Antifungal activity of chitosan in *Cladosporium cladosporioides* isolated from safflower

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NOTA CORTA

Actividad antifúngica de quitosano en *Cladosporium cladosporioides* aislado de cártamo

Resumen. El quitosano mostró inhibición sobre la germinación de esporas de *Cladosporium cladosporioides* a las 24 h (70.99 ± 12.53 y 32.72 ± 10.85%, QB y QA, respectivamente). El quitosano causó cambios morfométricos sobre las esporas, evidenciado por excesivo hinchamiento y disminución de la elongación del tubo germinal. Sin embargo, en comparación con los controles, el quitosano no inhibió las velocidades de extensión radial de los cultivos.

Palabras clave: control biológico, hongos patógenos, crecimiento radial, germinación de esporas.

Abstract. The chitosan showed inhibition of spore germination at 24 h on *Cladosporium cladosporioides* (70.99 ± 12.53 and 32.72 ± 10.85% for QB and QA, chitosans, respectively). Chitosan caused morphometric changes on spore evidenced by excessive swelling and delay on the germ tube. However, in comparison with the controls, the chitosan not inhibited the colony radial extension rate.

Key words: biological control, pathogenic fungi, radial growth, spore germination.

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Recent studies demonstrated that *Cladosporium* genera are associated to *Ramularia* and *Alternaria* in safflower leaves infected with false mildew (unpublished data, Quintana-Obregón *et al*.). Chitosan has been reported to inhibit and delay the growth of some common pathogens in plants and crops (Badawy and Rabea, 2011; El Hadrami *et al*., 2010). In studies previews, chitosan (3.4 g L^{-1}) showed inhibition on *Ramularia* and *Alternaria* (Quintana-Obregón *et al*., 2011a; 2011b). The goal of this study was to evaluate the *in vitro* antifungal activity of chitosan on *C. cladosporioides*.

Spores of *C. cladosporioides* grown at 25 °C, 12 h light-dark photoperiod, pH 5.5, and V8 medium. Subsequently, suspensions of spores were prepared after 96 h, using a sterile Tween 20 solution (0.1% v v^{-1}) and stirred for 5 minutes. The number of spores (mL) was determined using a Neubauer chamber. Two types of chitosan were evaluated, chitosan (Aldrich lot: 04924LH) with degree of deacetylation of 84% and molecular weight of 46.31 ± 5.2 kDa (QB), and chitosan (Fluka BioChemika, Lot: 436207/1) with deacetylation degree of 76% and molecular weight of 260.65 ± 10.9 kDa (QA). Flasks with V8 medium and chitosan with acetic acid (CH_3COOH) were autoclaved, cooled to 45 °C, and mixed. The concentration of chitosan in the V8 medium was 3.4 g L^{-1} and acetic acid 0.05M. Subsequently, 20 mL of mixture deposited on Petri dishes (9 cm in diameter) cooled at room temperature. Controls were V8 medium with and without acid.

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Petri dishes containing plugs of V8 medium (20x20x5 mm) were by spreading inoculated of a spore suspension containing 10^4 spores of *C. cladosporioides* incubated at 25 ºC. From each plug, 200 spores per plate (germinated and non-germinated) were randomly counted at different times using an optical microscope. Percentage of inhibition with respect to the acid control was calculated (Plascencia-Jatomea *et al*., 2003; El Ghaouth *et al*., 1992). Measurements in diameter and length of spores at 0, 8, and 24 h of incubation were made at 400x with the Image-Pro Plus v. 6.3 (Media Cybernetics, Inc., USA, 1993-2008). In addition, the increase in length and diameter of the spores were calculated using the equation $I = (x_i - x_0)$, where I is the increase in length or diameter, x_i is the average length or diameter, and x_0 is the average length or diameter in V8 medium (control H_2O) before the incubation.

Radial growth. A 6 mm well in the center of the culture medium in each Petri dish (6 cm in diameter) was with a sterile Pasteur pipette done, deposited inside a 10^5 spores solution. The diameters of the mycelia were manually at different times measured until the colony in the control reached the border of the plate. To identify the growth phases of the fungal colony an arithmetic and logarithmic growth (LOG_{10}) kinetics were plotted (Trinci, 1969). In addition, the rate of radial expansion of the mycelia U (µm h^{-1}) in the exponential phase (log) and stationary phase, were calculated from the slope by plotting the experimental colony radius against time, using the start and end time of each phase through the logarithmic plot of colony radius previously obtained.

A completely randomized design was used and the JMP 2004 program for the analysis of variance and Tukey multiple range test ($P < 0.05$) (JMP 5 vs. 5.0, SAS Institute Inc., USA) to rank the means of various treatments

Inhibition of spore germination by QA and QB was 32.72 ± 10.85 and 70.99 ± 12.53% after 24 h, respectively. The dimensions of the *C. cladosporioides* spores grown in V8 medium were 6.91 ± 1.50 µm of length and 3.43 ± 0.70 µm in diameter. There were no significant difference in the length and diameter of the spores at the first 8 h of incubation ($P<0.05$); however, after 24 h, an excessive increase (I) was observed in spores treated with chitosan, whereas in controls 80% was polarized and/or germinated (Figure 1, Table 1). During polarization and conidial development, spores from

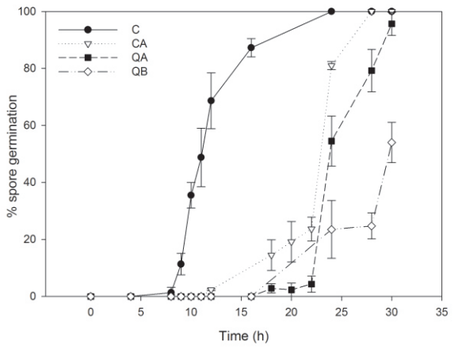

Figure 1. Kinetics of percentage of germination of spores *Cladosporium cladosporioides* in V8 medium at 25°C and photoperiods of 12 h.
The dates are averages of three replicates and standard deviation. C = control H_2O, CA = control acid (acetic acid 0.05M), QA = chitosan of 260 kDa with acetic acid 0.05M, QB = QA = chitosan of 46 kDa with acetic acid 0.05M.

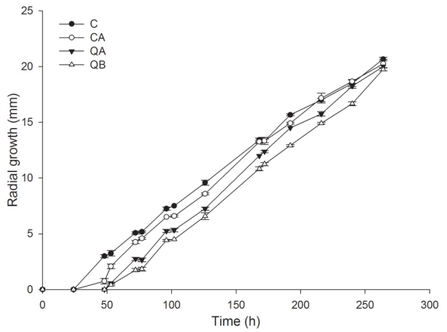

Figure 2. Radial growth kinetics *Cladosporium cladosporioides* in V8 medium at 25 ° C and photoperiods of 12 h.
The date are average of three replicates and standard deviation. C = control H_2O, CA = control acid (acetic acid 0.05M), QA = chitosan of 260 kDa with acetic acid 0.05M, QB = QA = chitosan of 46 kDa with acetic acid 0.05M.

Table 1. Effect of chitosan on *Cladosporium cladosporioides* spores, V8 medium at 25°C with 12 h photoperiod (light-dark)

	Control H_2O	Control acid*	QA* (3.4 g L^{-1})	QB* (3.4 g L^{-1})
8 h				
Spores germinated (%)	1.33±1.89^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}
Spores non-germinated (%)$^{\#}$	89.50±5.0^{b}	97.17±1.0^{a}	98.33±0.6^{a}	99.33±0.3^{a}
Swollen spores length (m)	7.51±0.96^{b}	7.90±1.39^{ab}	8.1±1.6^{a}	7.89±1.50^{ab}
Swollen spores diameter (m)	5.14±0.59^{a}	5.02±0.81^{a}	5.33±0.95^{a}	5.17±0.82^{a}
I in length of the spore	0.60	0.99	1.11	0.98
I in diameter of the spore	1.69	1.57	1.88	1.72
24 h				
Spore germinated (%)	100±0.0^{a}	81.0±1.41^{a}	54.5±8.79^{b}	23.5±10.15^{c}
Spore no germinated (%)$^{\#}$	0.0±0.0^{c}	18.5±0.71^{bc}	28.5±5.63^{b}	65.0±10.96^{a}
Inhibition of spores germinated (%)^{x}	NA	NA	32.72±10.85^{b}	70.99±12.53^{a}
Swollen spore length (m)	NA	NA	14.02±1.91^{a}	14.14±2.6^{a}
Swollen spores diameter (m)	NA	NA	12.74±1.54^{a}	12.72±2.04^{a}
Increased size in length of the spore	NA	NA	7.11	7.23
Increased size in diameter of the spore	NA	NA	9.29	9.27
30 h				
Spores germinated (%)	NA	NA	95.67±4.04^{a}	54.00±7.07^{b}
Spores no germinated (%)$^{\#}$	NA	NA	0.00±0.0^{b}	24.75±6.72^{a}
Inhibition of spores germinated (%)^{x}	NA	NA	4.33±4.04^{b}	46.00±7.07^{a}

*0.05 M CH_3COOH, $^{\#}$ Spores no germinated =100 - (spores germinated + spores polarizated).
^{x} In respect to control acid.
Means of three replicates and standard deviation. Superscript letters indicate statistical groups in rows. Tukey HDS test. P (0.05), JMP.
NA = not applicable

chitosan treatments showed an excessive swelling and delay in the elongation of the germ tube (24 h). After 30 h of incubation, the spores exhibited multiple polar initiation points (not quantified) and many germ tubes on the spore surface (not quantified) treated with chitosan QB and QA (before the germination, only 1-2 poles were in the control conditions observed). Before 8 h of incubation, the chitosan not affected the spores (Table 1). However, the presence of multiple polar in spore suggest a high level of nuclear division (polarization) and elongation of the germ tubes. The spore polarization is related to the formation of septa, division nuclear, mitosis and development of the cell wall (Bartiniki-Garcia and Lippman, 1977; Bartnicki-Garcia *et al*., 1968; Osherov and May, 2001) and chitosan affects the polarization and elongation of the germ tube (Plascencia-Jatomea *et al*., 2003). These changes may be due to interaction of the charges in the cell wall with chitosan amino groups to form a composite polyelectrolyte (Hirano and Nagao, 1989). In our study, both evaluated chitosans have a high deacetylation degree (76%), which indicates high density of positively charged amino groups in the molecule.

In radial growth, the results determined significant differences between water and the acid controls before 126 h of incubation (Figure 2). From the logarithmic kinetics obtained, in the controls were identified four stages of fungal growth (see captions of Figure 3). The radial expansion rates

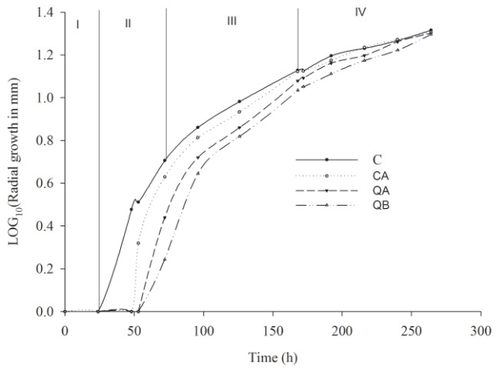

Figure 3. Logarithmic growth kinetics for radial *Cladosporium cladosporioides* in V8 medium at 25 °C and photoperiods of 12 h. Control is exemplified by the four stages of fungal growth: I. Phase lag; II. Log phase III. Slow down; IV. Phase of steady growth.

in the growth phase after 162 h in the QB was high (Table 2), and no significant differences were observed between their rates of the two growth phases, Phase II and Phase after 162 h (Table 2). After spore germination (Phase I in Figure 3), the tube hyphae developed germs which initiates the mycelial growth of the colony (Phase II in Figure 3). Radial expansion rates of the colony showed that chitosans was induced at higher rates after 162 h of incubation (Table 2). In addition, QB maintained the expansion rate, while that QA decreased (Table 2). However, fungi growth in the controls changed from the log phase to steady growth phase (Figure 3), that means a decrease in the radial expansion rates.

These morphometric changes in the radial growth of the colony might be due to defense mechanisms that fungus uses for adaptation to the culture medium, especially in treatment with low molecular weight chitosan. It is possible to maintain the growth rate in the log phase, which permits the colony to release a greater amount of enzymes (chitinases, deacetylases, and deaminase) which partially hydrolyze chitosan (Palma-Guerrero *et al*., 2010). This allows an optimal growth and movement to the steady growth phase.

Table 2. Radial extension rates of *Cladosporium cladosporioides* (R^2> 0.98)

Treatment	Phase log (Phase II)	Phase after 162 h
Control H_2O	88.33 ± 3.21^{bA}	$74.67 \pm 1.15^{cB*\#}$
Control acid*	115.00 ± 8.88^{aA}	$73.00 \pm 1.0^{cB*\#}$
QA* 3.4 g L^{-1}	106.67 ± 3.21^{aA}	$82.33 \pm 2.30^{bB*\#}$
QB* 3.4 g L^{-1}	92.00 ± 0.0^{bA}	90.00 ± 2.83^{aA}

* CH_3COOH 0.05 M. # see Phase IV in Figure 3.
Lower case letters indicate homogeneous groups in columns.
Capital letters indicate homogeneous groups in rows.

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