

Changes in Hyphal Morphology due to Chitosan Treatment in Some Fungal Species

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ABSTRACT

*In this work, changes in the hyphal morphology due to chitosan treatment in some fungal species were studied. Scanning electron microscope (SEM) observations revealed that chitosans with molar fraction of acetyl groups (F_A 0.16 and 0.18) and degree of polymerization (DP 1,089 and 1,242) had a direct effect on the morphology of the chitosan-treated fungi, reflecting its potential for causing a delay in the growth of *Alternaria alternata* ($500 \mu\text{g} \times \text{mL}^{-1}$), *Botrytis cinerea* ($1,000 \mu\text{g} \times \text{mL}^{-1}$), *Penicillium expansum* ($1,000 \mu\text{g} \times \text{mL}^{-1}$) and *Rhizopus stolonifer* ($500 \mu\text{g} \times \text{mL}^{-1}$). Mycelial aggregation and structural changes such as excessive branching, swelling of the cell wall and hyphae size reduction were observed in the micrographs.*

Key words: chitosan, alternative control of fungi, antifungal activity, plant pathogenic fungi, fungal morphology

INTRODUCTION

Chitin and chitosan (Fig. 1) and oligomers of glucosamine (GlcN) and N-acetyl glucosamine (GlcNAc) as shown in the Figure 2, have been of interest in the past few decades due to their broad range of potential industrial applications. However, only limited attention has been given to food application of these versatile polymers, oligomers and monomers (Shahidi, 1999). Chitin and chitosan are aminoglucoopyranans composed of N-acetylglucosamine (GlcNAc) and glucosamine (GlcN) residues. The polymers may be distinguished by their solubility in 1% aqueous acetic acid. Chitin, containing ca. > 40% GlcNAc residues ($F_A > 0.4$) is insoluble, whereas soluble polymers are named chitosan (Peter 2002a). Chitosan has three types of reactive functional

groups, an amino group as well as both primary and secondary hydroxyl groups at the C-2, C-3 and C-6 positions respectively (Furusaki et al. 1996). Method mostly used by the industry to produce chitosan from chitin is chemical deacetylation (alkaline hydrolysis); however, chitosan can be obtained by enzymatic deacetylation of chitin (Fig. 1).

Chitin is a natural biopolymer and it is a structural polysaccharide found in the exoskeleton of marine crustaceans (crab and shrimp shells) and insects. It is also widely found in fungi, occurring in *Basidiomycota*, *Ascomycota*, and *Oomycota*, as a component of cell walls and structural membranes of mycelia, stalks, and spores (Peter 2002b). After the discovery of antimicrobial activities of chitosans and its derivatives by Allan and Hadwiger (1979), Kendra and Hadwiger (1984) and Uchida and Ohtakara (1989), many

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researchers have conducted studies in this field (El Ghaouth et al. 1992; Roller and Covill 1999; Ait

Barka et al. 2004; Bautista-Baños et al. 2006; Oliveira-Jr et al. 2008).

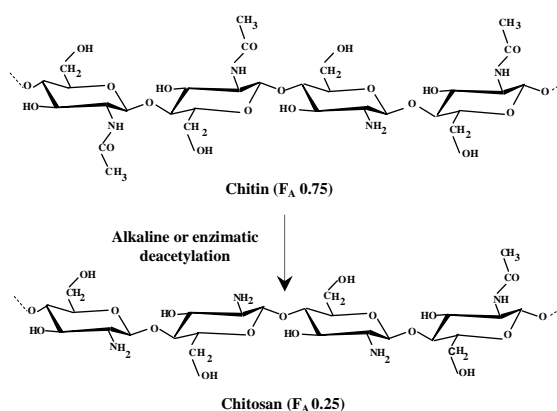


Figure 1 - Structure of chitin and chitosan

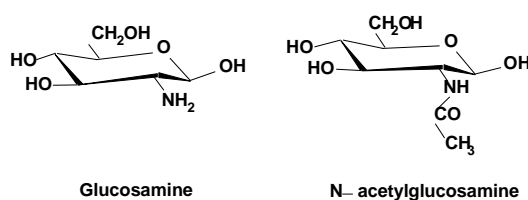


Figure 2 - Structures of glucosamine and N-acetylglucosamine monomers

The filamentous fungi *Alternaria alternata*, *Botrytis cinerea*, *Penicillium expansum* and *Rhizopus stolonifer* are the causal agents of rots in mango fruits (Prusky et al. 2002), strawberries (El Ghaouth et al. 1991a), apples (Morales et al. 2010) and peaches (Zhang et al. 2007), respectively. In vitro studies have demonstrated that chitosan cause growth inhibition of *Alternaria alternata*, *Botrytis cinerea*, *Penicillium expansum* and *Rhizopus stolonifer* (Oliveira-Jr. 2006). Chitosan coating on storability and quality of fresh strawberries protected them from infection by *Botrytis cinerea* and improved their quality (El Ghaouth et al. 1991b). There is a worldwide trend to explore new alternatives that can control postharvest pathogenic diseases, giving priority to methods that reduce disease incidence and avoid negative and side effects on human health as a result of the excessive application of synthetic fungicides. Thus, alternative approaches are necessary to maintain the marketable quality of fresh fruits.

The purpose of this work was to study the effect of chitosan on the hyphal morphology of *Alternaria alternata*, *Botrytis cinerea*, *Penicillium expansum* and *Rhizopus stolonifer*.

MATERIALS AND METHODS

Chitosans samples

Chitosan (molar fraction of acetyl groups F_A 0.16 and degree of polymerization DP 1,089) produced from alkaline deacetylation of chitin (Oliveira-Jr., 2006) and chitosan (F_A 0.18 and DP 1,242) from Primex (Siglufjordur, Iceland) were used. Chitosan F_A 0.16 and DP 1,089 were labeled as chitosan D and chitosan F_A 0.18 and DP 1,242 was labeled as chitosan P.

Microorganisms and cultivation

The filamentous fungi used in this study included *Alternaria alternata* (CCT 2816), *Penicillium expansum* (CCT 4680) and *Rhizopus stolonifer*

(CCT 2002). All of these strains were purchased from André Tosello Foundation Research and Technology (Campinas, SP, Brazil). *Botrytis cinerea*, an isolate from spoiled grape fruit, was provided by the Institute of Food Technology (Campinas, SP, Brazil). *Botrytis cinerea* and *Penicillium expansum* were cultured on potato dextrose agar (PDA) and in malt extract agar (MEA), supplemented with 2% (m/v) of glucose and peptone, respectively, while the *Rhizopus stolonifer* and *Alternaria alternata* were both cultured on MEA. In order to achieve the sporulation, the fungi were incubated in Petri dishes ($\varnothing=9\text{cm}$) for eight days for *A. alternata*, *B. cinerea* and *P. expansum* and for four days for *R. stolonifer* at 25°C at 100 cm beneath Hg lamps with a 12 h photoperiod. Spores were harvested by pouring the sterile water into the slant and stirring with a vortex for 20 seconds. The suspensions of spores and mycelia were filtered in the cotton. The concentration of spores was assessed by using a hemocytometer (Fuchs-Rosenthal Hell Linie) under light microscope (magnification 400 \times). The concentration of spores of *Rhizopus stolonifer* was adjusted to the 10,000 spores \times mL⁻¹ and for *B. cinerea*, *A. alternata* and *P. expansum* to the 20,000 spores \times mL⁻¹.

Chitosan solution and media preparation

Stock solutions of chitosans D and P (10 g \times L⁻¹) were transferred to 1.0 mL of acetic acid solution (40 mmol \times L⁻¹) in microtubes of 2.0 mL and shaken in a Vortex mixer for 1 h. The medium used was liquid malt extract (2% m/v) from Merck (Darmstadt, Germany). The chitosan concentrations in the media were 500 $\mu\text{g} \times$ mL⁻¹ for *A. alternata* and *R. stolonifer* and 1,000 $\mu\text{g} \times$ mL⁻¹ for *B. cinerea* and *P. expansum*. The final pHs were 4.9 (control medium), 5.4 (acetic acid, chitosan and medium) and 4.4 (control of acetic acid and medium). In previously autoclaved microtubes of 2.0 mL appropriate volume of chitosan stock or acetic acid solutions (50 μL for *A. alternata* and *R. stolonifer* and 100 μL for *B. cinerea* and *P. expansum*) was added. The microtubes containing 50 μL were supplemented with autoclaved distilled water to make the volume as 100 μL . In all the microtubes 890 μL of malt extract medium was added. The spore suspension was first vortex-mixed vigorously and 10 μL of spore suspensions were transferred to the microtubes. After inoculation, the microtubes were

closed and incubated at 25 °C under stirring of 200 o.p.m (orbits per minute) for three days for *R. stolonifer* and five days for *A. alternata*, *B. cinerea* and *P. expansum*. The experiments were carried out with three repetitions.

Scanning electron microscopy (SEM)

Scanning electron microscopy was carried out in accordance to the methodology described by Melo and Faull (2004) on the materials described below. In case of control suspension, the hyphae was removed from the media by using the clamp and the suspension treated with chitosans were filtered in Millipore membrane PTFE hydrophilic of pore size 0.45 μm and $\varnothing=13$ mm (São Paulo, SP, Brazil). The 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 in the same buffer for 1h and washed three times again in 0.1 mol L⁻¹ sodium cacodylate buffer pH 7.0. The material was then dehydrated in a crescent acetone series (10, 25, 40, 60, 75, 85, 95 and 100%, v/v) with 15min per change. The specimens were transferred to a critical point dryer to complete the drying process with carbon dioxide as a transition fluid. Then, the specimens obtained were mounted on aluminum stubs, with a double-stick carbon tape and sputter-coated with gold and observed using a field emission scanning electron microscope, Leo 982 (Zeiss + Leica).

RESULTS AND DISCUSSION

Results after five days of cultivation at 25°C for *A. alternata* (Fig. 3), *B. cinerea* (Fig. 4) and *P. expansum* (Fig. 5) and after three days for *R. stolonifer* (Fig. 6) showed that chitosan amendment caused aggregation and morphological changes of mycelia which were coated with chitosan. Aggregation, excessive mycelial branching and hyphae size reduction of all fungi treated with chitosan were observed. *A. alternata*, *B. cinerea* and *R. stolonifer* treated with chitosan besides to have the morphological changes mentioned before, also showed abnormal shapes and swelling in their mycelia (Figures 3, 4 and 6). El Ghaouth et al. (1992) reported that chitosan (3,000 $\mu\text{g} \times$ mL⁻¹, F_A 0,99, DP not shown) caused severe morphological changes in *R. stolonifer*

which was characterized by excessive branching and swelling of the cell wall; however, in *B. cinerea* and *A. alternata* that morphological changes were not observed. The chitosan ($500 \mu\text{g} \times \text{mL}^{-1}$ in *A. alternata* and *R. stolonifer*; $1,000 \mu\text{g} \times \text{mL}^{-1}$ in *B. cinerea* and *P. expansum*) labeled as sample D (F_A 0.16, DP 1,089) and labeled as sample P (F_A 0.18, DP 1,242) induced considerable morphological changes in all the four

fungi tested (Figures 3 to 6). These diverging results might have originated from different methods of chitosan preparation that had a significant effect on the DP and F_A of the resulting biopolymers and thereby, their antimicrobial activities.

Chitosans on the surface of *R. stolonifer* mycelia were observed in the scanning electron micrographs (Figures 6 C and D).

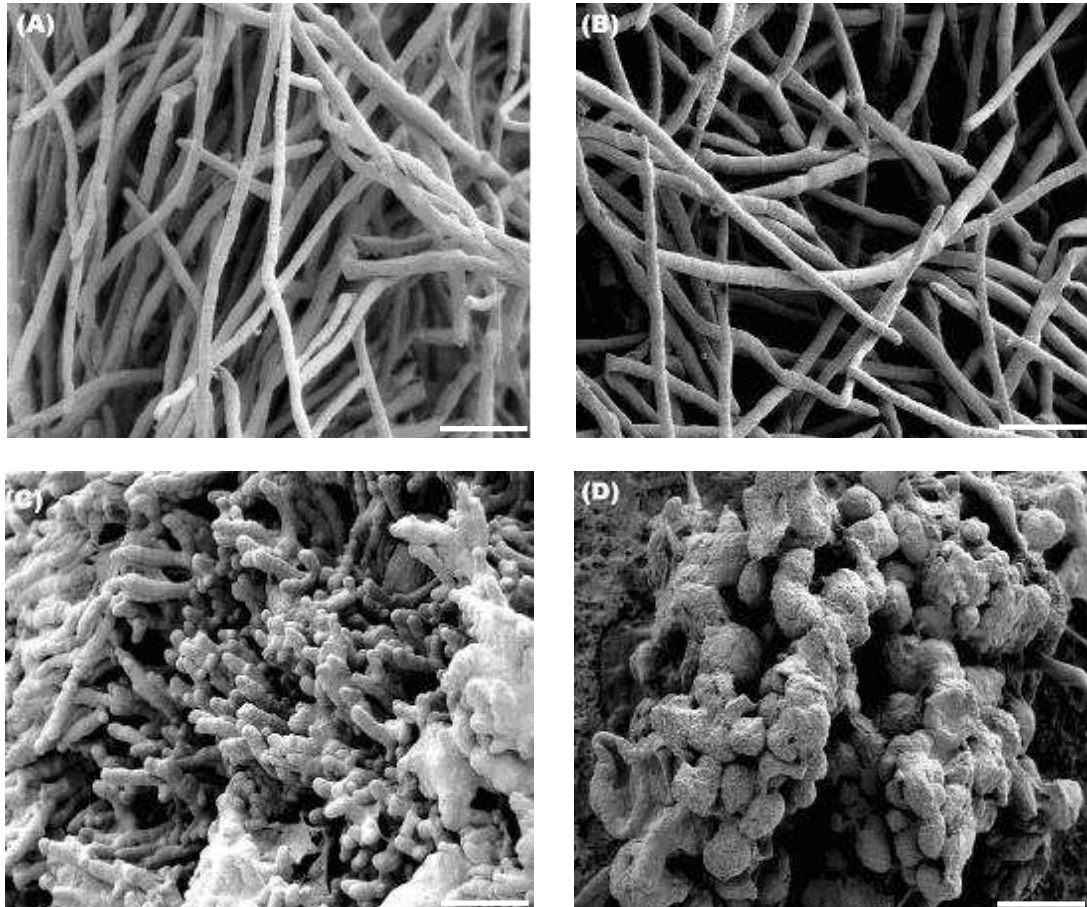


Figure 3 - Scanning electron micrographs of *Alternaria alternata* mycelia after 5 days of cultivation at 25 °C. (A) Control media, (B) acetic acid control ($40 \text{ mmol} \times \text{L}^{-1}$), (C) medium amended with chitosan D ($500 \mu\text{g} \times \text{mL}^{-1}$) and (D) medium amended with chitosan P ($500 \mu\text{g} \times \text{mL}^{-1}$). Bars = 20 μm .

In further studies, image analysis was used to measure the effect of chitosan on the morphology of fungi, such as *F. oxysporum* f. sp. *radicis-lycopersici*, and *S. sclerotiorum* treated with chitosan. These studies showed excessive mycelial branching, abnormal shapes, swelling, and hyphae

size reduction (Benhamou 1992; Cheah et al. 1997). Large vesicles or empty cells devoid of cytoplasm in the mycelium of *B. cinerea*, treated with chitosan, were observed by Ait Barka et al. (2004).

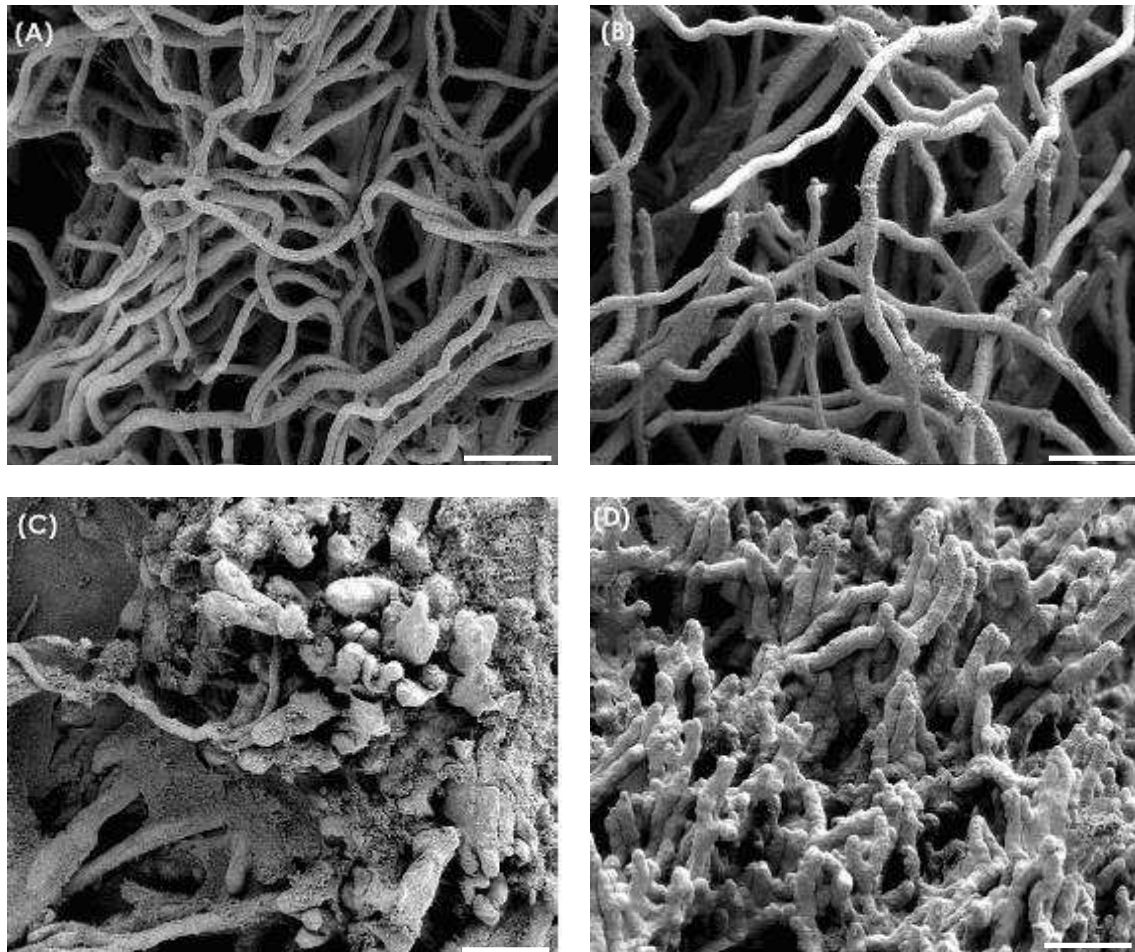


Figure 4 - Scanning electron micrographs of *Botrytis cinerea* mycelia after 5 days of cultivation at 25 °C. (A) Control media, (B) acetic acid control ($40 \text{ mmol} \times \text{L}^{-1}$), (C) medium amended with chitosan D ($1,000 \text{ } \mu\text{g} \times \text{mL}^{-1}$) and (D) medium amended with chitosan P ($1,000 \text{ } \mu\text{g} \times \text{mL}^{-1}$). Bars = 20 μm .

The micrographs showed that chitosan amendment caused aggregation of mycelia that were coated with this polymer. Similar morphological anomalies caused by chitosan coating on the mycelial surface of fungi studied, suggested that chitosan layer around the spores or hyphae could cause difficulty for the entry of nutrients in the cells.

The exact mechanism by which the higher chitooligosaccharides and chitosans exert antimicrobial activity is unknown. Based on the observation that the fungistatic activity was higher at lower pH, it was assumed that the toxicity was correlated, besides to optimum DP, to the cationic charge of the oligosaccharides (Torr et al. 2005). The present study indicated that reasons also could be important for the growth rates inhibition, i.e., enzymatic uptake of simple carbohydrates by the

permeases could temporally be blocked by the presence of the large oligosaccharides (Oliveira-Jr. et al. 2008). Several fungi systems (such as cellulase containing) are usually controlled by the 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 (Suto and Tomita 2001).

Chitin hydrolyzing enzymes could be similarly regulated, controlled by the inducers and short chain molecules. Amaretti et al. (2007) have demonstrated carbohydrate preferences in bacteria resulting from different distributions of carbon fluxes through the fermentative pathway, where it 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 (Oliveira-Jr. et al. 2008).

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 (Bautista-Baños et al. 2006; Fang et al. 1994; Shahidi et al. 1999). Roller and Covill (1999)

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 the 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 form the films (Bautista-Baños et al. 2006). The present results suggested that this barrier to water soluble nutrients could be most effective for chitosans of lower molar mass and low F_A , since it was observed that the water permeability of chitosan films was 50% reduced when molar mass of the original chitosan was reduced from 235 kDa (DP 1,383) to approximately 13.7 kDa (DP 45), which was also reported by Yoshida et al. (2008).

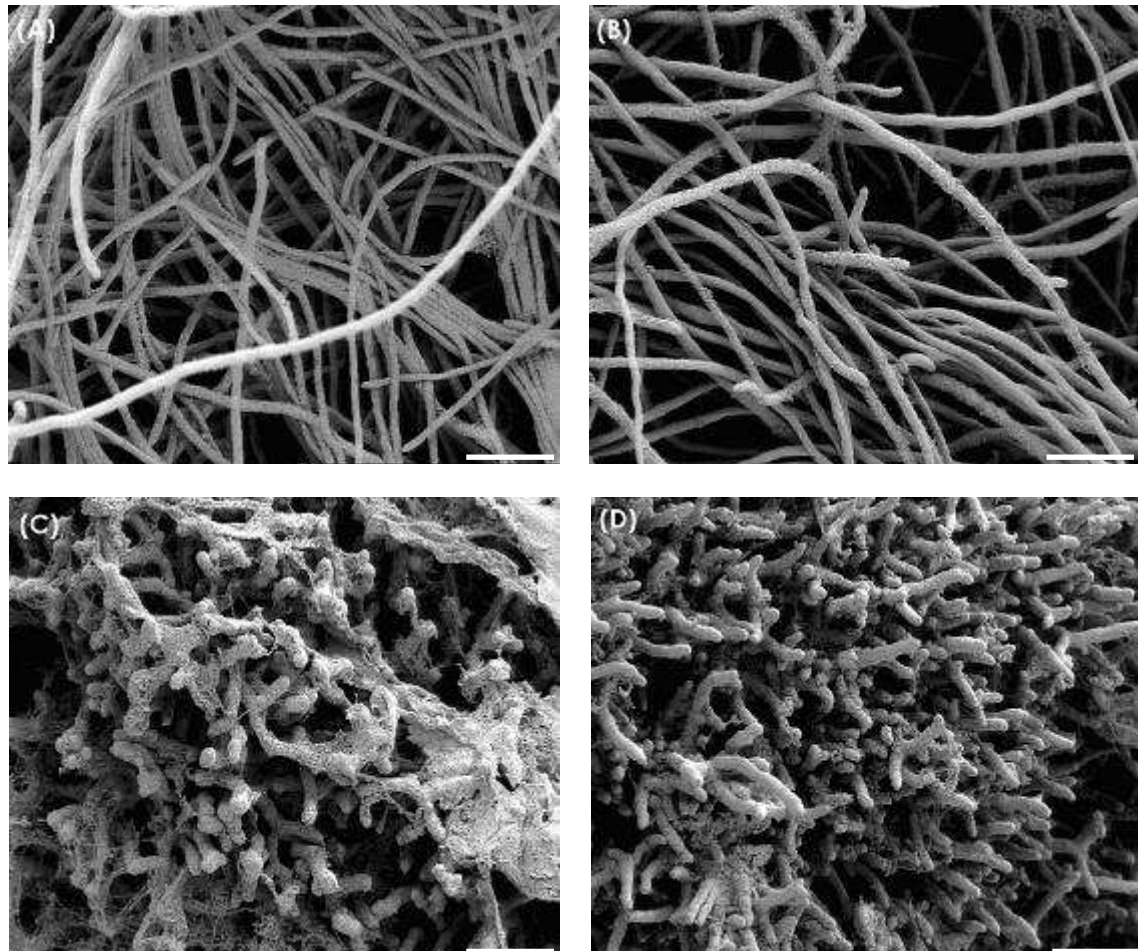


Figure 5 - Scanning electron micrographs of *Penicillium expansum* mycelia after 5 days of cultivation at 25 °C. (A) Control media, (B) acetic acid control (40 mmol \times L⁻¹), (C) medium amended with chitosan D (1,000 μ g \times mL⁻¹) and (D) medium amended with chitosan P (1,000 μ g \times mL⁻¹). Bars = 20 μ m.

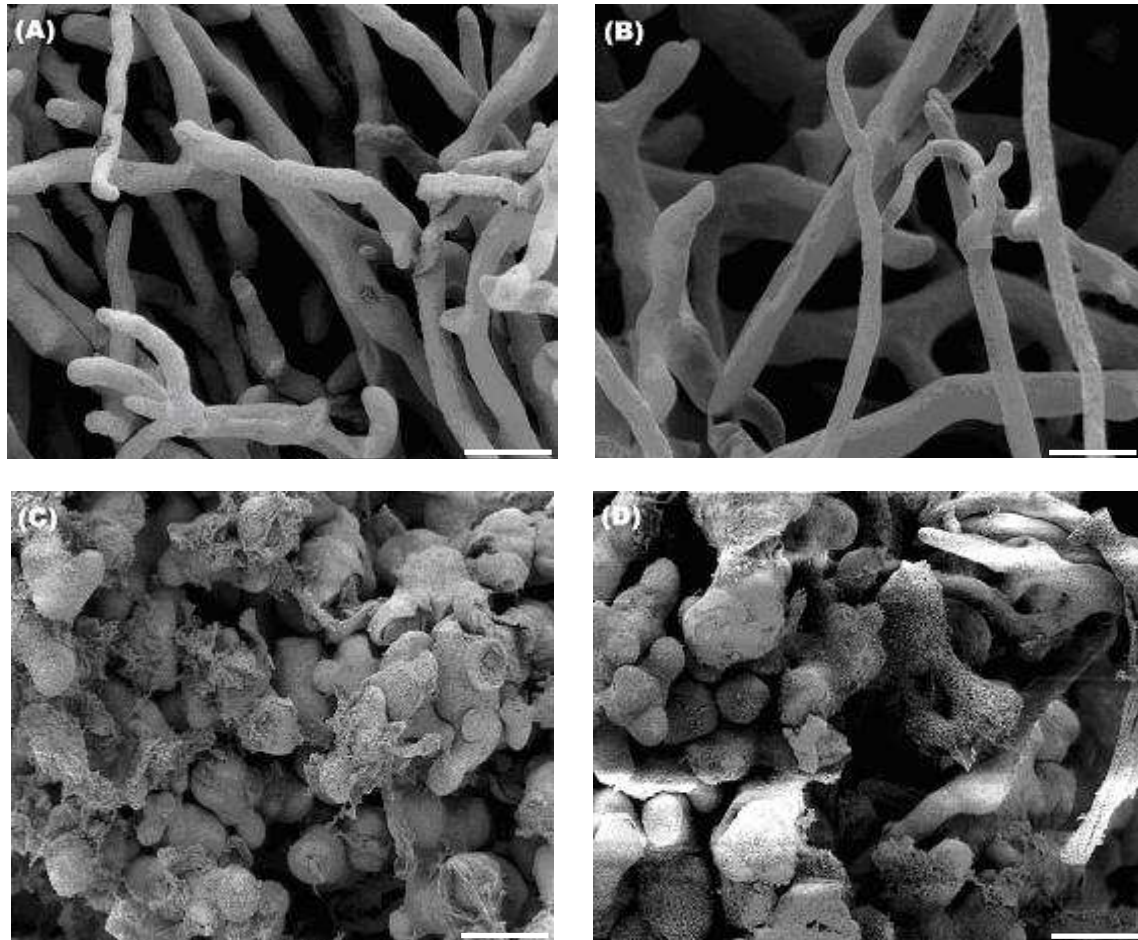


Figure 6 - Scanning electron micrographs of *Rhizopus stolonifer* mycelia after 3 days of cultivation at 25°C. (A) Control media, (B) acetic acid control ($40 \text{ mmol} \times \text{L}^{-1}$), (C) medium amended with chitosan D ($500 \mu\text{g} \times \text{mL}^{-1}$) and (D) medium amended with chitosan P ($500 \mu\text{g} \times \text{mL}^{-1}$). Bars = 20 μm .

Chitosan coating observed on the surface of the mycelia suggested that the fungal growth inhibition could be explained by a direct interaction of chitosan on the fungal cell wall as a consequence of polycationic nature of chitosan. Oliveira-Jr. (2006) have observed that chitosan samples with low F_A (high concentration of free amino groups protonated) and large DP (degree of polymerization) were most effective against the phytopathogenic fungi tested, while chitosan with high F_A did not have the ability to inhibit the fungal growth *in vitro*. In another study, Oliveira-Jr. et al. (2008) have demonstrated that chitooligosaccharides of eight degree of polymerization (DP) were not notably inhibitory to any of the fungi. On the other hand, higher chitooligosaccharides (DP 10 and DP 12) showed

initially inhibitory effects, which seemed to be more pronounced at a lower F_A .

The pH value of mycelial suspension, before its fixation and post-fixation, was about 5.4 and after this, it changed to 7.0. In the pH 5.4, chitosan was soluble in the mycelial suspension that was filtered in Millipore membrane (pore size 0.45 μm). Bound chitosan on the fungal cell wall was precipitated at a pH 7.0 and, then, chitosan coating was formed. This observation was supported by the scanning electron microscopy for all the fungi treated with chitosan. The micrographs of *P. expansum* previously treated with chitosan viewed in high magnification of 10,000 \times showed the chitosan coating formed on surface of the mycelia (Fig. 7 A).

The results demonstrated that chitosan acetate was

effective in restricting the fungal growth of filamentous fungi (Oliveira-Jr 2006) by causing a fungistatic inhibition effect as observed by the scanning electron microscopy.

In case of *A. alternata*, it was common to observe some spores with germ tubes inhibition as shown in Figure 7 B.

The aggregates of chitosans were observed in the micrographs on media amended with chitosans D and P on the surface of Millipore membranes that were fixed and post-fixed as described before

(Figures 8 A and C). As shown in the Figures 8 B and D, different aggregates of chitosans were observed in the micrographs on the media amended with the same chitosans on Millipore membrane surface. These chitosans were not fixed and post-fixed; they were filtered and dried at room temperature for one day. The difference observed in the aggregate of chitosans could be related to the pH 7.0 of the sodium cacodylate buffer used for SEM preparation that became chitosan insoluble.

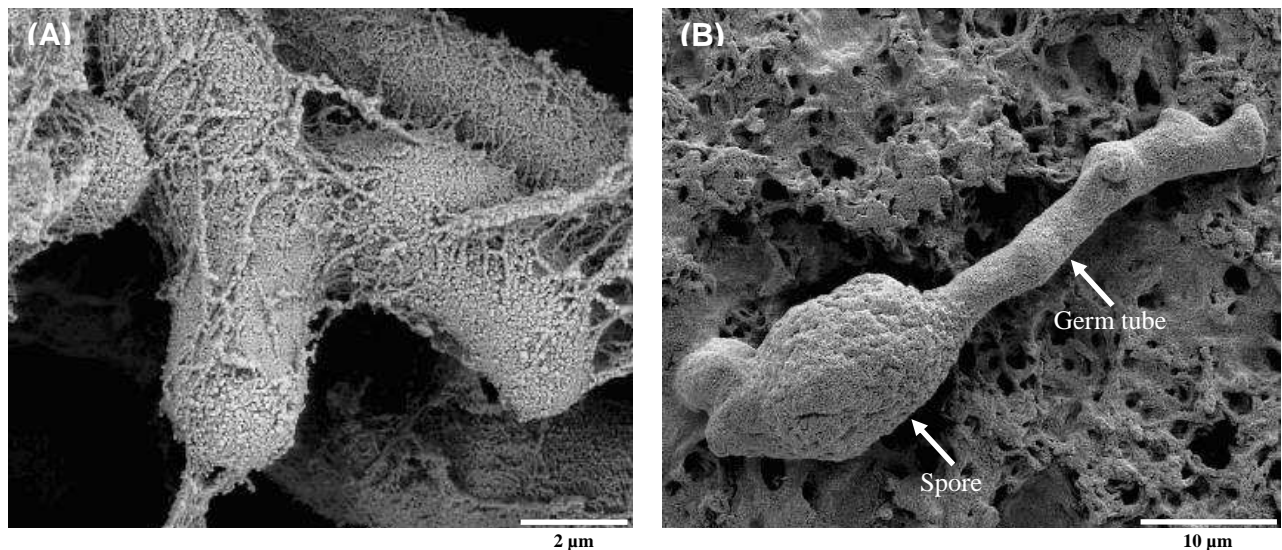
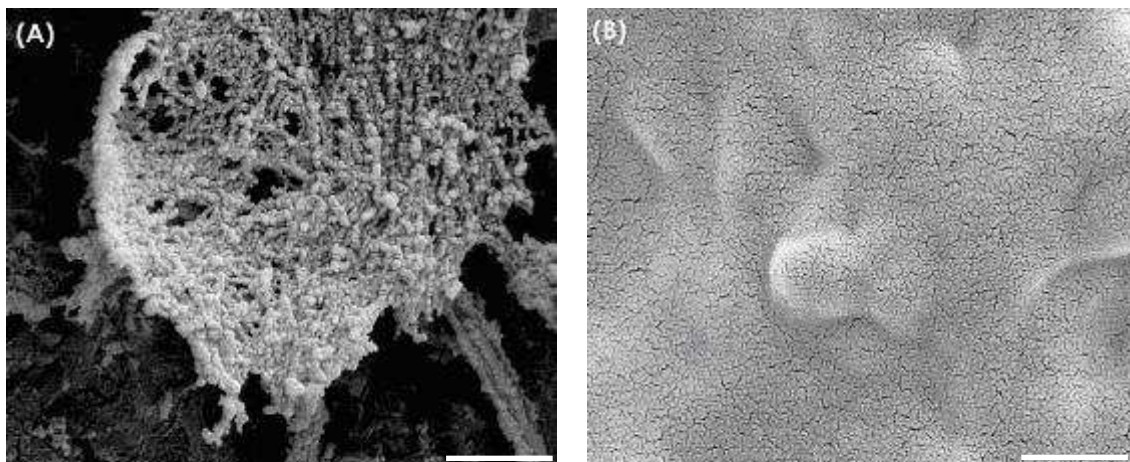


Figure 7 – (A) Scanning electron micrograph of *Penicillium expansum* mycelia after 5 days of culture at 25°C with medium amended with chitosan D ($1,000 \mu\text{g} \times \text{mL}^{-1}$). (B) Spore and germ tube of *Alternaria alternata* with medium amended with chitosan P ($500 \mu\text{g} \times \text{mL}^{-1}$). Figure 7 (A) bar = 2 μm ; Figure 7 (B) bar = 10 μm .



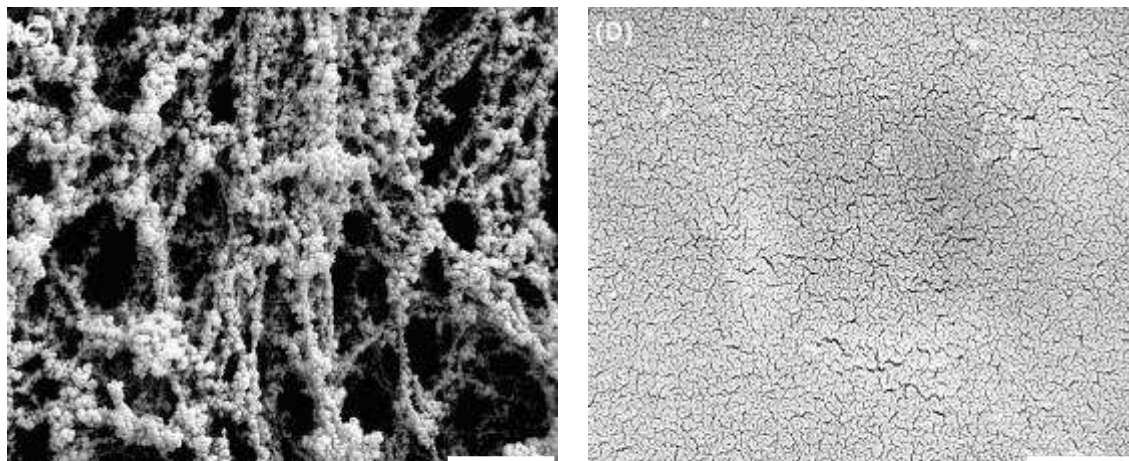


Figure 8 - Scanning electron micrographs of medium amended with $1,000 \mu\text{g} \times \text{mL}^{-1}$ of chitosan D (A) filtered and fixed as described in the item 2.6 (B) only filtered; $1,000 \mu\text{g} \times \text{mL}^{-1}$ of chitosan P (C) filtered and fixed as described in the item 2.6 and (D) only filtered. Bars = $2 \mu\text{m}$.

CONCLUSIONS

The SEM revealed that chitosan could be used to delay the fungal growth of the following filamentous fungi: *A. alternata*, *B. cinerea*, *P. expansum* and *R. stolonifer*. The micrographs showed that chitosans caused mycelial aggregation and structural changes as excessive branching, swelling of the cell wall and hyphae size reduction.

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