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Effect of Chitosan on *in vitro* Development and Morphology of Two Isolates of *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc.

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Abstract. *Colletotrichum gloeosporioides* is the casual agent of the disease known as anthracnose. This fungus infects a wide range of hosts during the pre and postharvest stages of various horticultural commodities. To date, chitosan a natural biodegradable compound, chitin derivative, has been tested in the control of pathogenic microorganisms. In this research, the fungicidal or fungistatic potential of chitosan with different degrees of polymerization (low, medium and high molecular weight) and concentrations (0.5, 1.0, 1.5, and 2.0%) was evaluated on the *in vitro* development of two isolates of *C. gloeosporioides* obtained from infected papaya fruit from the states of Veracruz and Guerrero, Mexico. The effect of chitosan on conidial morphology was also observed by image analysis. Results showed no difference in the fungicidal pattern among the three different types of chitosan applied. There was a higher fungicidal effect as chitosan concentration increased. The fungicidal potential of chitosan varied according to the treated isolate. Overall, sporulation was the variable most affected by this compound for both isolates. A hundred percent germination was observed when conidia were re-incubated on potato-dextrose-agar disks and a significant inhibition was obtained when re-incubated directly on chitosan solutions. The area of the chitosan-treated conidia was affected as well. In future studies, it is proposed to carry out more in depth research including the use of electronic and scanning microscopy, tests with higher concentrations of chitosan and the fungicidal evaluation of chitosan prepared by different methodologies.

Additional keywords: Image Analysis, natural compounds, anthracnose, chitin

Resumen. *Colletotrichum gloeosporioides* es el agente causal de la enfermedad conocida como antracnosis. Este hongo infecta a un amplio rango de hospederos durante la etapa pre y postcosecha de varios productos hortofrutícolas. Actualmente, se ha experimentado con el quitosano, compuesto natural, biodegradable, derivado de la quitina como una alternativa de control de los microorganismos patógenos. En esta investigación se evaluó el potencial fungicida o fungistático con diferentes grados de polimerización del quitosano (bajo, medio y alto peso molecular) y concentraciones (0.5, 1.0, 1.5 y 2.0%) en el desarrollo *in vitro* de dos cepas de *C. gloeosporioides* aisladas de frutos infectados de papaya provenientes de los estados de Veracruz y Guerrero, México. Se consideró también el efecto de este compuesto en la morfología de los conidios mediante el uso de análisis de imágenes. Los resultados demostraron que no hubo un patrón definido en el efecto fungicida entre los diferentes tipos de quitosano aplicado. A mayor concentración de quitosano hubo mayor efecto fungicida. El potencial fungicida del quitosano varió según la cepa tratada. En general, la esporulación fue la variable mayormente afectada por este compuesto en ambas cepas. Se observó un 100% de germinación cuando los conidios fueron re-incubados en discos de papa-dextrosa-agar, y una inhibición significativa en los conidios re-incubados directamente en solución de quitosano. El área de los conidios tratados con quitosano también fue afectada. Se propone que en futuros estudios se lleven a cabo investigaciones más profundas, incluyendo el uso de la microscopía electrónica de barrido y de transmisión, la aplicación de concentraciones mayores y la evaluación del efecto fungistático o fungicida del quitosano, siguiendo diferentes metodologías de preparación.

Palabras clave adicionales: Análisis de imágenes, compuesto natural, antracnosis, quitina

Chitosan is a deacetylated form of *N*-acetylchito-oligosaccarides containing poly *D*-glucosamine and is usually prepared from chitin. Chitosan is normally present in cell walls of crustaceans, insects, yeast and fungi, and due to its particular polycationic nature it has important industrial applications (Shepherd *et al*., 1997). The antimicrobial activity of chitosan is well observed in a wide variety of microorganisms. For example, mycelial growth of various fungi such as *Alternaria alternata* (Fr.:Fr.) Keissl., *Botrytis cinerea* Pers.:Fr., *Sclerotinia sclerotorium* (Lib.) de Bary and *Fusarium oxysporum* f. sp. *radicis-lycopersici* W.R. Jarvis and Shoemaker was inhibited when nutrient media was supplemented with chitosan (Benhamou 1992; Cheah *et al*., 1997; El Ghaouth *et al*., 1992a). In previous studies sporulation and conidial morphology of *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. were also affected by chitosan (Hernández-López, 2002). Numerous hypothesis have been associated with the fungicidal effect of chitosan, however, it is believed that chitosan affects fungal membrane, inducing leakage of cellular material affecting the balance between the biosynthesis and degradation of components of the cellular wall (Leuba and Stossel, 1986; El Ghaouth *et al*., 1992c). It is also hypothesized that chitosan improves the expression of genes involved in resistance (Hadwiger and Loschke, 1981). The fungicidal attributes of chitosan are influenced by various factors such as chitosan concentration, type of chitosan, degree of polymerization, degree of deacetylation, natural host constituency, nutrient composition, environmental conditions, and incubation period (Song *et al*., 2002). Several authors have concluded that chitosan concentration is highly correlated with a low or high fungal inhibition (Cheah *et al*., 1997; El Ghaouth *et al*., 1992b; 1994; Hernández-López, 2002; Reddy *et al*., 1999; Wade and Lamondia, 1994). It seems that the polycationic nature of chitosan and the length of the polymer chain is the key to these antifungal properties. Hirano and Nagao (1989), testing various types of chitosan (high and low molecular weight, chitosan oligosaccharides, and pectin acid) on 18 different fungal species, found that the best fungicidal activity on mycelia and spores occurred in media supplemented with high molecular weight chitosan. In that study, more than 30% inhibition of radial growth was observed with this type of chitosan on *Cladosporium cucumerinum* Ellis and Arth, *Rhizopus nigricans* Ehrenb., *Venturia inaequalis* (Cooke) G. Wint., *Valsa mali* Miyabe and Yamada, *B. cinerea*, *Rhizoctonia solani* Kühn, *Phomopsis fukushi* Tanaka and Endo and various species of *Fusarium* and *Alternaria*. However, when only mycelia was inoculated on supplemented potato-dextrose-agar with chitosan the low molecular weight form showed the highest mycelial inhibition on *V. mali*, *P. fukushi*, *A. alternata* and on two species of *F. oxysporum*. Other studies carried out with human pathogenic yeasts and bacteria consistently report that growth reduction is highly correlated with chitosan molecular weight and degree of polymerization (Savard *et al*., 2002; Song *et al*., 2002; Sudarshan *et al*., 1992). The main objectives of this work were to investigate the antifungal properties of low, medium or high molecular weight chitosan on mycelial growth, sporulation and conidial germination of two isolates of *C. gloeosporioides*, and to quantify for each isolate morphological differences such as area and the elliptical form factor of conidia by image analysis.

MATERIALS AND METHODS

Microorganisms. The isolates of *Colletotrichum gloeosporioides* were obtained from infected papaya (*Carica papaya* L.) fruit from two regions of Mexico: Alto Lucero, Veracruz and Atenango, Guerrero. Five-cm plugs of papaya tissue were disinfected with sodium hypochlorite (1%) and placed on the center of a Petri plate containing potato-dextrose-agar (PDA, Bioxon). After a seven day incubation period, the fungus was re-isolated and identified according to previous description (Barnett and Hunter, 1972). Monoconidial cultures were maintained in Petri plates containing PDA and incubated at 25 ± 2ºC.

Chitosan preparation. Chitosan, labeled as high (FW = 161 800,000 cps), medium (FW = 161 200, 000 cps), and low molecular weight (FW = 162 20,000 cps) was obtained from Sigma-Chemical Co. (St. Louis, MI, USA). To prepare 0.5, 1.0, 1.5 or 2.0% chitosan solutions, 0.15, 0.3, 0.45, and 0.6 g of chitosan were dissolved in 16 ml of distilled water with 0.6 ml of acetic acid, and heated to 35°C, while constantly agitating for 24 h. The pH was adjusted to 5.5 by adding sodium hydroxide 1M (El Ghaouth *et al*., 1991). Chitosan solutions were then autoclaved (15 lb/ pul^2 for 15 min). After solutions were cooled to room temperature, 1ml of each solution was spread with the aid of a sterile glass rod onto Petri plates containing sterile PDA. Plates were dried at ambient temperature and sealed for future use.

Inoculation and incubation. Petri plates were seeded with a 5 mm diameter mycelial plug taken from the margins of 8-10 d old cultures of *C. gloeosporioides* isolates. Control Petri plates contained only PDA. After 7 or 11 days of incubation at 25 ± 2°C mycelial growth and sporulation, respectively, were evaluated. Mycelial growth was measured at the end of the incubation period with the aid of a vernier. To collect conidia for sporulation, Petri dishes were rinsed with 10 ml water, the surface scrapped with a sterile glass rod and filtered through a cotton wool. Aliquots of 0.5 ml were transferred to a Neaubauer haemocytometer. Spore concentration was determined with a microscope (Nikon Mod. Alphaphot-2 YS2-H) at 40X. Six Petri plates were considered for mycelial evaluation and two for sporulation. For both isolates, germination was recorded on PDA disks or directly on chitosan solutions after 7, 9, 11, and 13 h incubation at room temperature. Aliquots of 50 µl of a spore suspension containing 2.1 x 10^5 were placed onto 20 mm diameter PDA

disks or in Eppendorf tubes (vol 0.5 ml) containing 0.3 ml of chitosan solutions. Germination was stopped by adding lactophenol-safranin once the germ tube was clearly observed.

Image analysis. The area (μm^2) and elliptical form factor (FFE) of conidia produced by the two isolates of *C. gloeosporioides* growing on PDA or PDA supplemented with chitosan were measured. Images of conidia were obtained using a Nikon, Alphaphot – 2 YS2 microscope with a charged coupled device camera CCD (Nikon, Coolpix 900). Magnification of the image was 10X. Images were analyzed using Meta Imaging series software, (version 4.0 for Microsoft Windows, Universal Imaging Corporation, USA).

Statistical analysis. Treatments were arranged in a completely randomized design. Mean separation by Tukey's multiple range test ($P < 0.05$) was carried out for mycelial growth, sporulation, and germination. For quantitative evaluations means and standard deviation of one hundred conidia per treatment were calculated. The experiment was repeated twice.

RESULTS

For both isolates (Veracruz and Guerrero) chitosan significantly ($P > 0.001$) reduced mycelial growth and sporulation, depending on molecular weight and concentration (Table 1). In general, for isolate Veracruz mycelial growth was reduced in all treatments, however, the lowest level of growth was observed when incubated in low molecular weight chitosan at 1.0 or 2.0% concentration, medium molecular weight chitosan at 2.0% concentration, and high molecular weight chitosan at 1.0%. For isolate Guerrero, the lowest mycelial growth was obtained on medium molecular weight chitosan at 0.5 and 1.0% concentration. Compared to the control, sporulation (4.2×10^4) for isolate Veracruz was stimulated when treated with low molecular weight chitosan at 0.5% (1.4×10^5), and markedly reduced when the isolate was grown on medium molecular weight chitosan at 1.5% (6.6×10^3). In general, sporulation of isolate Guerrero was similar among treatments and control, except for the high molecular weight chitosan treatment at 2.0% concentration, where it was markedly reduced (1.5×10^4). A hundred percent germination on PDA disks was observed on isolate Veracruz after 7 h incubation onwards (data not shown). For isolate Guerrero, germination was significantly different ($P > 0.001$) only after 7 or 9 h incubation (Table 2). For this isolate, after 7 h incubation, the lowest percentage germination (60%) was observed when treated with low molecular weight chitosan at 0.5%, whereas the highest germination was obtained in the control treatment (90%). After 9 h incubation, significant differences were observed. Generally, percentage germination was high among treatments (92.3-98.6%). Conidial germination on chitosan solution for both isolates was significantly different among treatments ($P > 0.001$) (Table 3). Overall, for both isolates germination was low during the four incubation periods for all treatments compared to the control. For isolate Veracruz, no germination was observed on low molecular weight

Table 1. Effect of low, medium or high molecular weight chitosan at various concentrations, on mycelial growth and sporulation of two isolates of *Colletotrichum gloeosporioides* incubated at 25 ± 2ºC.

Chitosan Concentration (%)	Isolate Veracruz[x]		Isolate Guerrero[x]	
	Mycelial growth[y] (mm) ($P > 0.001$)	Sporulation[z] spores/ml ($P > 0.001$)	Mycelial growth[y] (mm) ($P > 0.001$)	Sporulation[z] spores/ml ($P > 0.001$)
Low molecular weight				
0.5	81.5 b	1.4×10^5 a	83.3 abc	1.4×10^5 abc
1.0	80.8 cd	3.5×10^4 cde	84.1 ab	1.4×10^5 abc
1.5	81.5 bc	5.0×10^4 bc	83.7 abc	1.0×10^5 bc
2.0	78.1 cd	4.8×10^4 bc	83.0 abc	1.7×10^5 bc
Medium molecular weight				
0.5	81.2 bc	1.0×10^4 de	81.6 bc	1.6×10^5 abc
1.0	81.4 bc	1.5×10^4 e	81.3 c	1.3×10^5 abc
1.5	81.6 b	6.6×10^3 f	84.2 a	1.9×10^5 ab
2.0	79.3 cd	2.8×10^4 cde	84.8 a	1.7×10^5 abc
High molecular weight				
0.5	81.8 b	8.1×10^4 b	83.6 abc	1.5×10^5 abc
1.0	77.8 d	4.6×10^4 bcd	85.0 a	1.1×10^5 bc
1.5	81.6 b	2.2×10^4 cde	84.6 a	1.4×10^5 abc
2.0	79.2 cd	1.5×10^4 cde	84.8 a	1.5×10^4 cd
Control	85.0 a	4.2×10^4 cd	85.0 a	2.1×10^5 a

[x]Means separation within columns by Tukey's multiple range test at $P > 0.05$.
[y]Seven day incubation period.
[z]Eleven day incubation period.

Table 2. Percentage germination of *Colletotrichum gloeosporioides* isolate Guerrero incubated on potato-dextrose-agar disks for 7, 9, 11, and 13 h, after treatment with low, medium or high molecular weight chitosan at various concentrations.

Chitosan Concentration (%)	Germination (%)[z]			
	Incubation period (h)			
	7 (P > 0.001)	9 (P > 0.001)	11 (NS)	13 (NS)
Low molecular weight				
0.5	60.0c	96.3b	100a	100a
1.0	90.6b	92.3a	98.0a	100a
1.5	86.6b	97.0b	99.0a	100a
2.0	83.3ab	97.0b	98.0a	100a
Medium molecular weight				
0.5	70.0b	97.6b	99.0a	100a
1.0	77.3b	98.3b	99.0a	100a
1.5	75.0a	96.3b	99.0	100a
2.0	83.0ab	98.6b	98.0a	100a
High molecular weight				
0.5	74.3b	95.3a	100a	100a
1.0	89.6ab	99.0b	99.0a	100a
1.5	82.6ab	96.3b	98.0a	100a
2.0	87.3a	95.6b	99.0a	100a
Control	90.0b	98.0b	100a	100a

[z]Means separation within columns by Tukey´s multiple range test at P > 0.05.

chitosan at 2% concentration and high molecular weight chitosan at 1.5 and 2.0%. For isolate Guerrero, germination was also completely inhibited when treated with low or medium molecular weight chitosan both at 1.0 or 2.0% concentration, and high molecular weight chitosan at 1.5 or 2.0%. For both isolates, the area of the conidia was affected

Table 3. Percentage germination of two isolates of *Colletotrichum gloeosporioides* incubated on various types of chitosan solutions at different concentrations for 7, 9, 11, and 13 h.

Chitosan Concentration (%)	Germination (%)[z]							
	Isolate Veracruz				Isolate Guerrero			
	Incubation period (h) P < 0.001				Incubation period (h) P < 0.001			
	7	9	11	13	7	9	11	13
Low molecular weight								
0.5	0.25 c	0.75 b	1.25 b	1.25 b	1.0 b	1.0 c	1.0 c	1.0 b
1.0	1.25 c	2.25 b	3.0 b	3.0 b	0 b	0 c	0 c	0 b
1.5	0.6 c	0.6 c	0.6 c	0.6 c	0.6 b	0.6 c	1.2 b	1.2 b
2.0	0 c	0 c	0 c	0 c	0 b	0 c	0 c	0 b
Medium molecular weight								
0.5	0.25 c	1.25 b	1.5 b	1.5 b	6.0 b	6.0 b	11.3 a	11.3 b
1.0	1.5 b	2.0 b	2.3 b	3.2 b	0 b	0 c	0 c	0 b
1.5	1.0 c	1.0 b	1.0 b	1.0 b	1.0 b	1.0 c	1.0 c	1.0 b
2.0	0.5 c	0.5 b	0.5 c	0.5 c	0 b	0 c	0 c	0 b
High molecular weight								
0.5	0.7 c	1.2 b	2.5 b	2.5 b	2.5 b	3.6 c	3.6 c	3.6 b
1.0	1.7 b	2.7 b	2.7 b	2.7 b	0 b	0 c	0 c	0 b
1.5	0 c	0 c	0 c	0 c	0 b	0 c	0 c	0 b
2.0	0 c	0 c	0 c	0 c	0 b	0 c	0 c	0 b
Control	28.2 a	48 a	77 a	89.5 a	83.6 a	93.3 a	94.3 a	96.6 a

[z]Means separation within columns by Tukey´s multiple range test at P > 0.05.

Table 4. Effect of low, medium or high molecular weight chitosan and concentration on area (μm²) and elliptical form factor (FFE), of conidia of two isolates of *Colletotrichum gloeosporioides* after 7 day incubation period at 25 ± 2°C.

Chitosan Concentration (%)	Isolate Veracruz[z]		Isolate Guerrero[z]	
	Area	FFE	Area	FFE
Low molecular weight				
0.5	83.8 ± 10.4	1.9 ± 0.2	68.7 ± 7.9	2.2 ± 0.2
1.0	86.9 ± 11.6	1.8 ± 0.1	75.2 ± 11.4	2.3 ± 0.2
1.5	81.4 ± 18.4	2.0 ± 0.2	71 ± 10.3	2.3 ± 0.3
2.0	83.6 ± 9.6	1.9 ± 0.2	73 ± 11.7	2.1 ± 0.3
Medium molecular weight				
0.5	82.7 ± 13.7	1.8 ± 0.1	74.4 ± 13.5	2.2 ± 0.2
1.0	81.5 ± 9.6	1.9 ± 0.1	74.5 ± 11.5	2.2 ± 0.2
1.5	53.7 ± 14.9	2.1 ± 0.3	71.0 ± 9.5	2.3 ± 0.3
2.0	70.0 ± 15.1	1.8 ± 0.2	71.8 ± 7.6	2.3 ± 0.2
High molecular weight				
0.5	72.2 ± 14.3	2.0 ± 0.2	69.1 ± 12.7	2.2 ± 0.2
1.0	82.7 ± 17.1	1.9 ± 0.2	69.5 ± 11.8	2.3 ± 0.3
1.5	82.5 ± 10.2	1.8 ± 0.2	71.3 ± 10.4	2.4 ± 0.3
2.0	81.1 ± 13.7	1.9 ± 0.2	69.7 ± 8.3	2.1 ± 0.3
Control	71.6 ± 11.0	2.0 ± 0.2	68.0 ± 12.6	2.2 ± 0.3

[z]Values indicate media and standard deviations of 100 observations.

by type of chitosan and concentration, while the FFE was generally similar among treatments (Table 4). For isolate Veracruz, the smallest area (53.7 μm²) was observed with medium molecular weight chitosan at 1.5%, and except for the treatments medium and high molecular weight chitosan at 2.0 or 0.5% concentration, respectively, the area of the remaining treatments was higher than the control. The area of the conidia for all treatments of isolate Guerrero was higher than control treatment. For most treatments, both isolates showed an FFE close to 2.0 indicating that conidial form was elliptical.

DISCUSSION

Contrary to previous reports, in this study a defined fungicidal or fungistatic pattern among the various types of chitosan tested was not observed in both isolates of *C. gloeosporioides*. For all parameters tested, results were variable and did not show any relation with molecular weight; however, to some extent, chitosan concentration, regardless type of chitosan, influenced some of the variables tested as it was demonstrated with the low or zero conidial percentage germination of isolates Veracruz and Guerrero, and with the lower mycelial growth trend of isolate Veracruz compared to the remaining treatments and control, when incubated at 2.0% concentration. In previous studies, chitosan concentrations up to 2.5% completely inhibited mycelial growth of *C. gloeosporioides* (Hernández-López, 2002), confirming that concentration is a key factor for effective fungicidal action. Another explanation for the lack of fungicidal effect according to molecular weight, might be that in our methodology, chitosan solutions were rather plated on PDA Petri plates than mixed with PDA as reported by Hirano and Nagao (1989), El Ghaouth *et al*. (1991) and Benhamou (1992). In future experiments, higher chitosan concentrations and the comparison of different methodologies to prepare chitosan will be considered. In this investigation, differences in sporulation were more associated with different isolates than type of chitosan or concentration. However, for isolate Guerrero, all treatments reduced conidia formation. The conidial stimulation observed in isolate Veracruz treated with low molecular weight chitosan at 0.5% concentration, might be explained as a stress response originated by the action of chitosan. Similar stimulation has been reported by Reddy *et al*. (1998) when *A. alternata* was subjected to various concentrations of chitosan. In that study, sporulation was increased when the fungus was grown on high chitosan concentrations. Percentage germination of the two isolates tested was dramatically different according to the germination substrate, indicating that chitosan might have a fungistatic effect on PDA or a fungicidal effect on chitosan solutions. Isolate Veracruz had the smallest area and the lowest sporulation level when treated with medium molecular weight chitosan at 1.5% concentration, suggesting that perhaps spores were somewhat modified by chitosan. For further confirmation of possible alterations in conidia, observations with scanning and transmission electronic microscope might be relevant. Moreover, we think that many of the differences reported in our results might also be related to the typical

genetic variability of *C. gloeosporioides*, already evidenced by Hernández-Albíter (2003), indicating the importance of carrying out similar studies with other strains of *C. gloeosporioides*.

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