

GROWTH RATE INHIBITION OF PHYTOPATHOGENIC FUNGI BY CHARACTERIZED CHITOSANS

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ABSTRACT

The inhibitory effects of fifteen chitosans with different degrees of polymerization (DP) and different degrees of acetylation (F_A) on the growth rates (GR) of four phytopathogenic fungi (*Alternaria alternata*, *Botrytis cinerea*, *Penicillium expansum*, and *Rhizopus stolonifer*) were examined using a 96-well microtiter plate and a microplate reader. The minimum inhibitory concentrations (MICs) of the chitosans ranged from $100 \mu\text{g} \times \text{mL}^{-1}$ to $1,000 \mu\text{g} \times \text{mL}^{-1}$ depending on the fungus tested and the DP and F_A of the chitosan. The antifungal activity of the chitosans increased with decreasing F_A . Chitosans with low F_A and high DP showed the highest inhibitory activity against all four fungi. *P. expansum* and *B. cinerea* were relatively less susceptible while *A. alternata* and *R. stolonifer* were relatively more sensitive to the chitosan polymers. Scanning electron microscopy of fungi grown on culture media amended with chitosan revealed morphological changes.

Key words: chitosan, antifungal activity, fungal morphology, phytopathogenic fungi

INTRODUCTION

Chitin and chitosan are aminogluco-pyranans composed of N-acetylglucosamine (GlcNAc) and glucosamine (GlcN) residues, and are renewable resources currently being studied by academic and industrial groups (29) owing to their attractive properties and biological activities. Chitosans have been indicated for the preservation of foods (5, 34) juices (32) and other material from microbial deterioration due their action against different groups of microorganisms, such as bacteria (14, 15, 21, 35), yeast and fungi (1, 3, 4, 8, 9, 16, 18, 25, 28, 30, 33, 37, 42). Studies on coating of fruits and vegetables (6, 10, 11, 12, 13, 17) and defensive plant mechanism studies (39) have been described. However, most

studies describing inhibitory effects on the growth of microorganisms involved poorly characterized chitosans or only one or a few different degrees of polymerization (DP) and fractions of acetylation (F_A) of chitosans (3, 6, 8, 9, 10, 11, 13, 25, 33). Thus, important information is lacking on the influence of DP and F_A on the biological activity of chitosans against fungi of economic importance for post-harvest losses of fruits and vegetables. The phytopathogenic fungi evaluated in this study *B. cinerea*, *P. expansum*, *R. stolonifer*, and *A. alternata*, are responsible for strawberry (6, 9, 10), cucumber and bell pepper (11, 12), pear (22), apple (7), wheat (23), and tomato (3, 33) losses.

Growth of filamentous fungi is usually measured as an

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increase in dry mass (either stationary or shake flasks, and quantitative radial growth measurements on solid media. However, experiments testing the effect of several compounds on the growth of a fungus, can become very space-demanding and laborious, limiting the scale of studies.

The objective and novelty of this study was to examine the *in vitro* antifungal effect of fifteen chitosans with widely different DP and F_A against the phytopathogenic fungi *B. cinerea*, *P. expansum*, *R.stolonifer*, and *A. alternata*, which are responsible for important economic losses in Brazilian fruit exports (citrus, strawberries, grapes, papaya, apples amongst others) and to overcome the difficulties related to the conventional methods to measure biomass content, by using the microtiter plate technique.

MATERIALS AND METHODS

Chitosan samples

Chitosan samples were classified into four groups, according to their source and treatment (Table 1). Details on preparation and characterization of all chitosan tested are described in Oliveira-Jr (27). Group I contained 3 chitosans with different DP and the same F_A (the raw material was coded as A, from Polymar, Fortaleza, Brazil and was thermally depolymerized to produce samples B and C chitosans). Group II included 2 chitosans of same DP with different F_A obtained from alkaline deacetylation of chitin (Sigma Chemical Co., St. Louis, USA). Group III included chitosans with average DP of ca. 190, but F_A varying from 0.01 to 0.69. Chitosans of this group were prepared by partial homogeneous de-N-acetylation of highly acetylated chitosan polymers as described previously by Vander *et al.* (39), and generously provided by Dr. Kjell M. Vårum, Trondheim, Norway. The acetyl groups were distributed randomly along the linear polymer chains, and the polymers were all fully water soluble at slightly acidic pH. Group IV chitosans with a constant DP (obtained from mass average molar mass) of around 2,500 were prepared by re-N-

acetylation of a fully de-N-acetylated chitosan polymer (Table 1) as described by Lamarque *et al.* (19) and were provided by Dr. Alain Domard, Lyon, France. The acetyl groups were distributed randomly along the linear polymer chains, and the polymers were all fully water soluble at slightly acidic pH.

Table 1. Average degree of polymerization (DP) and fraction of acetylation (F_A) of chitosans (Group I) without treatment (A) and thermally treated for 3 and 10 h (B and C) and chitosans (Group II) obtained by partial alkaline deacetylation of chitin (D and E) and chitosans (X_1 to X_6) generated by partial homogeneous de-N-acetylation of chitin (Group III) and chitosans (Y_1 to Y_4) obtained by partial re-N-acetylation of polyglucosamine (Group IV).

	Group I			Group II		
Code	A	B	C	D	E	
DP ^a	1,383	366	45	1,171	1,089	
F_A	0.22	0.23 ^c	0.22	0.08	0.16	
	Group III					
Code	X_1	X_2	X_3	X_4	X_5	X_6
DP ^b	190	320	68	121	210	224
F_A	0.01	0.15	0.35	0.49	0.60	0.69
	Group IV					
Code	Y_1	Y_2	Y_3	Y_4		
DP ^a	2,580	2,608	2,528	2,518		
F_A	0.10	0.29	0.40	0.50		

^a DP_w (mass average molar mass)

^b DP_n (number average molar mass).

^c F_A determined by potentiometric titration. F_A of other samples were determined by high-field ¹H NMR (proton nuclear magnetic resonance spectroscopy)

Microorganisms and cultivation

A. alternata (CCT 2816), *P. expansum* (CCT 4680), and *R. stolonifer* (CCT 2002) were purchased from André Tosello Foundation (Campinas, Brasil). *B. cinerea*, an isolate from grape, was provided by the Department of Botany of the University of Munster. *B. cinerea* and *P. expansum* were cultured on potato dextrose agar (PDA) and in malt extract agar (MEA) supplemented with 2% (m/v) each of glucose and peptone, while *R. stolonifer* and *A. alternata* were both cultured on MEA. In order to achieve sporulation, the fungi

were incubated in Petri dishes ($\varnothing = 9$ cm) for 8 days for *A. alternata*, *B. cinerea*, and *P. expansum*, and for 4 days for *R. stolonifer* at 25°C at 100 cm under Hg lamps with a 12 h photoperiod. Water suspensions of spores and mycelia were filtered through cotton. The concentration of spores was assessed using a hemocytometer (Fuchs-Rosenthal Hell Linie) under optic microscopy (magnification 400×). The concentration of *R. stolonifer* spores was adjusted to 1×10^4 mL⁻¹ and those of *B. cinerea*, *A. alternata*, and *P. expansum* to 2×10^4 mL⁻¹.

Bioassays

Complete medium (CM), pH 4.3, was prepared as described by Pontecorvo (31), which contains approximately 6.2 g x L⁻¹ carbon and 0.6 g x L⁻¹ nitrogen, by considering the

contribution of yeast extract, peptone, casein and sucrose). Aliquots (150 µL) of sterile CM containing the required volume of chitosan ($2 \text{ mg} \times \text{mL}^{-1}$) for dose response and sterile water were dispensed into wells of 96-well polystyrene microtiter plates (Roth®) containing either 10 µL of a spore suspension of a test fungus or 10 µL of sterile water (blanks). All chitosan samples and concentrations tested against the phytopathogenic fungi used in this study are listed in Table 2. The plates were incubated at 25 °C under agitation, 200 o.p.m (orbits per minute), for up to three days for *R. stolonifer*, six days for *B. cinerea* and *A. alternata*, and five days for *P. expansum*. Fungal growth was assessed by measuring the optical density of the culture media at 405 nm at 24 h intervals for *A. alternata*, *B. cinerea*, and *P. expansum* and at 12 h intervals for *R. stolonifer*.

Table 2. Chitosan samples and concentrations tested against the phytopathogenic fungi *A. alternata*, *B. cinerea*, *P. expansum* and *R. stolonifer*.

Chitosan groups	Chitosan codes	Concentrations (µg × mL ⁻¹)	Fungi
I	A, B and C	0, 20, 40, 60, 100, 200, 300,	<i>A. Alternata</i> , <i>B. cinerea</i> , <i>P. expansum</i>
II	D and E	400, 600, 800, 900 and 1,000	and <i>R. stolonifer</i>
III	X ₁ , X ₂ , X ₃ , X ₄ , X ₅ and X ₆	0, 60, 100, 200, 300, 400,	<i>A. Alternata</i> and <i>B. cinerea</i>
IV	Y ₁ , Y ₂ , Y ₃ and Y ₄	600, 700, 800 and 900	

Three independent experiments were carried out for each condition, and the data are reported as means ±S.D. Statistical analysis was carried out with the OriginPro version 8 program (OriginLab Corporation, Northampton, Massachusetts, USA) and differences between the means were detected by the Tukey multiple comparison test. A standard curve was previously prepared to evaluate the correlation of absorbance values with dry weight of biomass, which was found to be linear for the range between zero and 4.0 for the fungi studied. According to Langvad (20), the absorbance measured in the microtiter plate reader is caused by light absorbance and light scattering.

Growth rate (GR) was calculated according to the following equation:

$$AUMGC = \sum_{i=1}^n [(X_{i+1} + X_i) / 2] [t_{i+1} - t_i]$$

where,

AUMGC = area under mycelial growth curve ($A_{\lambda=405\text{nm}} \times \text{day}^{-1}$)

X_i = the absorbance at the time on the i^{th} day

t_i = the time in days of assessment on the i^{th} day

n = the total number of observations

Minimum inhibitory concentration (MIC) was defined as the concentration of chitosan able to reduce GR values to zero.

Chitosans from Group I and from Group II were tested against all fungi studied and *A. alternata* and *B. cinerea*, were selected for further experimentation using Group III and Group IV chitosans. Therefore the antifungal activities of all chitosans were tested against *A. alternata* and *B. cinerea*, but only five chitosans were tested against *P. expansum* and *R. stolonifer*.

Scanning electron microscopy (SEM)

Scanning electron microscopy was carried out according to Melo and Faull (24). In case of control suspension the hyphae were removed from the media by using a clamp and the suspension treated with chitosans was filtered in Millipore PTFE hydrophilic membrane, pore size 0.45 μ m and \varnothing =13mm (São Paulo, Brasil). Samples were fixed by immersion in 2.5%(v/v) glutaraldehyde in 0.1 mol L⁻¹ sodium cacodylate buffer pH 7.0 for 1h, washed three times in 0.1 mol L⁻¹ sodium cacodylate buffer pH 7.0, post-fixed with 1%(m/v) osmium tetroxide diluted in the same buffer for 1h. They were then washed three times with the same buffer pH. Subsequently they were dehydrated in a series of crescent concentrations of acetone (10, 25, 40, 60, 75, 85, 95 and 100%v/v) with 15min resting time in each solution. The materials were dried in a CO₂ apparatus and sputter-coated with gold and viewed using a field emission scanning electron microscope, Leo 982 (Zeiss + Leica).

RESULTS

The growth curves (GC) of the filamentous fungi *R. stolonifer*, *B. cinerea*, *P. expansum*, and *A. alternata* showed that CM medium at pH 4.3 well suited the *in vitro* assays in the microtitre plates, within standard deviation values varying from 0.01 to 0.18 OD readings. It was also observed that 20% (v/v) of 40 mmol L⁻¹ acetic acid in CM (pH 3.9-4.2) did not significantly affect fungal GR and that chitosans markedly inhibited or completely prevented the growth of all four fungi tested. A dose-response relationship was generally observed for each fungus, with average fungal GR decreasing when the

concentration of chitosan increased.

Chitosans from Group I and from Group II were tested against all fungi studied and *A. alternata* and *B. cinerea*, were selected for further experimentation using Group III and Group IV chitosans. Therefore the antifungal activities of all chitosans were tested against *A. alternata* and *B. cinerea*, but only five chitosans were tested against *P. expansum* and *R. stolonifer*.

Figure 1 clearly shows the reduction of GR of *A. alternata*, from 10.91 \pm 0.23 to 1.44 \pm 0.26 A _{λ =405nm} x day⁻¹ when the concentration of chitosan A increased from zero to 60 μ g x mL⁻¹ (MIC=100 μ g x mL⁻¹). The sensitivity of the fungi against chitosan varied according to the strain, and is well depicted in this figure. The GR of *B. cinerea*, was significantly reduced with higher concentrations of chitosan A (900 μ g x mL⁻¹), from 3.22 \pm 0.35 to 0.45 \pm 0.21 A _{λ =405nm} x day⁻¹ and this reduction of GR values by the increase of chitosan concentration was similarly observed for the other fungi, *P. expansum* being the less susceptible in the presence of chitosan A, whose GR reduced from 9.78 \pm 0.44 to 2.17 \pm 0.11 A _{λ =405nm} x day⁻¹.

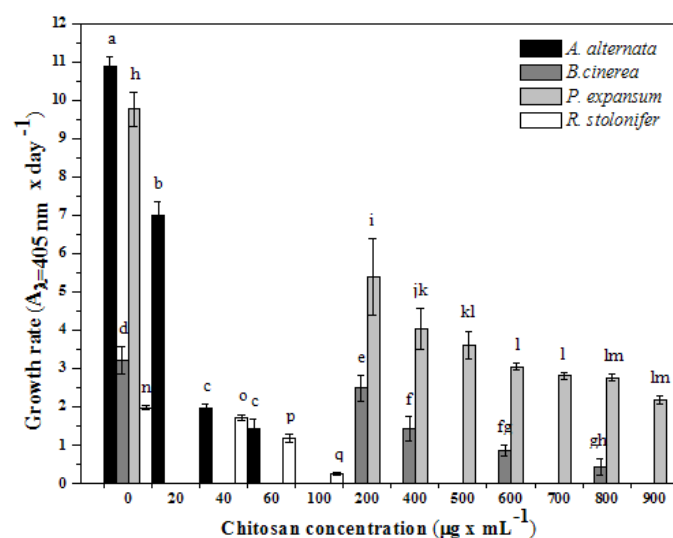


Figure 1. Growth rate of *A. alternata*, *B. cinerea*, *P. expansum* and *R. stolonifer* in presence of different concentrations of chitosan A (group I). ^{a-q}Means for the same fungus with different letters differ significantly ($p \leq 0.05$) according to the Tukey test.

Figure 2 shows the MIC values for Groups I and II chitosans. Chitosans with a constant F_A of ca. 0.22 but different DPs ranging from ca. 1,400 to 45, showed different antifungal activities against *A. alternata*, whose MICs were variable with decreasing DP. In contrast, the sensitivities of *B. cinerea* (MIC = 1,000 $\mu\text{g} \times \text{mL}^{-1}$) and *R. stolonifer* (MIC = 200 $\mu\text{g} \times \text{mL}^{-1}$) did not depend on the DP of these chitosans.

Group II chitosans, with a constant DP of ca. 1,100 but different F_A of 0.08 and 0.16, exhibited equal antifungal activities, with MICs of 100 $\mu\text{g} \times \text{mL}^{-1}$ for *A. alternata* and for *R. stolonifer*, and 900 $\mu\text{g} \times \text{mL}^{-1}$ for *B. cinerea*. Chitosans D and E did not completely inhibit the growth of *P. expansum* (data not shown), but their inhibitory effects were stronger than those of chitosan A from Group I. Therefore, the inhibitory effects of chitosans from Group I and Group II tended to increase with decreasing DP but were not markedly influenced by F_A of 0.08 to 0.22. In all cases, *A. alternata* and *R. stolonifer* were more sensitive to chitosan than *B. cinerea* and *P. expansum*. The less susceptible fungus, *P. expansum* was found to require concentrations of chitosans A, D and E higher than 900 $\mu\text{g} \times \text{mL}^{-1}$, whose MIC values could not be calculated (data not shown).

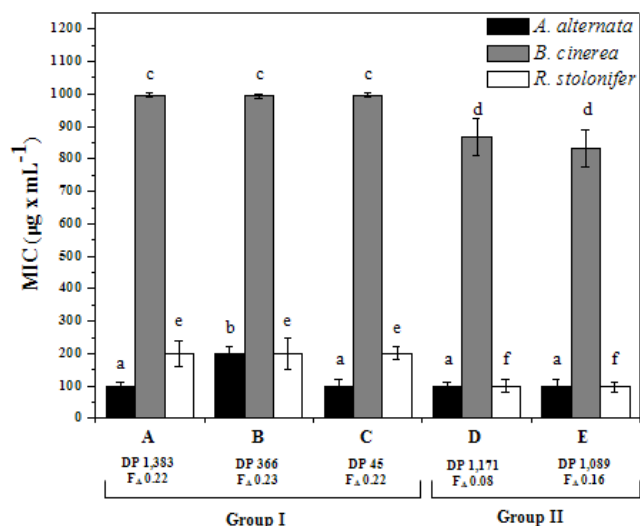


Figure 2. Minimum inhibitory concentrations of chitosans (Group I) A, B, and C with different DP (degree of polymerization) and chitosans (Group II) D and E with different F_A (fraction of acetylation) against *A. alternata*, *B. cinerea*, and *R. stolonifer*. ^{a-f}Means for the same fungus specie with different letters differ significantly ($p \leq 0.05$) according to the Tukey test.

The inhibitory effects of Group III chitosans, within similar DP (about 200) and F_A varying from 0.01 to 0.69 were evaluated against *B. cinerea* and *A. alternata* (Figure 3). Only chitosan X_1 (lowest $F_A = 0.01$) completely inhibited GR of *B. cinerea*, with a MIC of 800 $\mu\text{g} \times \text{mL}^{-1}$; complete inhibition was not obtained with the other chitosans of this group, up to concentration 800 $\mu\text{g} \times \text{mL}^{-1}$. In contrast, growth of *A. alternata* was completely suppressed by chitosans X_1 to X_4 , (F_A varied from 0.01 to 0.49) with increasing MICs. Again, complete inhibition was not obtained with samples X_5 and X_6 (F_A 0.060 and 0.69, respectively) using the maximum concentration of 800 $\mu\text{g} \times \text{mL}^{-1}$. Clearly, the antifungal activity of the Group III chitosans increased with decreasing F_A for both fungi and it was again observed that *A. alternata* was more sensitive than *B. cinerea* to chitosans.

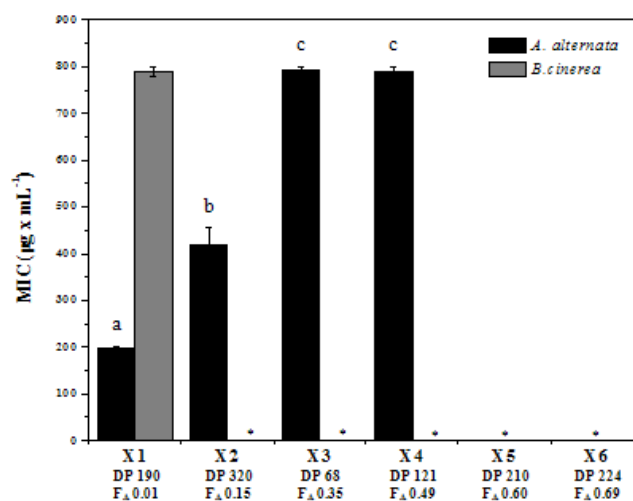


Figure 3. Minimum inhibitory concentrations of chitosans X_1 to X_6 (Group III) generated by partial homogeneous de-N-acetylation of chitin with similar DP and different F_A against *A. alternata* and *B. cinerea*. *MICs were not obtained for the chitosans against the fungi tested. ^{a-c}Means for the same fungus specie with different letters differ significantly ($p \leq 0.05$) according to the Tukey test.

The inhibitory effects of the Group IV chitosans (similar DP of about 2,500 and F_A from 0.10 to 0.50) against *B. cinerea*

and *A. alternata* can be seen in Figure 4. All chitosans were able to completely inhibit GR of both *B. cinerea* and *A. alternata* and MIC values could be calculated. Their inhibitory activity again increased with a decrease in F_A and *A. alternata* was found to be more sensitive than *B. cinerea*. Although the same trend in antifungal activities as that obtained with Group III chitosans was also noticed with Group IV chitosans, Group IV chitosans were more active than Group III chitosans.

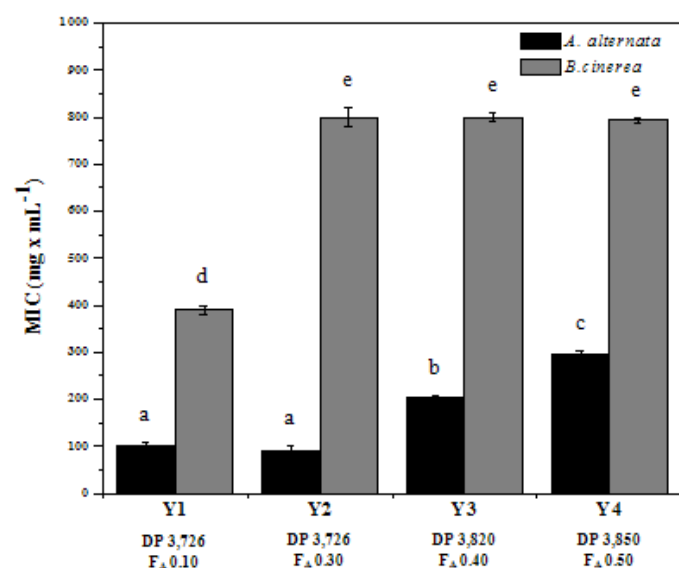
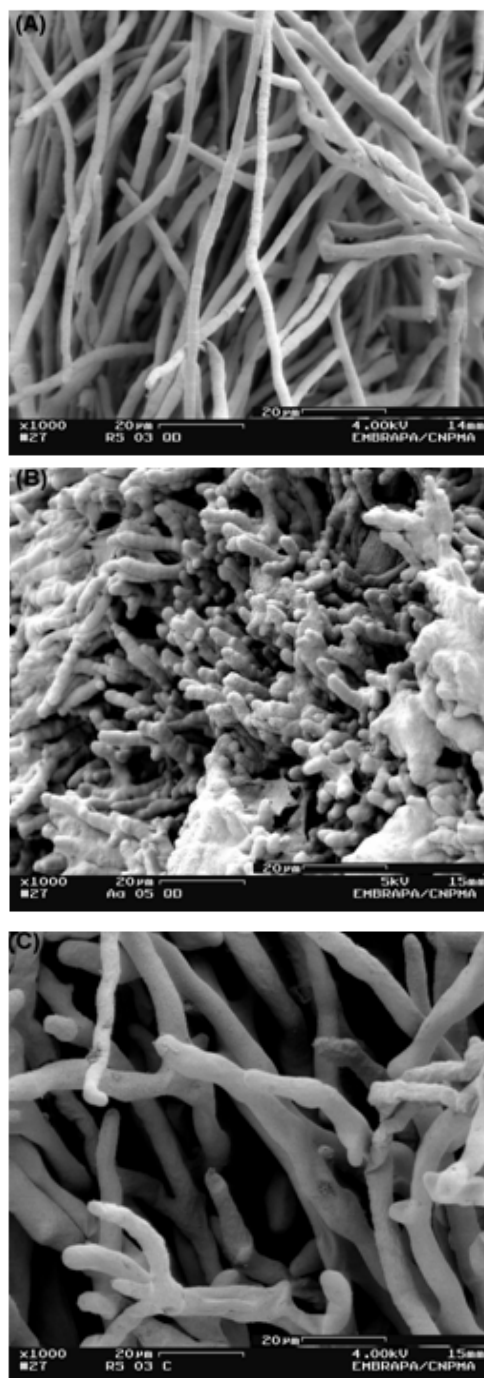


Figure 4. Minimum inhibitory concentrations of chitosans Y₁ to Y₄ (Group IV) obtained by partial re-N-acetylation of polyglucosamine with similar DP and different F_A against *A. alternata* and *B. cinerea*. ^{a-c}Means for the same fungus specie with different letters differ significantly ($p \leq 0.05$) according to the Tukey test.

Changes in fungal morphology due to the effect of chitosan

Chitosans were not only effective in restricting mycelial growth of *A. alternata*, *B. cinerea*, *P. expansum*, and *R. stolonifer*, but also induced marked morphological changes such as general excessive mycelial branching and hyphal size reduction and aggregation. Morphological anomalies caused by coating on the mycelia surface of the fungi studied suggest that

chitosan layer around the surface may make nutrient transport difficult. In the case of *A. alternata* and *B. cinerea*, abnormal shapes and swelling of the mycelia were observed in addition to the morphological changes, and also germ tube inhibition was found for *A. alternata* (Figure 5).



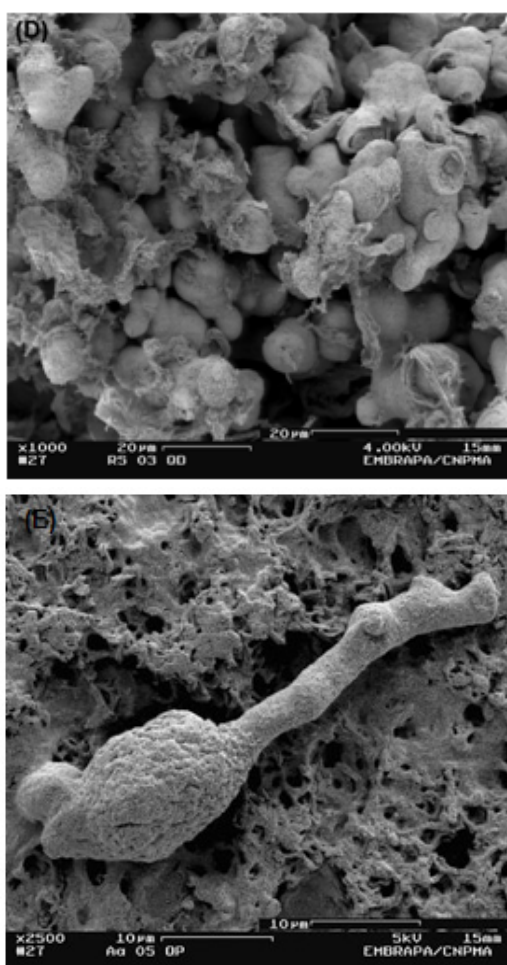


Figure 5. Scanning electron micrographs of mycelia of *A. alternata* (A) in the absence of chitosan, *A. alternata* (B) treated with $500 \mu\text{g} \times \text{mL}^{-1}$ chitosan A (DP 1,089 and F_A 0,16); *R. stolonifer* (C) in the absence of chitosan and *R. stolonifer* (D) treated with $500 \mu\text{g} \times \text{mL}^{-1}$ chitosan A (DP 1,089 and F_A 0,16). Magnification 1,000 \times . (E) Spore and germ tube of *Alternaria alternata* after 5 days of culture at 25 °C with medium amended with chitosan P ($1,000 \text{ mg} \times \text{mL}^{-1}$). Magnification at 2,500 \times .

DISCUSSION AND CONCLUSIONS

We have previously adapted the microtiter plate technique in our laboratory to study the antimicrobial activity of chitosan against plant pathogenic bacteria and fungi, now it has been

extended to this study on the growth of four fungi causing post-harvest damage to fruits and vegetables, in the absence or presence of up to fifteen different chitosans varying greatly in their DP and F_A . The present study corroborates our earlier observations (not published), namely that the antifungal activities of chitosans depend on both of these parameters, by employing the technique validated by Langvad (20). Our study here shows the advantage of miniaturization of analytical tools to evaluate GR and MIC of the four fungi, since conventional techniques require larger volumes of materials, samples, reagents, and are more time consuming.

The effect of F_A is best seen in chitosans of Group III and Group IV which span a wide range of F_A from ca. 0 to 0.7 in both cases, the antimicrobial activity of the chitosans decreased with increasing F_A . Consequently, fully de-acetylated polyglucosamine exhibited the highest antifungal activities. This effect was previously observed by Pacheco *et al.* (28) who have only evaluated a series of five different chitosans against the fungus *P. digitatum*.

The effect of DP is less unambiguous. Group I chitosans with a low F_A and spanning a DP range from 45 to ca. 1,400 clearly demonstrate that the antifungal activity increases with decreasing molecular weight. This is in accordance with our previous study (27) where the same trend was clearly seen with a series of polyglucosamines spanning a DP range from 20 to 2,500. However, when comparing the activities of chitosans of Group III and Group IV chitosans, the high DP chitosans (Group IV) were clearly more active than their low DP counterparts. The DP-dependent difference in inhibitory activity was more pronounced the higher the F_A of the chitosans in both studies. Considering this, we think that there is a possibility that the DP dependency of the inhibitory activity of chitosans changes with F_A , while highly de-acetylated chitosans exhibit their inhibitory activity best as small to medium sized polymers, the inhibitory activity of highly acetylated chitosans may be highest at very large polymer sizes. We are currently generating a series of chitosans

with a constant high F_A and spanning a DP range from small to very large polymers to investigate this possibility.

The relationship between DP of chitosans and chito-oligosaccharides and their antifungal activity has been investigated in some studies (3, 16, 25, 26, 28, 37, 38, 42). Few studies reported on the effect of F_A on the antifungal activity of chitosans, and even fewer on combined effects of both parameters (28).

Oligosaccharides of DP 9 and DP 14 which had been prepared by nitrous acid depolymerization of chitosan were active against *Leptographium procerum* and *Sphaeropsis sapinea*, but not against *Trichoderma harzianum*, while oligomers of DP 5 were inactive (37). On the other hand, *Fusarium solani* was inhibited by chito-oligosaccharides of DP ≥ 7 , while oligomers of DP 2 and DP 8 were not active against three *Fusarium* species (38). Low-DP chitosans, in particular chito-oligosaccharides with an average DP 20, were more effective than high-DP chitosans in inhibiting mycelial growth of a variety of phytopathogenic and wood inhabiting fungi (16, 38, 42). Different methods of chitosan preparation had a significant effect on the DP and F_A of the resulting biopolymers and thereby, their antimicrobial activities (28).

Oliveira-Jr *et al.* (26) have observed that chito-oligosaccharides of DP ≤ 8 are not notably inhibitory to any of the fungi, *A. alternata*, *B. cinerea*, *P. expansum*, and *R. stolonifer* and high-DP chito-oligosaccharides (DP ≤ 12) showed initially inhibitory effects. However, the complete inhibition for all fungi was not obtained by using chito-oligosaccharides. In contrast, as reported in our present study, *A. alternata*, *B. cinerea* e *R. stolonifer* were completely inhibited and growth reduction for *P. expansum* was observed by high-DP chitosans (DP 45 to 2,608). Higher antibacterial activity of chitosan compared with chitosan oligomers also was reported by several workers (32, 34, 38).

The novelty of our studies is that the antimicrobial activities of a very wide variety of chitosans against different fungi, including species belonging to different taxonomic

groups, namely is described here. While the growth of all of them was inhibited to some extent by the presence of chitosans, their sensitivity varied greatly. However, the relative antifungal activities of different chitosans were similar for all fungi studied, so that a general mechanism appears to be responsible for the observed growth inhibition.

The exact mechanism by which the higher chito-oligosaccharides and chitosans exert antimicrobial activity is unknown. Based on the other author's observation that the fungistatic activity is higher at lower pH, it was assumed that the toxicity is correlated, besides to optimum DP, to the cationic charge of the oligosaccharides (37). Our studies indicate that reasons also can be important for the growth rate inhibitions, i.e. enzymatic uptake of simple carbohydrates by permeases could temporally be blocked by the presence of the large oligosaccharides (27). Several fungi systems however, such as cellulase containing, are usually controlled by inducers, and glucose or catabolite repression, and the expression of enzymes to hydrolyze larger molecules to soluble oligosaccharides (low DP). After cellulose and large molecules are degraded a large amount of glucose is liberated, which causes catabolite repression (36). Chitin hydrolyzing enzymes could be similarly regulated, controlled by inducers and short chain molecules. Amaretti *et al.* (2) have demonstrated carbohydrate preferences in bacteria resulting from different distributions of carbon fluxes through the fermentative pathway, where substrate selectivity was observed based on the degree of polymerization, when shorter saccharides were the first to be consumed, while a delay was observed until longer oligosaccharides were utilized.

A number of possible mechanisms for the antimicrobial action of chitosan have been proposed, mostly based on the positive charge conferred by protonation of free amino groups at acidic pH, although the exact mechanism of action is still unknown. A polycationic chitosan or oligomer can potentially interact with negatively charged fungal cell membrane components (i.e., proteins, phospholipids), thus interfering with

the normal growth and metabolism of the fungal cells (4, 13, 34). Roller and Covill (32) reported that amino groups in chitosan have the ability to interact with a multitude of anionic groups on the yeast cell wall surface, thereby forming an impervious layer around the cell. Because of its property to form films, chitosan may thus act as a barrier (i.e. anionic groups) and consequently, reducing their availability to a level that will not sustain growth of the pathogen (4). Our results suggest that this barrier to water soluble nutrients may be most effective for chitosans of lower molar mass and low F_A , since we also have observed (40) that the water permeability of chitosan films is 50% reduced when molar mass of the original chitosan is reduced from 235 kDa (DP 1,383) to approximately 13.7 kDa (DP 45).

In conclusion, in the present study we have shown that the combined effects of F_A and DP are important variables in the bioactivity of chitosans. The chitosan coating on the surface of the mycelia and severe structural alterations in the fungal mycelia, observed by scanning electron microscopy, suggest that fungal growth inhibition could be explained by the direct interaction of chitosan on the fungal cell wall. Results of this study indicate that chitosan samples with low F_A were most effective against the phytopathogenic fungi tested, while chitosan with high F_A did not have the ability to inhibit fungal growth *in vitro*. Complete inhibition was obtained for the fungi *B. cinerea*, *R. stolonifer*, and *A. alternata*, and growth reduction for *P. expansum*, using chitosan samples with different F_A and DP.

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REFERENCES

- Allan, C.R.; Hadwiger, L.A. (1979). The fungicidal effect of chitosan on fungi of varying cell composition. *Exp. Mycol.* 3, 285-287.
- Amaretti, A.; Bernardi, T.; Tamburini, E.; Zanoni, S.; Lomma, M.; Matteuzzi, D.; Rossi, M. (2007) Kinetics and metabolism of *Bifidobacterium adolescentis* MB 239 growing on glucose, galactose, lactose, and galactooligosaccharides. *Appl. Environ. Microbiol.* 11, 3637-3644.
- Badawya, M. E. I.; Rabea, E. I. (2009) Potential of the biopolymer chitosan with different molecular weights to control postharvest gray mold of tomato fruit. *Postharvest Biol. Technol.* 51, 110-117.
- Bautista-Baños, S.; Hernandez-Lauzardo, A.N.; Velázquez-del Valle, M.G.; Hernández-López, M.; Bravo-Luna, L.; Ait Barka, E.; Bosquez-Molina, E.; Wilson, C.L. (2006). Chitosan as a potential natural compound to control pre and postharvest diseases of horticultural commodities. *Crop Prot.* 25, 108-118.
- Beales, N.; Smith, J. (2007). Antimicrobial Preservative-Reduced Foods. In: Smith, J.(eds). *Technology of Reduced Additive Foods*. Wiley, London, England. p.84-105.
- Bhaskara Reddy, M.V.; Belcaceci, K.; Corcuff, R.; Castaigne, F.; Arul, J. (2000). Effect of pre-harvest chitosan sprays on post-harvest infection by *Botrytis cinerea* and quality of strawberry fruit. *Postharvest Biol. Technol.* 20, 39-51.
- de Capdeville, G.; Wilson, C. L.; Beer, S. V.; Aist, J. R. (2002) Alternative disease control agents induce resistance to blue mold in harvested 'Red Delicious' apple fruit. *Phytopathology.* 92, 900-908.
- El Ghaouth, A.; Arul, J.; Asselin, A.; Benhamou, N. (1992). Antifungal activity of chitosan on postharvest pathogens: Induction of morphological and cytological alterations in *Rhizopus stolonifer*. *Mycol. Res.*, 96, 769-779.
- El Ghaouth, A.; Arul, J.; Grenier, J.; Asselin, A. (1992). Antifungal activity of chitosan on two postharvest pathogens of strawberry fruits. *Phytopathology.* 82, 398-402.
- El Ghaouth, A.; Arul, J.; Ponnampalam, R.; Boulet, M. (1991). Chitosan coating effect on storability and quality of fresh strawberries. *J. Food Sci.* 56, 1618-1620.
- El Ghaouth, A.; Arul, J.; Ponnampalam, R.; Boulet, M. (1991). Use of chitosan coating to reduce water loss and maintain quality of cucumber and bell pepper fruits. *J. Food Process. Preservat.* 15, 359-368.
- El Ghaouth, A.; Arul, J.; Wilson, C.; Benhamou, N. (1997). Biochemical and cytochemical aspects of the interactions of chitosan and *Botrytis cinerea* in bell pepper fruit. *Postharvest Biol. Technol.* 12, 183-194.
- Fang, S.W.; Li, C.F.; Shih, D.Y.C. (1994). Antifungal activity of chitosan and its preservative effect on low sugar candied kumquat. *J. Food Prot.* 56, 136-140.
- Fujimoto, T.; Tsuchiya, Y.; Terao, M.; Nakamura, K.; Yamamoto, M.

- (2006) Antibacterial effects of Chitosan solution against *Legionella pneumophila*, *Escherichia coli*, and *Staphylococcus aureus*. *Int. J. Food Microbiol.* 112, 96-101.
15. Gil, G.; del Monaco, S.; Cerrutti, P.; Galvagno, M. (2004). Selective antimicrobial activity of chitosan on beer spoilage bacteria and brewing. *Biotechnol. Lett.* 26, 569-574.
 16. Hirano, S.; Nagao, N. (1989). Effects of chitosan, pectic acid, lysozyme and chitinase on the growth of several phytopathogens. *Agric. Biol. Chem.* 53, 3065-3066.
 17. Jiang, Y.M.; Li, Y.B. (2001). Effects of chitosan coating on postharvest life and quality of longan fruit. *Food Chem.* 73, 139-143.
 18. Kendra, D.F.; Hadwiger, L.A. (1984). Characterization of the smallest chitosan oligomer that is maximally antifungal to *Fusarium solani* and elicits pisatin formation by *Pisum sativum*. *Exp. Mycol.* 8, 276-281.
 19. Lamarque, G.; Lucas, J.M.; Viton, C.; Domard, A. (2005). Physicochemical behavior of homogeneous series of acetylated chitosans in aqueous solution: Role of various structural parameters. *Biomacromolecules.* 6(1), 131-142.
 20. Langvad, F. (1999). A rapid and efficient method for growth measurement of filamentous fungi. *J. Microbiol. Methods.* 37, 97-100.
 21. Liu, H.; Du, Y.; Wang, X.; Sun, L. (2004). Chitosan kills bacteria through cell membrane damage. *Int. J. Food Microbiol.* 95, 147-155.
 22. Mari, M.; Iori, R.; Leoni, O.; Marchi, A. (1996) Bioassays of glucosinolate-derived isothiocyanates against postharvest pear pathogens. *Plant Pathol.* 45, 753-760.
 23. McRoberts, N.; Lennard, J. H. (1996) Pathogen behaviour and plant cell reactions in interactions between *Alternaria* species and leaves of host and nonhost plants. *Plant Pathol.* 45, 742-752.
 24. Melo, I. S., Faull, J. L. (2004). Scanning electron microscopy of conidia of *Thichoderma stromaticum*, a biocontrol agent of witches broom disease of cocoa. *Braz. J. Microbiol.* 35, 330-332.
 25. Meng, X.; Yang, L.; Kennedy, J. F. Tian, S. (2010) Effects of chitosan and oligochitosan on growth of two fungal pathogens and physiological properties in pear fruit. *Carbohydr. Polym.* 81, 70-75.
 26. Oliveira-Jr E.N.; El Gueddari, N.E.; Moerschbacher, B.M.; Peter, M.G.; Franco, T.T. (2008). Growth of Phytopathogenic Fungi in the Presence of Partially Acetylated Chitoooligosaccharides. *Mycopathologia.* 166, 163-174.
 27. Oliveira-Jr. E.N. (2006). *Caracterização dos efeitos de quitosanas na inibição de fungos fitopatogênicos*. Campinas, Brasil, 147p. (PhD. Thesis. School of Chemical Engineering. UNICAMP).
 28. Pacheco, N.; Larralde-Corona, C.P.; Sepulveda, J; Trombotto, S.; Domard, A.; Shirai, K. (2008). Evaluation of chitosans and *Picchia guilliermondii* as growth inhibitors of *Penicillium digitatum*. *Int. J. Biol. Macromol.* 43, 20-26.
 29. Peter, M.G. (2002). Chitin and chitosan from animal sources. In: Vandamme, E.J.; De Baets, S.; Steinbüchel, A.(eds). *Biopolymers, Polysaccharides II: Polysaccharides from Eukaryotes*. Wiley-VCH, Weinheim, Germany, p.481-574.
 30. Placencia-Jatomea, M.; Viniegra, G.; Olayo, R.; Castillo-Ortega, M.M.; Shirai, K. (2003) Effect of chitosan and temperatura on spore germination on *Aspergillus niger*. *Macromol. Biosci.* 3, 582-586.
 31. Pontecorvo, G. (1953). The genetics of *Aspergillus nidulans*. *Adv.Genet.* 5, 150-151.
 32. Roller, S.; Covill, N. (1999). The antifungal properties of chitosan in laboratory media and apple juice. *Int. J. Food Microbiol.* 47, 67-77.
 33. Sánchez-Domínguez, D.; Ríos, M. Y.; Castillo-Ocampo, P.; Zavala-Padilla, G.; Ramos-García, M.; Bautista-Baños, S. (2011) Cytological and biochemical changes induced by chitosan in the pathosystem *Alternaria alternata*–tomato. *Pest. Biochem. Physiol.* 99, 250–255.
 34. Shahidi, F.; Arachchi, J.K.V.; Jeon, Y.J. (1999). Food applications of chitin and chitosans. *Trends Food Sci. Technol.* 10(2), 37-51.
 35. Sudharshan, N. R.; Hoover, D.G.; Knorr, D. (1992). Antibacterial action of chitosan. *Food Biotechnol.* 6, 257-272.
 36. Suto, M.; Tomita, F. (2001) Induction, catabolite repression mechanisms of cellulase in fungi. *J. Biosci. Bioeng.* 92, 305–311.
 37. Torr, K.M.; Chittenden, C.; Franich, R.A.; Kreber, B. (2005). Advances in understanding bioactivity of chitosan and chitosan oligomers against selected wood-inhabiting fungi. *Holzforchung.* 59, 559-567.
 38. Uchida, Y.; Izume, M.; Ohtakara, A. (1988) Preparation of chitosan oligomers with purified chitosanase and its application. In: Skjåk-Bræk G., Anthonsen T., Sandford P.(eds). *Chitin and chitosan: sources, chemistry, biochemistry, physical properties and applications*. Elsevier, London, England, p.373–382.
 39. Vander, P.; Varum, K.M.; Domard, A.; El Gueddari, N.E.; Moerschbacher, B.M. (1998). Comparison of the ability of partially N-acetylated chitosans and chitoooligosaccharides to elicit resistance reactions in wheat leaves. *Plant Physiol.* 118, 1353-1359.
 40. Yoshida, C.M.P.; Oliveira-Jr, E.N.; Franco, T.T. (2008). Chitosan tailor-made films: the effects of additives on barrier and mechanical properties. *Packing Technol. Sci.* 22, 161 – 170.
 41. Yu, T.; Li, H.Y.; Zheng, X.D. (2007). Synergistic effect of chitosan and *Cryptococcus laurentii* on inhibition of *Penicillium expansum* infections. *Int. J. Food Microbiol.* 14, 261-266.
 42. Zhang, M.; Tan, T.; Yuan, H.; Rui, C. (2003) Insecticidal and fungicidal activities of chitosan and oligo-chitosan. *J. Bioact. Compat. Polym.* 18, 391-400.