. piriformis* M119J showed the most abnormal swelling in presence of CHOS DP_n 40, but all abnormally swollen conidia showed further germ tube elongation. *In vivo* testing showed that CHOS DP_n 23 was the most effective in reducing flower infection by two isolates of *B. cinerea*. Our results show that CHOS inhibit fungal germination and growth and that the effect depends highly on the level of polymerization of the oligomers.

Keywords: *Botrytis cinerea*; *Mucor piriformis*; chitosan; chito-oligosaccharides (CHOS); antifungal; plant protection

Introduction

The pathogenic fungus *Botrytis cinerea* Pers.ex Fr. (anamorph of *Botryotinia fuckeliana*) causes gray mold in over 200 plant species, mainly dicotyledonous plants, resulting in severe pre- and postharvest losses in agricultural crops like berries, fruits and vegetables (Williamson et al. 2007; Rosslenbroich and Stuebler 2000; Tronsmo 1991). The pathogenic fungus *Mucor piriformis* Fischer also causes postharvest rots on strawberries as well as on several other fruit crops (Sholberg 1990). Pathogens may develop resistance towards chemical fungicides, creating a need for even more chemicals (Holmes & Eckert 1999). However, as existing chemical fungicides may be harmful for mammals and the environment, there is a need to reduce their use. Attempts have been made to reduce gray mold using biological or cultural control methods (Card et al. 2009), but these often give less consistent results than chemical control. There is a need for antifungal products that cause no harm to the environment and are non-toxic to mammals (Parvu et al. 2010). Chitosan (deacetylated chitin) has long been known to have such properties (Trotel-Aziz et al. 2006).

Chitin is a linear biopolymer consisting of ß 1,4-linked *N*-acetyl-D-glucosamine (GlcNAc) residues and it is one of the most abundant biopolymers in nature. The fraction of acetylation *(FA)* of chitin is usually above 0.90, meaning there are few D-glucosamine (GlcN) units present. Chitin is found as a structural polymer in fungi, crustaceans, arthropods, insects, and nematodes (Goody 1990). Chitosan, a linear polymer made by partial deacetylation of chitin, is a heteropolymer consisting of *N*-acetyl-D-glucosamine (GlcNAc) and D-glucosamine (GlcN) residues (Goody 1990). Chitosan can be hydrolysed into shorter chains to produce chito-oligosaccharides (CHOS). Both chitosan

and CHOS show little or no toxicity towards mammalian cells (Jung et al. 1999; Singla and Chawla 2001), are biodegradable, and may protect plants by direct antimicrobial activity or by eliciting plant defence mechanisms (Hadwiger 1979; Hadwiger and Beckman 1980). The antifungal activity of chitosan is influenced by many factors, such as its concentration (Palma-Guerrero et al. 2008), degree of polymerization (DP) or molecular weight (Kendra and Hadwiger 1984), fraction of acetylation (F_A) (Stössel and Leuba 1984), pH, and ionic strength of the media (Jung et al. 1999: Wang 1992).

Previous studies on the effect of molecular weight on the antimicrobial activity of chitosan have given diverging results. While some studies found that polymeric chitosan (high molecular weight) had higher antifungal activity than low molecular weight chitosan (Eikenes et al. 2005; Meng et al. 2010), other studies reported the opposite result (Kim et al. 2005; Xu et al. 2007). However, as most studies involved chitosan with only one or very few different molecular weights that were not obtained from the same source or by the same method, these data may not give comparable results. The aim of this study is therefore to use well-defined chitosan with respect to molecular weight and determine which show the greatest inhibitory effect against the two common plant pathogens *B. cinerea* and *M. piriformis*. Two types of experiments were carried out: an *in vitro* microtiter plate assay on conidial germination and hyphal growth of *B. cinerea* and *M. piriformis*, and an *in vivo* assay on strawberry flower infection by *B. cinerea*.

Materials and Methods

Enzymatic production and analysis of chito-oligosaccharides (CHOS)

Chitosan (KitoNor; fraction of acetylation (F_A) 0.15; viscosity average molecular weight (MWv) 33.4 kDa and DP_n 206, was obtained from Norwegian Chitosan (Gardermoen, Norway). CHOS with varying level of polymerization were produced from chitosan (DP_n 206) by enzymatic hydrolysis for different lengths of time, using chitosanase ScCsn46A as described by Heggset and coworkers (Heggset et al. 2010). Briefly, chitosanase ScCsn46A, originally from *Streptomyces coelicolor* q9rj88, was purified from the culture supernatant of a recombinant *Escherichia coli* BL21Star (DE3) strain, following the published protocol, without removal of the $(His)_6$-tag after purification. The enzyme was dialyzed against Tris-HCl (20 mM) at pH 8 and stored at 4°C.

Hydrolysis of chitosan to produce CHOS

Chitosan (DP_n 206, 10 mg mL^{-1}) in buffer (0.04M NaAc, 0.1M NaCl, pH 5.5) was incubated at 37°C and shaken at 225 rpm for about 10 min until the chitosan was dissolved. The pH was further adjusted to 5.5 with NaOH (0.5M). Chitosanase ScCsn46A[26] (0.5 µg mg^{-1} chitosan) was added to the chitosan solution and the mixture was incubated for 10-50 minutes at 37°C and 225 rpm. The enzymatic reaction was stopped by decreasing the pH to 2.5 with HCl (0.5M), followed by immersing the tube in boiling water for at least 10 minutes to inactivate the enzymes permanently. The resulting CHOS samples were dialyzed against dH_2O for 48 hours (water was changed every 12 hours) using a cellulose membrane (Float-A-Lyzer® MWCO 500 Da, from Spectrum Labs, Texas, USA) to remove buffer salts from the sample. Dialyzed samples were sterile filtrated through Filtropur S 0.2 µm sterile filters (Sarstedt, Germany), lyophilized and stored at 4°C (Aam et al. 2010).

^{1}H-NMR analysis of CHOS

Lyophilized CHOS (10 mg) were dissolved in deuterium oxide (D_2O, 0.5 ml) and the pH was adjusted to 4.2 with sodium deuteroxide (NaOD) and deuterium chloride (DCl) prior to lyophilization. This process was repeated once. Finally the lyophilized CHOS sample was dissolved in D_2O (700 µL) and ^{1}H-NMR analysis was performed on a 300 MHz Varian Gemini instrument (Varian, USA) at 85^{o}C (^{1}H-NMR spectra is shown in Fig. 1). The DP_n was calculated with the equation (Dα+Dβ+D+Aα+Aβ+A)/(Dα+Dβ+ Aα+Aβ), where Dα, Dβ, Aα and Aβ are the integral of the reducing end signals of the α and β anomers of the deacetylated (D) and acetylated (A) units respectively, D is the integral of the signals from deacetylated units GlcN) in internal and non-reducing end positions, and A is the integral of the signals from acetylated units (GlcNAc) in internal and non-reducing end positions (Sørbotten et al. 2005).

Separation of CHOS DP 3-10 by size exclusion chromatography (SEC)

CHOS DP_n 5, made from enzymatic hydrolysis of chitosan (DP_n 206) with ScCsn46A, was separated on three Superdex^{TM} 30 columns (XK columns from GE Healthcare) with an overall dimension of 2.6 × 180 cm. The flow rate of the mobile phase (0.15 M NH_4Ac, pH 4.5) was maintained at 0.8 ml min^{-1} (Sørbotten et al. 2005). A refractive index detector (Gilson model 133, UK) was used to monitor the relative amounts of the CHOS fractions. CHOS (100 mg) sample was applied in each run. CHOS DP 3-10 was collected from several separate runs and pooled followed by dialysis to remove salts from the buffer. The sample was then sterile filtrated through Filtropur S 0.2 µm sterile filters, and lyophilized.

Matrix assisted laser desorption/ ionization time of flight mass spectrometry

MS spectra were acquired using an Ultraflex^{TM} TOF/ TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) with gridless ion optics under control of Flexcontrol 4.1. For sample preparation, 1 µL of isolated CHOS and 2 µL of matrix solution (15 mg mL^{-1} 2,5-dihydroxybenzoic acid) were mixed and 1 µL of the mixed solution was spotted on a target plate (Cederkvist et al. 2008). The spotted samples were dried at room-temperature. The MS experiments were conducted using an accelerating potential of 20 kV in the reflector mode.

Fungal pathogens

The fungal pathogens used in this experiment were *Botrytis cinerea* isolate BC 101, isolated from infected strawberry fruit in Grimstad, Norway; *B. cinerea* isolate BCBD, from a chickpea leaf in Gazipur, Bangladesh and *M. piriformis* isolate M199J, from an infected strawberry fruit at Hobøl, Norway. For the *in vitro* and *in vivo* bioassays, conidia were collected from cultures grown on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI) under regular laboratory light for two weeks at 23 ± 1°C. The conidia were suspended in sterile water and were filtered through sterile cotton to remove fragments of mycelia. Concentrations of conidia in aqueous suspensions were determined by hemocytometer counter at 400 × magnification (Leica, DM RBE, Germany) and adjusted to the required concentrations.

In vitro bioassay; inhibition of conidia germination and hyphal growth

The effect of chitosan and CHOS on germination of *B. cinerea* (isolates BC 101 and BCBD) and *M. piriformis* M199J was tested in a modified synthetic medium (MSM) with pH 5.3 and the following final concentrations: 2.5 mM NH_4NO_3; 0.28 mM $CaCl_2 \cdot 2H_2O$; 0.16 mM $MgSO_4 \cdot 7H_2O$; 0.002 mM $MnSO_4 \cdot 4H_2O$; 0.002 mM $ZnSO_4 \cdot 7H_2O$; 1 mM KH_2PO_4; 0.06 mM $FeC_6H_5O_7 \cdot 5H_2O$ and 55.5 mM glucose. Experiments were set up by adding 100 µl of chitosan or CHOS in 2X MSM to 100µl conidial suspension (4×10^4 conidia mL^{-1} in water) in wells in a flat-bottom 96-well microtiter plate (Nunc™, Roskilde, Denmark). There were three replicate wells of each treatment. The microtiter plates were incubated at 23 ± 1°C for 24 hours. The germination percentage 12 and 24 hours after inoculation (HAI) was visually estimated at 400× magnification using an invert microscope (Fluovert FU, Ernst Leitz Wetzlar GmbH, Wetzlar, Germany). The conidia were counted as germinated when the germ tube length was at least as long as the diameter of the conidium. The germination of *M. piriformis* M199J conidia was measured differently since all germinated conidia exposed to chitin or CHOS showed abnormal swelling with amoeba-like structure and one or more protruded portions. When the length of the protruded part was at least as long as the diameter of the swelled conidia the conidia was considered germinated. The germination inhibition percentage was calculated by the following equation:

Germination inhibition (%) = $\{(a-b)/a\} \times 100$

Where, a = germinated conidia in the control (conidia in MSM)

b = germinated conidia in the presence of chitosan or CHOS in MSM

The pH of the conidia suspension in the microtiter wells was between 5.2 and 5.3 at the start of the experiment, and remained about the same 24 hours after inoculation.

Germination and further germ tube elongation were also documented by photographs (10-15, from each treatment with Canon D-400 camera, Japan) taken through the invert microscope at 400× magnification (Fluovert FU, Ernst Leitz Wetzlar GmbH, Wetzlar, Germany) 12 and 44 hours after inoculation.

In vivo bioassay: Inhibition of infection of strawberry flowers by Botrytis cinerea

Chitosan (DP_n 206) and CHOS (DP_n 9, 23, 40 and 48) were tested for their ability to reduce infection by *B. cinerea* BC 101 and *B. cinerea* BCBD of newly opened strawberry (*Fragaria* × *ananassa* cv. Corona) flowers. The strawberry plants were grown in the greenhouse under controlled temperature (18^{o}C at day; 12^{o}C at night), light (16 hours, light intensity: 150 µmols m^{-2} sec^{-1}) and relative humidity (65%) conditions. Eighteen flowers per treatment (six replications of three flowers) were cut off with a 1½-2 cm stem and placed in empty pipette tip racks set in plastic containers filled with 1-2 cm water. Conidia suspension (final concentration: 1×10^6 conidia/ml) was mixed with different test ingredients in sterile water, or with sterile water (control reaction). Flowers were infected by applying 10 µL of the sample at the base of three petals on each flower with a pipette. The treatments were randomized. The plastic containers were covered with aluminium foil and incubated at 23 ± 1°C. The relative humidity was 90-95%, measured by a thermo-hygrometer (Lambrecht, Germany). The experiment was repeated twice. The infection was recorded as visible necrotic regions on the abaxial surface of the flowers under the inoculation point daily for eight days, and the area under the disease

progress curve (AUDPC) was calculated on the basis of the cumulative daily infection by the following equation:

$$AUDPC = \sum (D_i - D_{i-1}) \times \{S_{i-1} + 0.5 (S_i - S_{i-1})\}$$

Where, D_i = Days of the i^{th} assessment and S_i = Proportion of the i^{th} infected inoculation point.

The protection index (in %) was calculated by using the AUDPC values in the following equation (Bardin et al. 2008):

$$100 \times (AUDPC_{control} - AUDPC_{treatment})/AUDPC_{control}$$

Where $AUDPC_{control}$ represent flowers inoculated with *B. cinerea* BC 101 or BCBD conidia alone and $AUDPC_{treatment}$ represents flowers inoculated with *B. cinerea* BC 101 or BCBD conidia premixed with chitosan or CHOS .

Data analysis

In the microtiter plate assay, the percentage of germination inhibition of pathogens by chitosan and CHOS were transformed by arcsine transformation and tested by one way ANOVA (only non transformed data are presented). In the strawberry flower assay, the AUDPC was calculated based on the cumulative daily infections from one to eight days, and tested by one way ANOVA. When appropriate, means were separated by Tukey's Honestly Significant Difference method. All statistical analysis were done by Microsoft Office Excel 2007 and Minitab 16 (MINITAB, USA).

Results

Production and characterization of CHOS

CHOS fractions were produced by degrading chitosan (DP_n 206 and F_A of 0.15) with ScCsn46A, as described in the materials and methods section. By varying the incubation time, CHOS fractions with DP_n values between 75 and 5 were produced. DP_n was used as an indicator of molecular weights for all CHOS samples since this is straightforwardly determined by ^{1}H NMR analysis.[27] The ^{1}H-NMR spectra of the CHOS fractions showed that all reducing ends were completely deacetylated (signals at 5.43 ppm for -Dα and 4.92 ppm for -Dβ) (Fig. 1). There were no signals for new acetylated reducing ends (signal for -A α at 5.19 ppm and for DA β at 4.74). A CHOS fraction of DP_n of 5 was further purified by size exclusion chromatography (SEC). CHOS of DP 3-10 were collected and pooled together to serve as the fraction with lowest molecular weights.

Effects of chitosan and CHOS on the ability to inhibit germination and germ tube elongation *in vitro*

The inhibitory effects on conidial germination of chitosan and CHOS with different DP_n (80 µg mL^{-1}) against *B. cinerea* and *M. piriformis* are shown in Table 1. All three isolates experienced a higher degree of inhibition at 12 vs. 24 hours after inoculation (HAI). *M. piriformis* was sensitive (in terms of germination inhibition) to a wider range of CHOS (DP_n 9-75) while *B. cinerea* BC 101 was only markedly inhibited by CHOS DP_n 23 and 40. The results clearly show a size dependency for the antifungal effect of CHOS.

The antifungal activity was also tested for the original chitosan (F_A 0.15, DP_n 206) and CHOS DP3-10 as controls for high and low molecular weight fractions. At 80 µg mL^{-1}, there was no significant difference between DP 3-10 and chitosan in terms of

germination inhibition, but at a high concentration (1300 µg mL^{-1}) DP 3-10 showed significantly higher germination inhibition than chitosan of all tested pathogens 24 hours after inoculation (Table 2).

The effects of DP_n on the ability of chitosan and CHOS to inhibit conidial germination and further germ tube elongation were observed visually (Figs. 2 and 3). Chitosan (DP_n 206) and CHOS (DP_n 40) caused abnormal swelling of *M. piriformis* (Fig. 2), but no such effect was observed on the two *B. cinerea* isolates. However, CHOS DP_n 40 caused granular inclusions in the cytoplasm of all tested pathogens. In the presence of CHOS DP_n 40, germ tubes from both *B. cinerea* isolates ceased to grow after germination and no further growth was observed 44 hours after inoculation (Fig. 3). The *M. piriformis* M199J conidia treated with CHOS DP_n 40 were abnormally swollen 12 hours after inoculation (Fig. 2) but continued to grow; however, many non-germinated and non-swollen conidia were also present. Chitosan (DPn 206) and CHOS DP_n 9 did not affect germ tube elongation of the tested pathogens (Fig. 3).

Strawberry flower bioassay

CHOS DP_n 23 followed by CHOS DP_n 40 were the most effective chitooligosaccharides in reducing flower infection by *B. cinerea* (BC 101 and BCBD) (Table 3 and Fig. 4). *In vivo,* both *B. cinerea* isolates caused 100% infection of strawberry flowers six days after inoculation in the control and when treated with chitosan DP_n 206 and CHOS DP_n 9 (500 µg mL^{-1}) (Fig. 4). CHOS DP_n 23 and CHOS DP_n 40 reduced the *B. cinerea* BC 101

infection to 60% and 30% respectively, and the *B. cinerea* BCBD infection to 43% and 20% respectively at six days after inoculation (data not shown).

Discussion

Molecular weight, and consequently the degree of polymerization, as well as the degree of acetylation, are important factors affecting the antifungal activity of chitosan (Aam et al. 2010; Kendra and Hadwiger 1984; Oliveira Junior et al. 2012; Rahman 2013). Our previous *in vitro* and *in vivo* studies on chitosan with different F_A showed that chitosan with low F_A (0.11 and 0.18) was more inhibitory than chitosan with higher F_A (0.39) (Rahman 2013). Chitosan with F_A 0.15 was therefore selected for the present study, in which well-defined chitosan and CHOS fractions of different DP/DP_n obtained from the same chitosan were used to test for antifungal activity against *Botrytis cinerea* and *Mucor piriformis in vitro* and *in vivo*. The antifungal activity of chitosan and CHOS varied with the DP_n in both bioassays, and CHOS 23 and 40 were the most effective of the tested CHOS in inhibiting germination and further germ tube elongation of the tested pathogens *in vitro*. Antifungal activity of chitosan has been previously reported to depend on DP, where 6 kDa (DP_n around 40) was the most effective on a range from 5 to 27 kDa in inhibiting *Candida krusei* (Gerasimenko et al. 2004). In our study, chitosan with high polymerization (DP_n 206, MWv 34.4 kDa) and low polymerization (CHOS DP 3-10) were only effective in inhibiting germination and further germ tube elongation of *B. cinerea* and *M. piriformis* at a high concentration (1300 µg mL^{-1}) and CHOS DP 3-10 was more effective than chitosan (DP_n 206). This corresponds to a previous study stating that CHOS with low DP (DP 3-9) were more effective in inhibiting *B. cinerea* than chitosan with high DP (molecular weight 300-500 kDa) (Xu et al. 2007).

The effectiveness of chitosan and CHOS also depends on the cell wall composition of the tested pathogen (Allan and Hadwiger 1979). The cell walls of the ascomycetous fungi (*B. cinerea*) contain chitin, whereas the zygomycetous fungi (*M. piriformis*) contain both chitin and chitosan (Ruiz-Herrera 1992). Our study shows that *M. piriformis* is more sensitive to the tested chitosans than *B.cinerea.* However, previous research showed contradictory results regarding the correlation between chitosan's antifungal activity and fungal cell wall composition. Allan and Hadwiger documented that fungi with cell walls containing chitosan (*Mucor* spp.) were not sensitive to chitosan (1000 µg mL^{-1}) (Allan and Hadwiger 1979). However, using the same chitosan concentration, El-Ghaouth showed germination and growth inhibition of other zygomycetous fungi (*Rhizopus stolinifer* and *M. racemosus*) with chitosan in their cell wall (El-Ghaouth et al. 1992). Our results showed that the sensitivity of fungal pathogens to chitosan varied with the DP. *M. piriformis* M199J was sensitive (more than 50% inhibition of germination 24 hours after inoculation) to CHOS with a wide range of DP (DP_n 15-48), *B. cinerea* BCBD was sensitive to CHOS with a narrower range of DP (DP_n 15 - 40) and *B. cinerea* BC 101 was only sensitive to DP_n 23 and 40.

Microscopic observations confirmed that the DP of CHOS was important for its ability to inhibit germination and growth (further germ tube elongation) of the tested pathogens, since CHOS DP_n 40 showed more cytoplasmic disorder and abnormal swelling of conidia than other tested DP_n. *B. cinerea* conidia developed granular substances in the cytoplasm in the presence of CHOS DP_n 40 at low concentration (80 µg mL^{-1}) while higher concentrations of CHOS DP 3-10 (1300 µg mL^{-1}) and chitosan DP_n 206 (≥2500 µg mL^{-1}) were required for the same effect. Similar morphological changes have previously been found in *B. cinerea* treated with chitosan (Ait et al. 2004). Still, *B. cinerea* conidia, even at a high chitosan concentration (5000 µg mL^{-1}), did not show

abnormal swelling, which has also been observed in a previous study (El-Ghaouth et al. 1992). In contrast, all our tested DP of chitosan and CHOS, except the lowest DP (CHOS DP_n 9 and DP 3-10), caused abnormal swelling of *M. piriformis* conidia. The swelling varied with the concentration and DP_n of chitosan and CHOS where the most abnormal swelling (amoeba-like structure) occurred in *M. piriformis* treated with CHOS DP_n 40. Also, other studies have confirmed that the DP of chitosan influenced the extent of abnormal swelling of fungal conidia. Chitosan with high DP (molecular weight 30.7 kDa, DP ≈ 150) caused more abnormal swelling of *Rhizopus stolonifer* conidia than chitosan with low DP (molecular weight 17.4 kDa , DP ≈ 90) (Hernández-Lauzardo 2008) and low DP (exact molecular weight not mentioned) at a high concentration (15000 µg mL^{-1}) changed the shape of *Rhizoctonia solani* conidia (Bautista-Baños et al. 2004).

No lyses of abnormally swollen *M. piriformis* conidia were observed and all swollen conidia continued to grow (further elongation of germ tube after germination). Also, El-Ghaouth et al. reported excessive branching and abnormal swelling of *R. stolonifer* treated with chitosan (1500 µg mL^{-1}, molecular weight not mentioned), and no alternation nor lysis of the cells (El-Ghaouth et al. 1992). Other reasons for further growth of the abnormally swelled *M. piriformis* conidia could be that the cells with abnormal swelling belonged to a more resistant subpopulation or survived as the chitosan concentration was reduced through binding to other cells (Rhoades and Roller2000). The reason why conidia of *M. piriformis*, but not *B. cinerea*, showed abnormal swelling could be due to the inherent different cell wall compositions. It could be that the application of chitosan affected new cell wall synthesis of *M. piriformis* and resulted in abnormal swelling of conidia.

In the strawberry flower assay, CHOS DP_n 23 was the most effective in reducing flower infection by *B. cinerea* in line with what was observed *in vitro*. In addition to the direct antifungal effects of chitosan seen in the *in vitro* bioassay, chitosan may protect flowers indirectly by eliciting plant defense mechanisms against pathogens (Palma-Guerrero et al. 2008). Previous studies showed that chitosan acts as an elicitor of multiple defense responses of higher plants (Aziz et al. 2006; Vander et al. 1998), and that the eliciting effect depends on the DP of chitosan (Lin et al. 2005). Thus, in our study CHOS DP_n 23 may have been more effective in inducing defense responses in strawberry than chitosan (DP_n 206).

In conclusion, the objective of our study was to find the most effective DP of chitosan and CHOS against *B. cinerea* and *M. piriformis.* Our study demonstrated that the DP_n of chito-oligosaccharides (CHOS) affected their antifungal activity both *in vitro* and *in vivo.* CHOS were more effective in inhibiting plant pathogens than chitosan (DP_n 206), and the most effective DP of CHOS was in the range of DP_n 15-40. CHOS in this range may be a potential environmentally friendly product that can be used in biodynamic and organic agri- and horticulture.

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Table 1. Effect of chitosan (DP_n 206; F_A 0.15) and CHOS (both 80 µg mL^{-1}) on germination inhibition (GI)[a] (expressed as percent of control in MSM) of *Botrytis cinerea* (BC 101 and BCBD) and *Mucor piriformis* M199J 12 and 24 hours (h) after inoculation.

Chitosan/ CHOS	DP_n [b]	*B. cinerea* BC 101		*B. cinerea* BCBD		*M. piriformis* M199J	
		GI % 12 h	GI % 24 h	GI % 12 h	GI % 24 h	GI % 12 h	GI% 24 h
Chitosan	206	13 b[a]	0 b[a]	5 c[a]	0 c[a]	44 c[a]	0[a] e
CHOS	75 ± 7	12 b	3 b	7 c	0 c	99 a	30 d
	58 ± 3	10 b	2 b	7 c	1 c	99 a	51 c
	48 ± 3	13 b	4 b	6 c	5 c	99 a	50 c
	40 ± 1	90 a	77 a	98 a	99 a	99 a	95 a
	23 ± 3	82 a	80 a	98 a	99 a	99 a	89 a
	15 ± 1	15 b	4 b	100 a	99 a	99 a	66 b
	11 ± 1	7 b	4 b	94 b	45 b	70 b	7 e
	9 ± 1	1 b	1 b	0 c	0 c	21 d	0 e
	DP3-10	3 e	3 c	1 c	0 c	32c	1 c

[a] Means in columns without common letters are significantly different according to Tukey's method at $P< 0.01$.

[b] DP_n data are mean ± standard deviation of three experiments

All data are means of three experiments.

Table 2. Effect of different concentration of chitosan DP_n 206 and CHOS DP 3-10 on germination inhibition (GI) (expressed as percent of control in MSM) of *Botrytis cinerea* (BC 101 and BCBD) and *Mucor piriformis* M199J 12 and 24 hours (h) after inoculation.

Chitosan/CHOS	Concentration ($\mu g\ mL^{-1}$)	*B. cinerea* BC 101		*B. cinerea* BCBD		*M. piriformis* M199J	
		GI % 12 h	GI % 24 h	GI % 12 h	GI % 24 h	GI % 12 h	GI % 24 h
DP_n 206	1300	54 b[a]	36 b[a]	42 b[a]	30 b[a]	10a[a]	3 c[a]
	600	31 cd	26 b	30 b	18 bc	10a	2 c
	80	19 d	4 c	4 c	3 c	50b	1 c
DP 3-10	1300	85 a	88 a	77 a	67 a	100a	98 a
	600	41 bc	43 b	75 a	26 b	10a	83 b
	80	3 e	3 c	1 c	0 c	32c	1 c

[a] Means in columns without common letters are significantly different according to Tukey's method at $P<0.01$

All data are means of two experiments

Table 3. Inhibition of disease development on strawberry flowers inoculated with a mixture of *Botrytis cinerea* (BC 101 or BCBD) conidia and chitosan (DP_n 206) or CHOS with different DP_n (9, 23, 40 and 48) (all 500 µg mL^{-1}). Control indicates conidia in sterile water.

Chitosan/CHOS	*B. cinerea* BC 101		*B. cinerea* BCBD	
	AUDPC[a]	Protection index (%)	AUDPC[a]	Protection index (%)
Control	4.4 a[b]	-	4.4 a[b]	-
DP_n 206	4.1 a	6 d[b]	3.6 b	18 d[b]
DP_n 48	3.3 b	25 c	2.8 c	36 c
DP_n 40	1.8 c	59 b	1.5 d	66 b
DP_n 23	0.7 d	85 a	0.7 e	84 a
DP_n 9	4.0 a	8 c	3.5 b	21 d

[a] The AUDPC was used to calculate the protection index. The AUDPC values were calculated from cumulative disease incidences recorded up to eight days after inoculation (DAI) with incubation at 23 ± 1°C

[b] Means in columns without common letters are significantly different according to Tukey's method at $P< 0.01$.

All data are means of three experiments

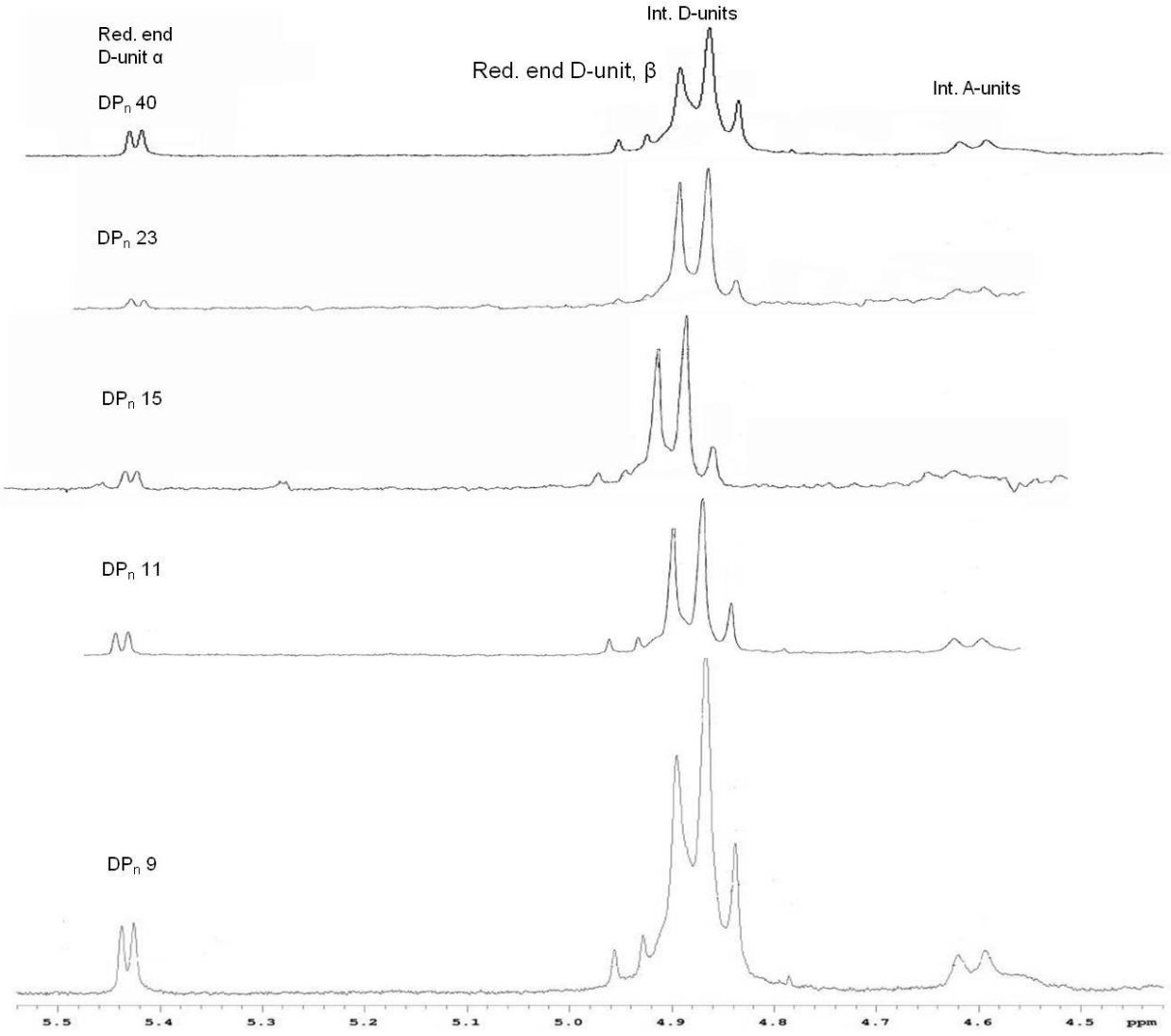

Figure 1. ^{1}H-NMR spectra of CHOS after hydrolysis of KitoNor chitosan (F_A 0.15) with ScCsn46A for DP_n of 40, 23, 15, 11, and 9, respectively (same spectra for DP_n of 75, 58, and 48 are omitted for clarity).

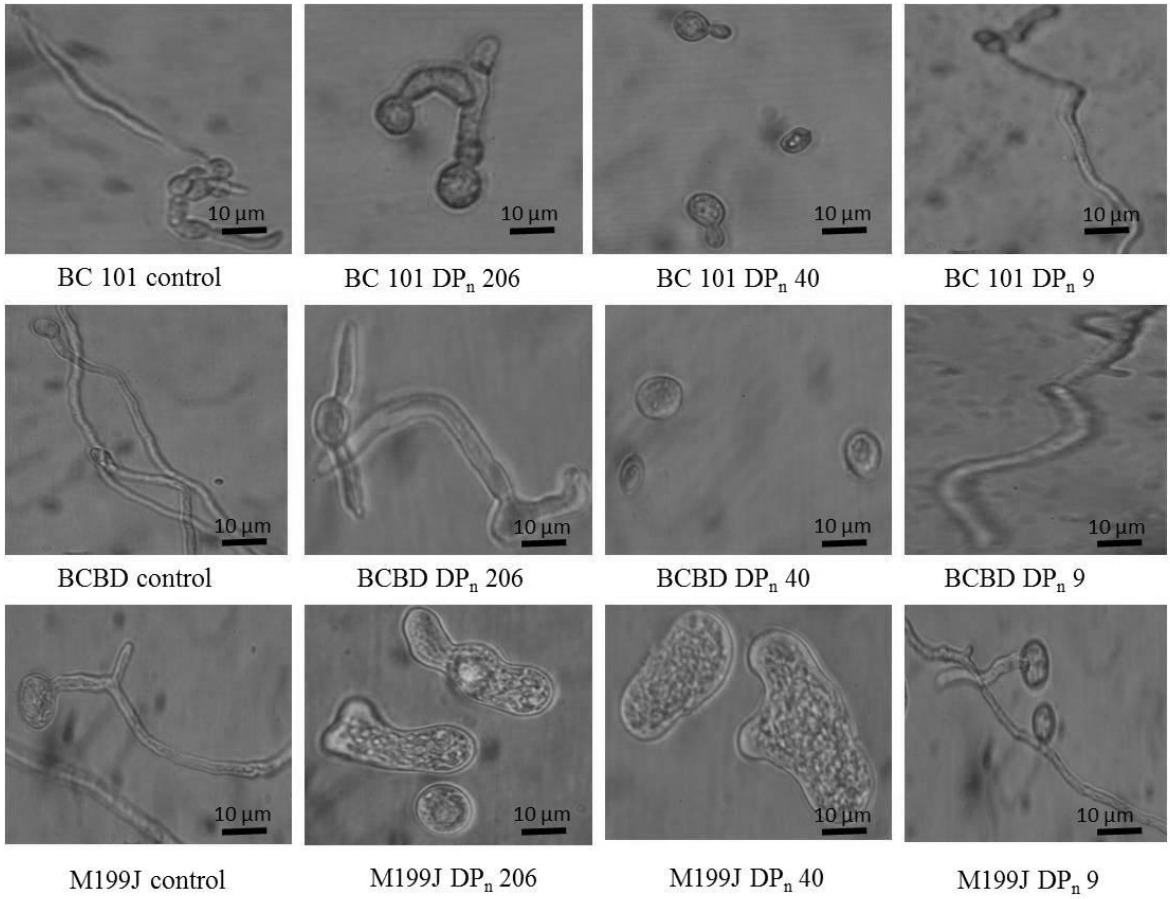

Figure 2. Effect of chitosan DP_n 206 and CHOS DP_n 9 and 40 (all 80 µg mL^{-1} in MSM) on conidia germination and further germ tube elongation of *Botrytis cinerea* (BC 101 and BCBD), and *Mucor piriformis* M199J (12 HAI, 23 ± 1°C). BC 101: *B. cinerea* BC 101; BCBD: *B. cinerea* BCBD, M199J: *M. piriformis* M199J. Control is conidia in MSM.

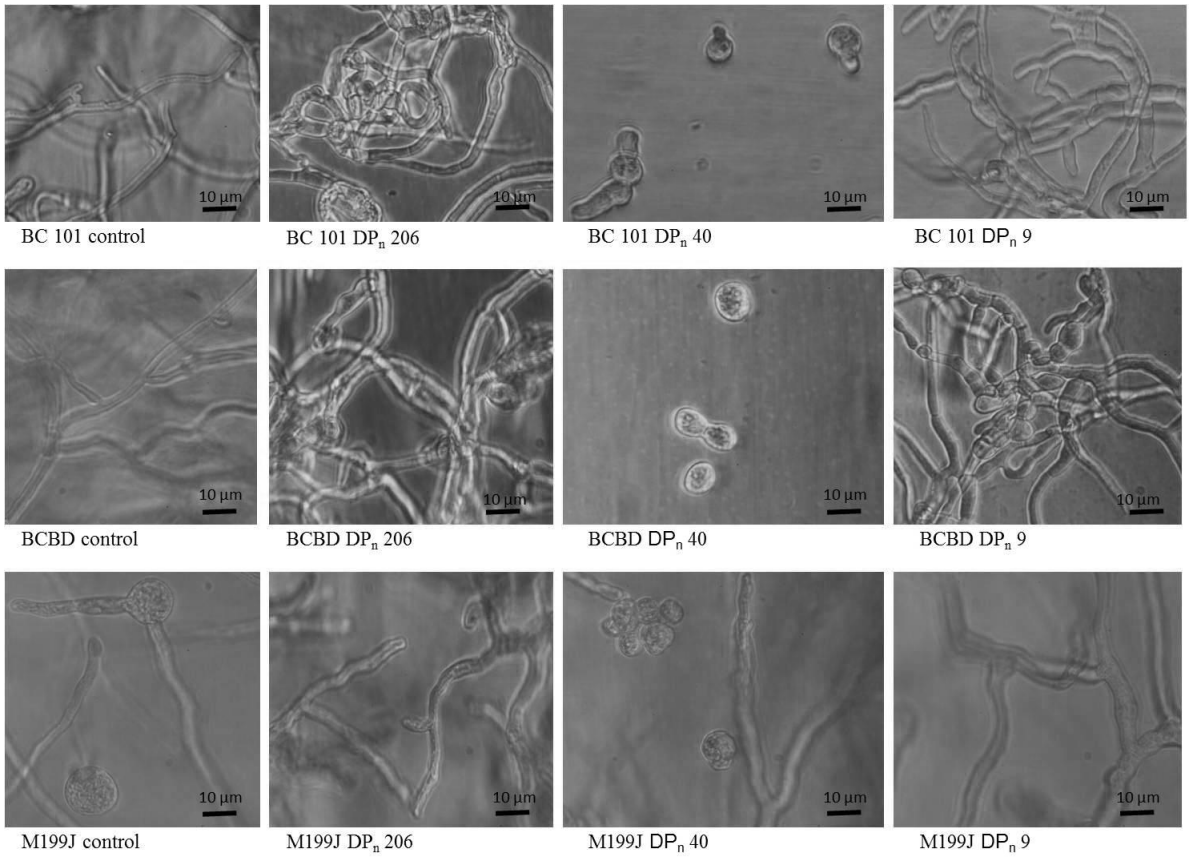

Figure 3. Effect of chitosan (DP_n 206) and CHOS (DP_n 9 and 40) (80 µg mL^{-1} in MSM) on germ tube elongation of *Botrytis cinerea* (BC 101 and BCBD), and *Mucor piriformis* M199J (44 hours after inoculation at 23 ± 1°C). BC 101: *B. cinerea* BC 101; BCBD: *B. cinerea* BCBD, M199J: *M. piriformis* M199J. Control is conidia in MSM.

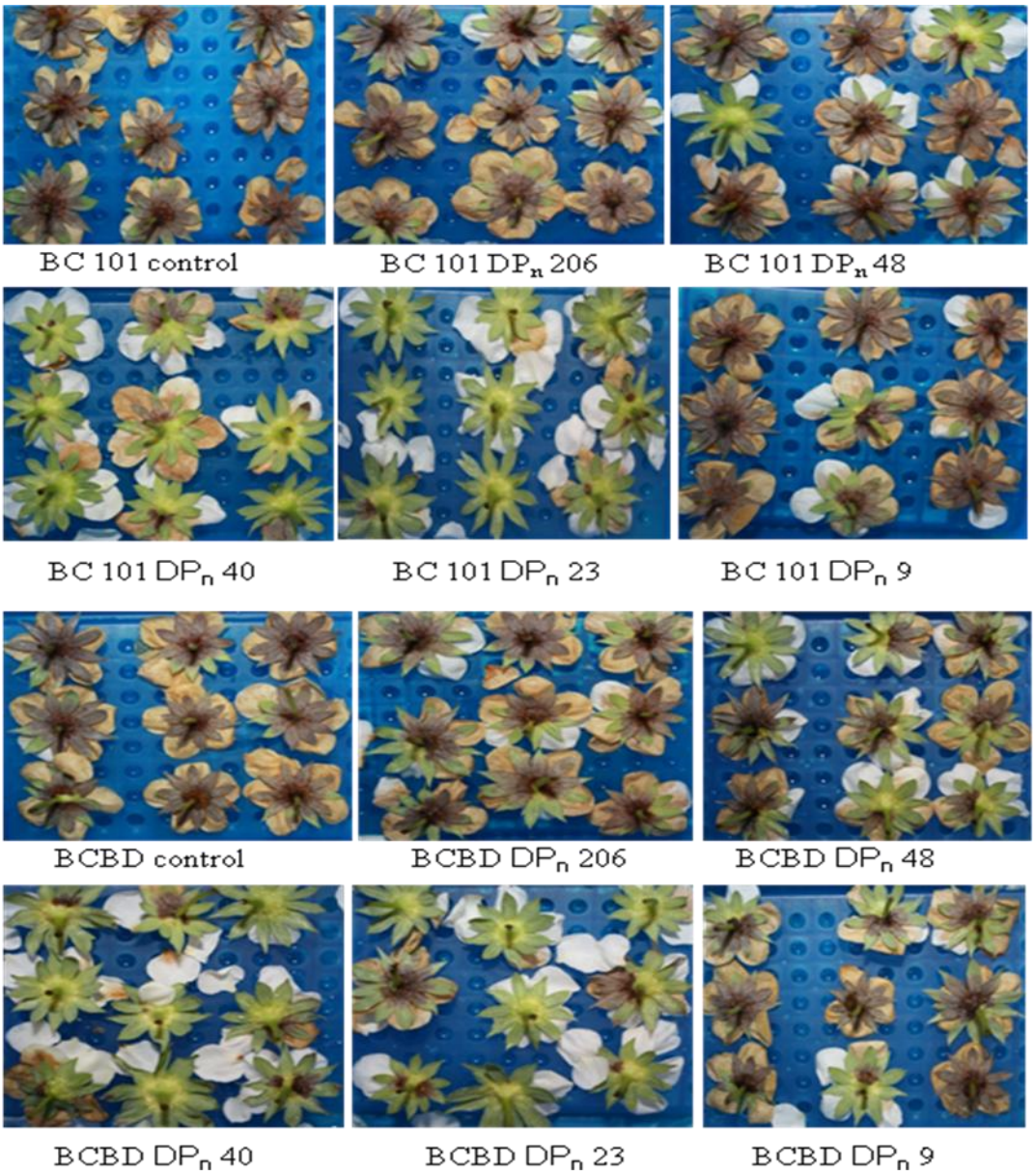

Figure 4. Antifungal effect of chitosan and CHOS. The photos illustrate the inhibitory effect of chitosan (DP_n 206) and CHOS DP_n 9, 23, 40 and 48 (500 µg mL^{-1}) on disease caused by *Botrytis cinerea* (BC 101 and BCBD) applied to detached strawberry flowers six days after inoculation. Control was conidia in sterile water. The flowers were considered 100% infected when all three inoculation points displayed necrotic signs. All treatments included 18 flowers, but only nine flowers are shown here.