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Carbohydrate Polymers 73 (2008) 541–547
Carbohydrate Polymers
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Antifungal effects of chitosan with different molecular weights on *in vitro* development of *Rhizopus stolonifer* (Ehrenb.:Fr.) Vuill

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Received 14 June 2007; received in revised form 14 November 2007; accepted 21 December 2007
Available online 31 December 2007

Abstract

Determination of the molecular weight of three types of chitosan was carried out by HPSEC-RI. The effect of low, medium and high molecular weight chitosan was evaluated on development of three isolates of *Rhizopus stolonifer*. Image analysis and electronic microscopy observations were done in spores of this fungus. Germination of *R. stolonifer* in potato dextrose broth with chitosan was also evaluated. Results pointed out that the low molecular weight chitosan was more effective for inhibition of mycelial growth while the high molecular weight chitosan affected spore shape, sporulation and germination. Studies of scanning and transmission electron microscopy revealed numerous and deeper ridge ornamentations of the chitosan-treated spore.

Keywords: Chitosan; Image analysis; Antifungal activity; Spore ornamentations

1. Introduction

Rhizopus stolonifer (Ehrenb.:Fr.) Vuill., is the causal agent of rhizopus rots disease of various fruits and vegetables (Stevens et al., 2004; Zhang, Wang, Zheng, & Dong, 2007). This fungus infects ripe fruit after harvest, unless fruit in the field have major injuries or split pits (Föster, Driever, Thompson, & Adaskaveg, 2007). During several years synthetic fungicides have been used to control this microorganism. However, in several studies it has been shown that the compounds used in these fungicides caused strain resistance representing a potential risk for the environment and human health (Adaskaveg, Föster, & Sommer, 2002; Northover & Zhou, 2002). The search of natural alternatives for the control of postharvest commodities has been improved, and option such as chitosan has been evaluated (Tripathi & Dubey, 2004).

Chitosan is the *N*-deacetylated derivative of chitin, is a natural polymer composed of β-(1,4)-2-acetamido-2-deoxy-D-glucose and β-(1,4)-amino-2-deoxy-D-glucose units. The positive charge of chitosan confers to this polymer numerous and unique physiological and biological properties with great potential in a wide range of industries such as pharmacology, medicine and agriculture (Bautista-Baños et al., 2006; Rinaudo, 2006). The antimicrobial activity of chitosan has been considered in a wide variety of microorganisms. Diverse studies have shown the effect of molecular weight and concentration of chitosan on antibacterial activity (Liu et al., 2006; No, Park, Lee, & Meyers, 2002; Zheng & Zhu, 2003). On the other hand, the antifungal activity and its ability to extend the storage life of fruits and vegetables has been demonstrated (Chien, Sheu, & Lin, 2007a; Chien, Sheu, & Lin, 2007b; Hernández-Muñoz, Amenar, Ocio, & Gavara, 2006). It was found that chitosan depending on type and concentration caused mycelial growth inhibition (25 al 90.5%) in *Penicillium digitatum* and *Penicillium italicum* (Chien & Chou, 2006). Hirano and Nagao (1989) testing high and low molecular weight chitosan on different fun-

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doi:10.1016/j.carbpol.2007.12.020

gal species found that the best fungicidal activity on mycelia occurred in media supplemented with low molecular weight chitosan. In other studies, the results showed no differences in the fungicidal pattern among the three different types of chitosan, however there was a higher fungicidal effect as chitosan concentration increased (0.5–2.0%) (Bautista-Baños et al., 2005).

Chitosan was very effective in inhibiting spore germination, germ tube elongation and radial growth of *R. stolonifer*, additionally this biopolymer induced morphological changes (El Ghaouth, Arul, Grenier, & Asselin, 1992a). The spore morphology of *R. stolonifer* presented variations in area, form and optical density in chitosan solutions (Hernández-Lauzardo, Hernández-Martínez, Velázquez-del Valle, Guerra-Sánchez, & Melo-Giorgana., 2007). Chitosan and its derivatives have been considered as versatile biopolymers in agriculture applications, nevertheless its potential uses as an antimicrobial compound need to be studied in depth (Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003). To date, there are few studies about the antifungal effect of chitosan with different molecular weights on phytopathogen fungi. Therefore, the aim of this work was to evaluate the antifungal effect of chitosan with different molecular weights on *in vitro* development (mycelial growth, sporulation, morphology and germination spores) of three isolates of *R. stolonifer*.

2. Materials and methods

2.1. Chitosan solutions

To prepare stock solution (10 mg mL^{-1}) 2 g of chitosan (Sigma–Aldrich, St. Louis, MO, USA) of low, medium and high molecular weight were dissolved in 100 mL of distilled water with 2 mL of acetic acid (stirrer for 24 h), the volume was adjusted to 200 mL with distilled water. The pH was adjusted to 5.6 by adding sodium hydroxide 1 M (El Ghaouth, Arul, & Ponnampalam, 1991). Chitosan solutions were autoclaved for 15 min. The corresponding aliquots were taken to obtain different chitosan concentrations (1.0, 1.5 and 2.0 mg mL^{-1}).

2.2. High performance size exclusion chromatographic-refractive index (HPSEC-RI) study

Dextran standards (Sigma–Aldrich, St. Louis, MO, USA) of diverse molar mass (2.0×10^6, 2.82×10^5, 1.88×10^5, 6.5×10^4 and 4.0×10^4 g/mol) were used for obtain a calibration curve. Dextran were dissolved in HPLC-grade water at 25 °C, filtered using 5 μm nylon syringe filters (Daigger & Company, Inc., vernon Hills, IL) and injected 50 μL into the HPSEC system.

The chitosan of low, medium and high molecular weight (2 g) were solubilized in 100 mL of HPLC-grade water and 2 mL of acetic acid at 25 °C for 24 h. Thereafter, 3 mL of the chitosan solution was mixed with 27 mL of HPLC-grade water and this solution was filtered using 5 μm nylon syringe filter (Daigger & Company, Inc., vernon Hills, IL). The solution was injected (50 μL) onto the HPLC AT 1100 equipment (Angilent Technology, Deutchland GmbH Waldbronn, Germany) with the GPC-SEC PL aquagel-OH mixed, 8 μm column (7.5 mm ID × 300 mm) (Agilent Technologies Deutchland GmbH Waldbronn, Germany). The column and the RI detector were maintained at 30 °C. The eluent was HPLC-grade water, carefully degassed and filtered before use through Durapore GV (0.2 μm) membranes. The flow rate was 1.0 mL min^{-1}. The data analysis was realized using the GPC software of Agilent (Agilent Technologies Deutchland GmbH Waldbronn, Germany).

2.3. Assays of effectivity with different molecular weights and concentrations of chitosan on in vitro development of R. stolonifer

2.3.1. R. stolonifer strains

Three isolates of *R. stolonifer* were obtained from naturally infected tomatoes (*Lycopersicon esculentum* Mill) harvested from three different regions of Morelos State, México: Cuautla (R1), Oaxtepec (R2) and Yautepec (R3). Infected fruit was placed in moist chambers at 25 ± 2 °C until symptoms appeared (ca. four days). Portions of the infected tissue were placed on Petri plates containing Potato Dextrose Agar (PDA) and re-inoculated on tomato fruit to obtain pure cultures. To obtain monosporic cultures, serial dilutions were prepared from pure cultures and individual spores were collected and grown on PDA at 25 ± 2 °C for their identification (Hernández-Lauzardo, Bautista-Baños, Velázquez-del Valle, & Trejo-Espino, 2006).

2.3.2. Bioassays

Mycelial discs (5 mm) of each pure culture (R1, R2 and R3) were placed in the center of Petri plates containing PDA with different treatments (low, medium and high molecular weight) chitosan at 1.0, 1.5 and 2.0 mg mL^{-1} concentrations). Control Petri plates contained only PDA. The test plates were incubated at 25 ± 2 °C during 72 h. All experiments were repeated two times with five replicates. Treatments were arranged in a completely randomized design.

2.3.3. Effect of chitosan on mycelial growth of R. stolonifer

The mycelial growth was measured when mycellium reached the edges of the control plates with a digital vernier and expressed as average diameter (mm).

2.3.4. Effect of chitosan on sporulation of R. stolonifer

Petri plates were rinsed with 10 mL distilled water, the surface scrapped with a sterile glass road and filtered through a cotton wool. The procedure was repeated two times. Spore counting was done using a Neubauer haemocytometer and light microscopy (Nikon, Alphaphot-2YS2) at (40×).

2.4. Microscopic studies of the spores of R. stolonifer

2.4.1. Light microscopy

Images of spores were obtained using a light microscope (Nikon, Alphaphot-2 YS2) with a charged coupled video camera (DL 330 DAGE-MTI). Magnifications of the images were 40×. Images were analyzed using Meta Imaging series software, (version 4.0 for Microsoft Windows, Universal Imaging Corporation). The elliptical form factor (EFF) (dimensionless) was measured on 100 observations (n) per isolate. Relative frequency (RF) of shapes spores of *R. stolonifer* was calculated with the following formula: $RF = [Frequency/n] \times 100\%$, frequency was determinate with a histogram. Ranges of classes (EFF) were given as follow: globoses (0–1.15), ellipsoidal (1.16–1.30) and angular ($\geqslant$1.31) (Hernández-Lauzardo et al., 2006).

2.4.2. Scanning electron microscopy (SEM)

Spores of 72 h incubation period grown on PDA were fixed with 6% glutaraldehyde for 24 h at room temperature, rinsed three times with phosphate buffers 0.02 M and subsequently fixed with 2% osmium tetraoxide for 2 h at 20 °C and dehydrated in a graded ethanol series for 5 min each, CO_2 dried (SAMDRI-780B Tousimis) and sputter-coated with gold palladium in a Nanotech sputter coated (BAL-TEC SDC 050). Samples were kept in a desiccator until examination with a scanning electron using a microscope Carl Zeiss DSM 940 operated at 30 kV.

2.4.3. Transmission electron microscopy (TEM)

Similar procedure (Section 2.4.2.) was used until spores dehydrated. Afterwards, the samples were embedding on Epon810 and the ultrafine tissue (80 nm) at room temperature was cut on an ultra-microtome RMC-MT7000 and diamond knife. Once the tissue was mounted on a copper grid a post-staining was done (uranyl acetate during 30 min, lead citrate during 20 min). Samples were kept in a desiccator until examination with a transmission electron using a microscope Carl Zeiss EM 910 operated at 120 kV (Benhamou, 1992).

2.5. Effectivity of chitosan on spore germination of R. stolonifer in liquid medium

Aliquots of 50 μL of a spore suspension (1×10^6 spores mL^{-1}) were placed in Eppendorf tubes containing 500 μL of PDB with different chitosan concentrations (1.0, 1.5 and 2.0 mg mL^{-1}). The samples were incubated at 25 ± 2 °C during 6 h. Germination of 100 spores per sample was determined microscopically (40×). A spore was considered germinated when the length of the germ tube equaled or exceeded the length of the spore (El Ghaouth et al., 1992a).

2.6. Statistical analysis

Data were analyzed through ANOVA (Sigma Stat version 2.0). Medians separation by Tukeys's multiple range test ($p < 0.05$) was carried out for mycelial growth, sporulation and spore germinations.

3. Results and discussion

3.1. Molecular characteristics of chitosan

Chromatograms of chitosan (Fig. 1) and molecular characteristics (Table 1) showed defined peaks with unimodal distribution. The molecular weight ranged between 1.74×10^4 and 3.07×10^4 Da for chitosan low and high molecular weight, respectively. The molecular weight and the distribution of the polysaccharide is very important in the functional properties that can give in the system where is added. The polydispersity assessed by the ratio Mw/Mn is a distribution measurement of the size of the polymers present in the sample. The Mw/Mn values (Table 1) were in a range from 1.23 to 1.40, showing that the methodology used for determining chitosan molar mass can be suggested for the study of its structural characteristics. Other studies where chitosan was added as antibacterial, the molecular weight ranged between 5.5×10^4 and 15.5×10^4 Da (Liu et al., 2006) and 0.5×10^4 and 30.5×10^4 Da (Zheng & Zhu, 2003). However, the methodology for obtaining the molecular weight and distribution was not reported. The use of HPSEC-RI methodology can be feasible for molecular studies of chitosan; expensive detectors (such as MALLS, Viscosity, etc.) are not required.

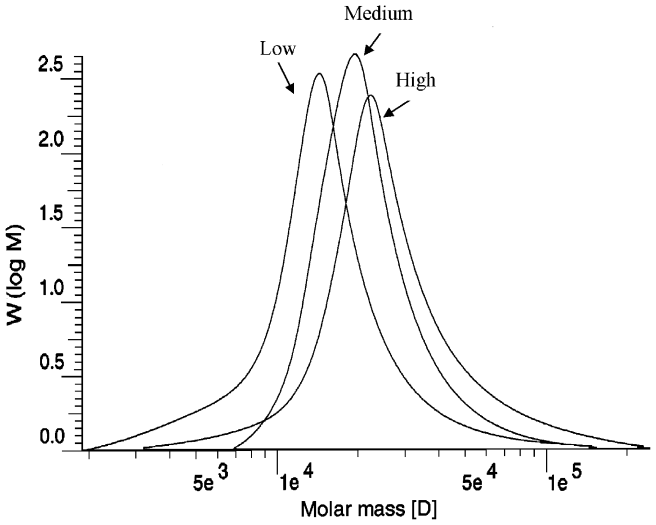

Fig. 1. HPSEC-RI chromatograms of chitosan with low, medium and high molecular weight.

Table 1
Weight-average (Mw) molar masses of chitosan obtained by HPSEC-RI

Chitosan	Mw (10^4×) Da	Mw/Mn
Low	1.74	1.40
Medium	2.38	1.23
High	3.07	1.31

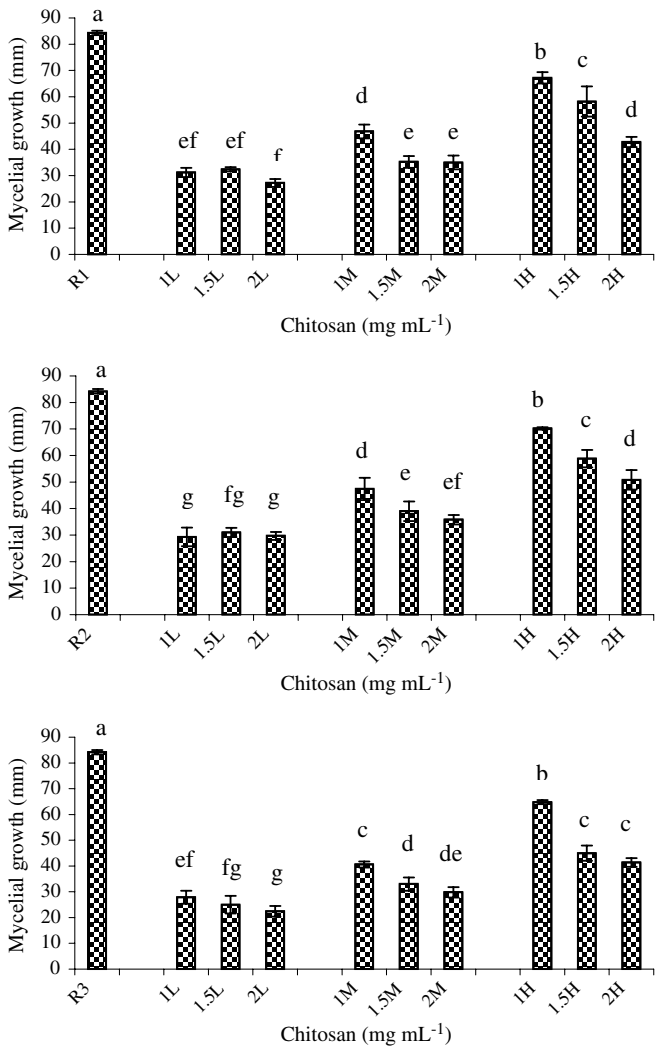

Fig. 2. Effect of low (L), medium (M) or high (H) molecular weight chitosan at various concentrations, on mycelial growth of three isolates of *Rhizopus stolonifer*. Bars represent standard deviations of the means. Values followed by different letters are significantly different according to Tukey's multiple range test at $P < 0.05$.

Table 2
Effect of low, medium or high molecular weight chitosan at various concentrations, on sporulation of three isolates of *Rhizopus stolonifer*

Chitosan (mg mL^{-1})	(Spores 10^5 mL^{-1})		
	R1	R2	R3
Control	8.5 ± 2.15 a	7.1 ± 0.96 a	9.0 ± 1.63 a
Low molecular weight			
1.0	4.3 ± 1.22 bc	4.6 ± 1.02 b	3.3 ± 0.70 b
1.5	2.4 ± 1.46 cd	1.4 ± 0.71 cd	2.4 ± 0.65 b
2.0	1.2 ± 0.99 d	1.4 ± 0.50 cd	1.2 ± 0.39 c
Medium molecular weight			
1.0	4.9 ± 0.58 b	4.2 ± 1.50 bc	0.9 ± 0.35 c
1.5	2.5 ± 0.87 cd	2.8 ± 1.70 c	0.7 ± 0.41 c
2.0	2.4 ± 0.73 cd	1.9 ± 0.44 cd	0.4 ± 0.10 c
High molecular weight			
1.0	1.0 ± 0.95 d	0.5 ± 0.26 de	0.5 ± 0.23 c
1.5	0.9 ± 0.33 d	0.2 ± 0.09 de	0.2 ± 0.10 c
2.0	0.6 ± 0.32 d	0.1 ± 0.05 e	0.2 ± 0.10 c

3.2. Effect of chitosan on mycelial growth of R. stolonifer

The mycelial growth of the three isolates of *R. stolonifer* was reduced on media amended by the three types of chitosan and by all concentrations (Fig. 2). The highest inhibitory effect was observed with low molecular weight chitosan. Overall, strain R3 presented a defined tendency of inhibition as chitosan concentration increased. In our study, chitosan high molecular weight had the lowest inhibitory effect on the three isolates tested. Contrary to these results, the inhibitory effect on mycelial growth of *Fusarium oxysporum* f. sp. *vasinfectum*, *Alternaria solani* and *Valsa mali* occurred when these fungi grew on media with high molecular weight chitosan ($Mw = 2.0 \times 10^5$ Da) (Guo et al., 2006). In another study, low molecular weight chitosan at different concentrations (0.01–1%) markedly inhibited mycelial growth of *Botrytis cinerea* and *Penicillium expansum* (Liu, Tian, Meng, & Xu, 2007).

3.3. Effect of chitosan on sporulation of R. stolonifer

Compared to the non-treated strains of *R. stolonifer*, it was evident that chitosan affected sporulation (Table 2). Different results were observed on each isolates tested. In general, the high molecular weight chitosan affected sporulation more than low and medium molecular weight chitosan. There was not observed a relative effect with concentration. In previous studies, differences in sporulation of *Colletotrichum gloeosporioides* were more associated with isolates than type of chitosan or concentration. In that study, there was not a fungicidal pattern according to degree of polymerization of chitosan (Bautista-Baños et al., 2005).

3.4. Relative frequency of spores of R. stolonifer treated with chitosan

It is reported that shape of spores of *R. stolonifer* presents three distinctive forms: globose, ellipsoidal and angular (Hernández-Lauzardo et al., 2006; Schipper, 1984). In our microscopic study, similar forms were observed by measurements of EFF in spore (Fig. 3). High molecular weight chitosan caused the highest variation in EFF. The highest relative frequency of globose spores was observed with high molecular weight chitosan for all concentrations tested. A similar effect was observed in spores treated with low and medium molecular weight chitosan at 2.0 mg mL^{-1} concentration. In previous studies, changes in shape of spores of *R. stolonifer* by low molecular chitosan addition at 1.5% concentration were also found; however, the relative frequencies were not reported (Bautista-Baños, Hernández-López, & Bosquez-Molina, 2004). In further studies, it is reported that in conidia of *C. gloeosporioides* low, medium and high molecular weight chitosan-treated, there were not alterations in their shapes. In that study, for all parameters tested, results were variable and did not show any

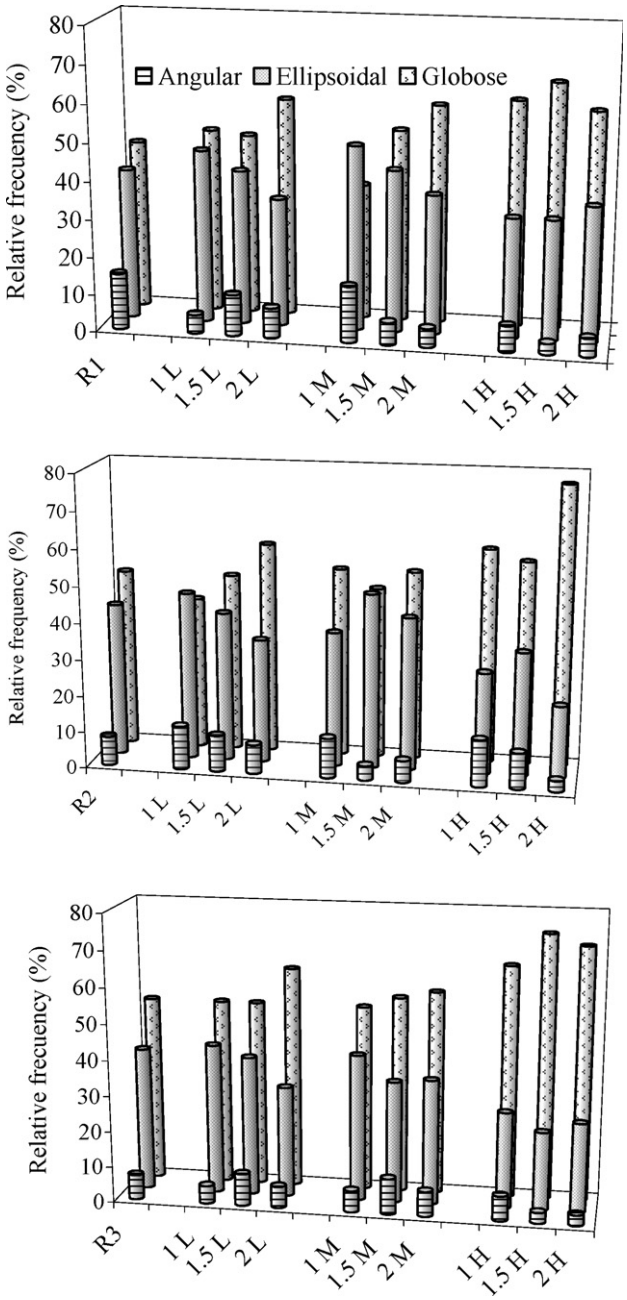

Fig. 3. Shape of spores of *R. stolonifer* treated with low (L), medium (M) or high (H) molecular weight chitosan at various concentrations.

relation with the molecular weight applied (Bautista-Baños et al., 2005).

3.5. SEM and TEM observations of spores of R. stolonifer treated with chitosan

In previous investigations, it is observed by SEM that *R. stolonifer* spores presented distinctive surface ornamentations consisted of continuous ridges alongside the spores (Schipper, 1984; Hernández-Lauzardo et al., 2006). In this study, the typical ridges changed after chitosan treatment. Compared with the non-treated spores, it was highlighted numerous (Fig. 4A and B) and deeper (Fig. 4C and D) ridge formations alongside the chitosan-treated spore. This behaviour was similar in spores treated with all types and concentrations of chitosan. Ultrastructural studies have also confirmed alterations to hyphae (swellings and convolutions, loosened cell walls) of *R. stolonifer* by chitosan (El Ghaouth, Arul, Asselin, & Benhamou, 1992b). To our knowledge this is the first report of the effect of chitosan on ornamentation of spores of *R. stolonifer*.

Table 3
Effect of low, medium or high molecular weight chitosan at various concentrations, on spore germination of three isolates *of Rhizopus stolonifer*

Chitosan (mg mL^{-1})	Spore germination (%)		
	R1	R2	R3
Control	91.5 a	90 a	90 a
Low molecular weight			
1.0	11.5 b	11.0 b	9.0 b
1.5	11.0 b	10.0 b	8.0 bc
2.0	10.0 b	7.0 c	2.0 c
Medium molecular weight			
1.0	11.5 b	11.0 b	7.0 bc
1.5	10.0 b	9.0 b	5.0 c
2.0	8.0 c	9.0 b	2.0 c
High molecular weight			
1.0	0.0 d	0.0 d	0.0 d
1.5	0.0 d	0.0 d	0.0 d
2.0	0.0 d	0.0 d	0.0 d

Different letters within columns indicate significant differences at $P < 0.05$, according to Tukey's multiple range test.

3.6. Spore germination of R. stolonifer treated with chitosan

Spores germination of *R. stolonifer* was affected by all chitosan treatments (Table 3). Low and medium molecular weight chitosan markedly reduced germination, but no significant effects were found among concentrations and a complete inhibition of germination was observed in spores treated with high molecular weight chitosan. In other study a define pattern associated with molecular weight of chitosan and its effect on spore germination was not clear (Bautista-Baños et al., 2005). Therefore it is necessary to carry out more basic studies about the mode of action and the effect of the physicochemical properties of chitosan with different degrees of polymerization on various stages of development of fungi.

4. Conclusions

The molecular weight of chitosan influenced *R. stolonifer* development at various stages of growth. The low molecular weight chitosan was more effective for inhibition of

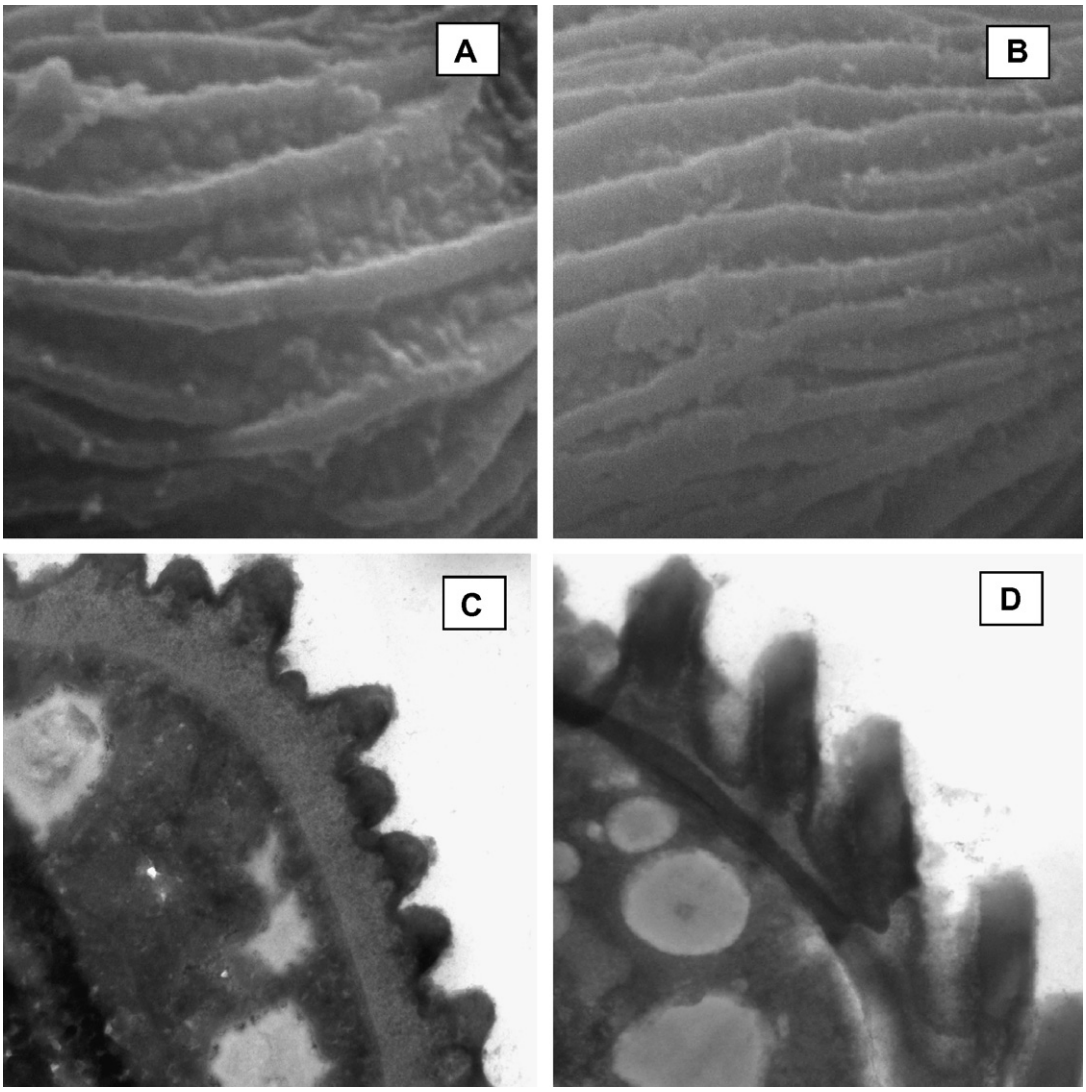

Fig. 4. Scanning electron micrographs (A and B) and transmission electron micrographs (C and D) (20,000× and 25,000×, respectively) of spores of *R. stolonifer* treated without (A and C) or with (B and D) chitosan.

mycelial growth, while the high molecular weight chitosan affected more the development of the spores (sporulation, shape and germination). Changes in ornamentation of spores chitosan-treated were observed.

Acknowledgements

We are grateful to the National Polytechnic Institute for financial support and Eng. Patricia Castillo for technical work in SEM and TEM studies.

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