

Effect of chitosan on three isolates of *Rhizopus stolonifer* obtained from peach, papaya and tomato

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Effect of chitosan on three isolates of *Rhizopus stolonifer* obtained from peach, papaya and tomato.

Abstract — Introduction. *Rhizopus stolonifer* is the causal agent of *Rhizopus* rot disease in various fruit and vegetables. **Materials and methods.** The effect of chitosan was evaluated *in vitro* on mycelial growth, sporulation, morphological characteristics and germination of spores of three isolates of *R. stolonifer* (from peach, papaya and tomato). The effect of chitosan on controlling *Rhizopus* decay in peach, papaya and tomato fruit *in situ* in comparison with the synthetic fungicide dichloran was also studied. **Results and discussion.** Our results showed that the mycelial growth and sporulation of the three isolates were markedly inhibited at all tested chitosan concentrations. The highest antifungal indexes and sporulation reduction were observed with chitosan at 2 mg·mL⁻¹. In our study, the morphological characteristics of the spores of *R. stolonifer* showed different behavior depending on the evaluated isolates. In general, the highest effect on germination was observed at the chitosan concentration of 2 mg·mL⁻¹. Our results demonstrated that chitosan was effective in reducing the percentage of infection and the severity index on peach, papaya and tomato fruit compared with those of non-treated control. The chitosan was not more effective than dichloran in reducing the percentage of infection. The results of the study suggest that chitosan (2 mg·mL⁻¹) is a good alternative for the control of *Rhizopus* decay on peach, papaya and tomato fruit; it could be considered as a potential agent in natural alternatives to control postharvest diseases.

Mexico / *Prunus persica* / *Carica papaya* / *Lycopersicon esculentum* / fruits / postharvest decay / fungal diseases / *Rhizopus stolonifer* / chitosan / antifungal properties

Effet du chitosane sur trois isolats de *Rhizopus stolonifer* obtenus de pêche, papaye et tomate.

Résumé — Introduction. *Rhizopus stolonifer* est l'agent causal de la moisissure à *Rhizopus* dans différents fruits et légumes. **Matériel et méthodes.** L'effet du chitosane a été évalué *in vitro* sur la croissance du mycélium, la sporulation, les caractéristiques morphologiques et la germination des spores de trois isolats de *R. stolonifer* obtenus de pêche, papaye et tomate. L'effet du chitosane pour contrôler *in situ* la moisissure à *Rhizopus* sur fruits de pêche, papayer et tomate a également été étudié par rapport à un fongicide synthétique, le dichloran. **Résultats et discussion.** Nos résultats ont montré que la croissance du mycélium et la sporulation des trois isolats ont été fortement inhibées à toutes les concentrations de chitosane testées. L'indice antifongique le plus élevé et la sporulation la plus faible ont été observés avec le chitosane à 2 mg·mL⁻¹. Dans notre étude, les caractéristiques morphologiques des spores de *R. stolonifer* se sont révélées différentes selon les isolats évalués. En général, la dose de 2 mg de chitosane·mL⁻¹ a été la plus efficace pour contrôler la germination de *R. stolonifer*. Les résultats ont démontré que le chitosane est efficace pour réduire le pourcentage d'infection et l'indice de sévérité sur les pêche, papaye et tomate par rapport au témoin non traité. Le chitosane n'a pas été plus efficace que le dichloran pour réduire le pourcentage d'infection. Les résultats de l'étude suggèrent que le chitosane à 2 mg·mL⁻¹ serait une bonne solution pour le contrôle de la moisissure à *Rhizopus* sur les pêche, papaye et tomate ; ce traitement pourrait potentiellement être utilisé pour lutter naturellement contre les maladies post-récolte.

Mexique / *Prunus persica* / *Carica papaya* / *Lycopersicon esculentum* / fruits / maladie post-récolte / maladie fongique / *Rhizopus stolonifer* / chitosane / propriété antifongique

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1. Introduction

Rhizopus stolonifer (Ehrenb.:Fr.) Vuill. is the causal agent of *Rhizopus* rot disease in various fruits and vegetables such as peach (*Prunus persica* Batsch.), papaya (*Carica papaya* L.) and tomato (*Lycopersicon esculentum* Mill.) [1–3]. *Rhizopus stolonifer* is a good colonizer of plant debris and infects harvest fruits, often destroying the entire contents of a box within a few days by hydrolysis with tissue-macerating ability [4]. Over the past years, synthetic fungicides have been used to control this microorganism. However, it has been shown that some compounds used in these fungicides have caused strain resistance, representing a potential risk for the environment and human health [5, 6]. Thus, there is a worldwide trend to explore natural products in order to reduce the use of synthetic fungicides, and options such as chitosan have been evaluated [7].

Chitosan is the N-deacetylated derivative of chitin, a natural polymer composed of β -(1, 4)-2-acetamido-2-deoxy-D-glucose and β -(1, 4)-amino-2-deoxy-D-glucose units; it has become a promising alternative to control postharvest fungal rotting [8]. The positive charge of chitosan confers upon this polymer several physiological and biological properties with great potential in a wide range of industries such as pharmacology, medicine and agriculture [9]. The application of chitosan as an antifungal agent and as an elicitor of plant defense has been demonstrated [10]. The antifungal effect of chitosan has been observed against several fungi and its activity depends on its deacetylation degree, molecular weight and concentration [11–13]. In some studies, it has been found that chitosan reduced the radial growth and caused morphological changes in *R. stolonifer* [11].

The results of other studies showed that chitosan inhibited sporulation and germination and induced changes in the ornamentations of the *R. stolonifer* spores [14]. However, there are few reports on antifungal effects of chitosan on *in vitro* and *in situ* development of *R. stolonifer*. Therefore, our objectives were to evaluate the effect of chi-

tosan on mycelial growth, sporulation, morphological characteristics and germination of spores of *R. stolonifer* and investigate the effect of chitosan on controlling *Rhizopus* rot caused by *R. stolonifer* in peach, papaya and tomato fruit.

2. Materials and methods

2.1. *Rhizopus stolonifer* isolates and culture conditions

Three isolates of *R. stolonifer* were obtained from peach fruit, papaya fruit and tomato fruit harvested in fields in Morelos, México. Infected fruit were placed in moist chambers at 25 °C until symptoms appeared. Portions of the infected tissue were placed on Petri plates containing Potato Dextrose Agar (PDA) and re-inoculated on peach, papaya and tomato fruit to obtain pure cultures. Monosporic cultures were obtained by serial dilutions prepared from pure cultures and individual spores were collected and grown on PDA at 25 °C for their identification [15, 16].

2.2. Chitosan solutions

Chitosan of low molecular weight (from crab shells, viscosity=20–200 cps, % deacetylation = 75–85, Mw = 17.4 kDa.) was purchased from Sigma-Aldrich, St. Louis, MO, USA. To prepare stock solution (10 mg·mL⁻¹), two grams of chitosan were dissolved in 100 mL of distilled water with 2 mL of acetic acid (stirred for 24 h), and the volume was adjusted to 200 mL with distilled water. The pH was adjusted to 5.6 by adding sodium hydroxide 1 M [17]. Chitosan solution was autoclaved for 15 min. The corresponding aliquots were taken to obtain different chitosan concentrations [(1.0, 1.5 and 2.0) mg·mL⁻¹].

2.3. *In vitro* antifungal assay

Mycelial discs (5 mm) of each pure culture of *R. stolonifer* were placed in the center of Petri plates containing PDA with different

concentrations of chitosan [(1.0, 1.5 and 2.0) mg·mL⁻¹]. Control Petri plates contained only PDA. The plates were incubated at 25 °C for 72 h.

2.4. Effect of chitosan on mycelial growth

The mycelial growth was measured when the mycelium reached the edges of the control plates and was expressed in terms of average diameter (mm). The Antifungal Index (AI) was calculated as follows: $AI (\%) = - (Da / Db) \times 100$, where *Da* is the diameter of the growth zone in the test plates and *Db* is the diameter of the growth zone in the control plates [18].

2.5. Effect of chitosan on sporulation

Petri plates were rinsed with 10 mL of distilled water. The surface was scraped with a sterile glass rod and filtered through cotton wool. The procedure was repeated two times. Spore counting was done using a Neubauer chamber and light microscopy (40 ×). Data were analyzed and expressed as spores·mL⁻¹.

2.6. Microscopic studies on sporangiospores

To evaluate the morphological characteristic of spores, 10 × images of spores of each *R. stolonifer* isolate were obtained using a light microscope (Nikon, Alphaphot-2 YS2) with a video camera (DL 330 DAGE-MTI). Images were analyzed using Meta Imaging series software. The total area (μm²) and elliptical form factor (EFF) (dimensionless) of spores were measured on 100 observations per isolate [16].

2.7. Effect of chitosan on spore germination

Aliquots of 50 μL of a spore suspension (1 × 10⁵ spores·mL⁻¹) were placed in Eppendorf tubes containing 500 μL of potato dextrose broth (PDB) with different chitosan concentrations [(1.0, 1.5 and

2.0) mg·mL⁻¹]. Control tubes contained only PDB. The samples were incubated at 25 °C for 8 h. Germination of 100 spores per sample was determined microscopically (40 ×) at 10 h of incubation [19].

2.8. *In situ* antifungal assay

Peach, papaya and tomato fruit were collected from a regional market in Cuautla, Morelos (México). Fruit were selected based on size and absence of physical injuries or disease infection. Fruit were disinfected with 1% (w/v) sodium hypochlorite for 10 min then rinsed with distilled water and air-dried. The fruit were randomly distributed (15 per treatment). They were wounded, dipped in chitosan solutions (2 mg·mL⁻¹) or dichloran (1 mg·mL⁻¹) for 15 min and air-dried. The fruit were sprayed with spore solutions of each isolate (1 × 10⁶ spores·mL⁻¹). Control fruit were sprayed after the distilled water treatment. Fruit were kept in humidified chambers for 5 d at 25 °C. After the storage period, percentage infection, disease severity (1–5, where 1 = 0% of fruit surface rotten, 2 = 1% to 25%, 3 = 26% to 50%, 4 = 51% to 75% and 5 = 76% to 100%) and percentage weight loss were evaluated.

2.9. Statistical analyses

All experiments were repeated at least two times with five replicates (*in vitro*) or three replicates (*in situ*). Data were analyzed through ANOVA (Sigma Stat version 2.0). Means separation by Tukey's multiple range test ($P < 0.05$) was carried out in all experiments.

3. Results

3.1. Antifungal index of chitosan on mycelial growth of *R. stolonifer*

The mycelial growth of the three isolates of *R. stolonifer* was markedly inhibited at all chitosan concentrations (*table 1*). Similar results were observed on each isolate tested.

Table I.

Antifungal Index of chitosan on mycelial growth of *Rhizopus stolonifer* isolated from peach, papaya and tomato fruits (values are followed by standard deviations of the means).

Chitosan (mg·mL ⁻¹)	Antifungal Index (%) (48 h)		
	Peach	Papaya	Tomato
Control (0.0)	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a
1.0	29.44 ± 0.51 b	19.62 ± 0.72 b	13.30 ± 0.71 b
1.5	38.92 ± 0.68 c	27.32 ± 0.53 c	17.34 ± 0.91 c
2.0	53.81 ± 0.59 d	34.74 ± 0.22 d	20.04 ± 0.49 d

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

Table II.

Effect of chitosan on sporulation of three isolates of *Rhizopus stolonifer* from peach, papaya and tomato fruits (values are followed by standard deviations of the means).

Chitosan (mg·mL ⁻¹)	Sporulation ($\times 10^5$ spores·mL ⁻¹) (72 h)		
	Peach	Papaya	Tomato
Control	5.9 ± 0.06 a	2.9 ± 0.13 a	8.8 ± 0.06 a
1.0	2.6 ± 0.15 b	2.1 ± 0.08 b	3.6 ± 0.07 b
1.5	1.4 ± 0.08 c	1.6 ± 0.07 c	3.0 ± 0.07 c
2.0	0.6 ± 0.05d	1.0 ± 0.05 d	0.9 ± 0.13 d

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

For all isolates of *R. stolonifer*, the antifungal index showed significant statistical differences at (1.0, 1.5 and 2.0) mg·mL⁻¹ chitosan concentrations. A relative effect of the concentration was observed. The highest antifungal indexes (20.04 to 53.81) were observed with chitosan at 2.0 mg·mL⁻¹. In general, chitosan at 2.0 mg·mL⁻¹ showed a similar effect in the three isolates.

3.2. Effect of chitosan on sporulation of isolates of *R. stolonifer*

The chitosan affected sporulation of the peach, papaya and tomato isolates (table II). The values of spores·mL⁻¹ showed significant statistical differences at all chitosan

concentrations evaluated. There was a demonstrated relative effect with concentration. The highest inhibitory effect was observed with chitosan at 2.0 mg·mL⁻¹ [(0.6, 1.0 and 0.9) spores $\times 10^5$ ·mL⁻¹, respectively].

3.3. Effect of chitosan on morphological characteristics of the spores of *R. stolonifer*

In our study, the morphological characteristics of the spores of *R. stolonifer* showed different behavior depending on the evaluated isolates of *R. stolonifer* (table III). There were no significant statistical differences in the total area of spores of papaya and tomato isolates treated with chitosan at (1.0,

Table III.

Effect of chitosan on total area and elliptical form factor (EFF) of spores of three isolates of *Rhizopus stolonifer* obtained from peach, papaya and tomato growing on potato dextrose broth at different chitosan concentrations for 72 h (values are followed by standard deviations of the means).

Chitosan (mg·mL ⁻¹)	Morphological measured total area (μm ²)			Morphological measured EFF (dimensionless)		
	Peach	Papaya	Tomato	Peach	Papaya	Tomato
Control	62.83 a	94.06 a	72.17 a	1.21 a	1.23 a	1.21 a
1.0	71.53 b	84.66 a	70.11 a	1.21 a	1.16 a	1.20 a
1.5	72.95 b	94.20 a	68.84 a	1.20 a	1.18 a	1.18 a
2.0	67.04 ab	88.90 a	71.44 a	1.16 a	1.23 a	1.16 a

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

Table IV.

Effect of chitosan on spore germination for three isolates of *Rhizopus stolonifer*, obtained from peach, papaya and tomato fruits, grown on a potato dextrose broth medium for 8 h (values are followed by standard deviations of the means).

Chitosan (mg·mL ⁻¹)	Germination of spores (%)		
	Peach	Papaya	Tomato
Control (0.0)	90.33 ± 4.80 a	82.83 ± 3.48 a	80.83 ± 0.75 a
1.0	52.16 ± 1.16 b	82.50 ± 7.17 a	62.16 ± 1.47 b
1.5	46.00 ± 1.26 c	44.50 ± 3.01 b	55.50 ± 1.64 c
2.0	7.66 ± 2.33 d	12.00 ± 1.41 c	38.33 ± 2.06 d

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

1.5 and 2.0) mg·mL⁻¹. On the contrary, the peach isolates showed significant statistical differences in the total area of their spores [(71.53, 72.95 and 67.04) μm², respectively]. The value of control was 62.83 μm² for this late isolate. The elliptical form factor was not affected by treatments with chitosan.

3.4. Effect of chitosan on spore germination

The peach and tomato isolates showed similar responses regarding the average of spore germinations (table IV). All the concentrations of chitosan used in this study were effective in reducing the spore germination of these isolates.

Spore germination was affected in the papaya isolate only at the (1.5 and 2.0) mg chitosan·mL⁻¹ concentrations. In general, the highest effect was observed at 2.0 mg chitosan·mL⁻¹ [(7.66, 12.00 and 38.33)% for peach, papaya and tomato isolates, respectively].

3.5. *In situ* antifungal assay of chitosan on peach, papaya and tomato fruit

For the *in situ* antifungal assay, the lowest percentage of infection was obtained with dichloran treatment [(41.33, 61.33 and 26.70)% for peach, papaya and tomato

Table V.

Effect of chitosan ($2 \text{ mg}\cdot\text{mL}^{-1}$) and dichloran, *in situ* antifungal assay using peach, papaya and tomato fruits (values are followed by standard deviations of the means).

Treatments	Percentage infection	Severity Index	Percentage of weight loss
Peach fruit			
Control	100.00 ± 0.00 a	3.77 ± 0.05 a	20.25 ± 4.70 a
Chitosan	66.20 ± 0.34 b	2.17 ± 0.04 b	23.02 ± 3.73 a
Dichloran	41.33 ± 0.57 c	1.57 ± 0.05 c	21.01 ± 4.98 a
Papaya fruit			
Control	86.87 ± 0.23 a	2.07 ± 0.05 a	7.82 ± 1.17 a
Chitosan	73.43 ± 0.51 b	1.73 ± 0.06 b	7.12 ± 1.42 a
Dichloran	61.33 ± 1.16 c	1.63 ± 0.05 b	8.15 ± 1.32 a
Tomato fruit			
Control	80.40 ± 0.53 a	2.57 ± 0.05 a	7.05 ± 3.59 a
Chitosan	33.43 ± 0.51 b	1.67 ± 0.05 b	7.52 ± 3.16 a
Dichloran	26.70 ± 0.27 c	1.76 ± 0.06 b	6.49 ± 1.40 a

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

isolates, respectively, *table V*). Compared with the non-treated control, chitosan was effective in reducing the percentage of infection of peach (66.20%), papaya (73.43%) and tomato (33.43%) isolates.

The results of the severity index depending on the isolates were evaluated. There were significant statistical differences in the values of the severity index in the presence of chitosan compared with control and with dichloran. The papaya and tomato isolates exhibited similar behavior: there were no statistically significant differences between the treatment with chitosan or dichloran.

The values of weight loss percentage obtained in all isolates of *R. stolonifer* were similar and did not depend on the treatment. In general, chitosan at $2.0 \text{ mg}\cdot\text{mL}^{-1}$ delayed the postharvest decay of peach, papaya and tomato fruit caused by *R. stolonifer*.

4. Discussion

In our study, we observed an antifungal activity of chitosan with different concentrations. These results demonstrated that the

concentration of chitosan is correlated directly with its inhibitory effect. Similar results were obtained by other authors who used chitosan to control *Botrytis cinerea* and *Penicillium expansum* [20, 21]. However, it is known that the antifungal action of chitosan is influenced by several factors, including the nutrient composition of the culture media [10].

We also observed that, the sporulation depending on the isolates of *R. stolonifer*, the results might have several variations [14]. On the other hand, in *Colletotrichum gloeosporioides*, there has been a reported difference in sporulation which was associated with the differences in isolates instead of the type or concentration of chitosan [22]. The results that we obtained and those found in the literature suggest that the effect of chitosan depends on the isolates evaluated.

In our study, the effect of chitosan on morphological characteristic of spores was independent of the concentration used. On the other hand, there is information concerning the shape of spores of *R. stolonifer* with three distinctive forms (globose, ellipsoidal and angular) [15] but, in our study,

similar forms were observed by measurements of the elliptical form factor in spores. There were no statistically significant differences in elliptical form factor observed from spores of the three isolates considered. Our result therefore differs from those of other studies. In previous investigations, changes in the elliptical form factor of spores of *R. stolonifer* by chitosan at different concentrations were found [14]. There are few reports on the change in the morphological characteristics of the *R. stolonifer* spores effected by chitosan and it is necessary to continue this research.

Additionally, the spore germination was affected by chitosan treatment. The highest effect was observed at 2 mg·mL⁻¹ for peach, papaya and tomato isolates. In previous studies, spore germination of *R. stolonifer* was markedly inhibited with chitosan [14]. In other investigations, strains of *Fusarium oxysporum* and *Verticillium dahliae* showed increased sensitivity to chitosan; spore germination in the presence of chitosan was completely inhibited [23]. We suggest the effect of chitosan in *in vitro* cultures depends on many factors such as type of isolates, culture medium and chitosan concentrations used.

In general, chitosan at 2.0 mg·mL⁻¹ delayed the postharvest decay of peach, papaya and tomato fruit caused by *R. stolonifer* in *in situ* assays. In previous studies, chitosan [(5 and 10) mg·mL⁻¹] delayed the development of postharvest disease caused by *Monilinia fructicola* in peach fruit [24]. Other authors reported that chitosan (20 mg·mL⁻¹) could be used to reduce deteriorative processes, maintain quality and increase the shelf life of fresh-cut papaya stored at 5 °C [25]. There are also studies on the use of chitosan in controlling postharvest diseases in tomato fruit. It is reported that chitosan at 5–10 mg·mL⁻¹ was successful in controlling gray and blue mold caused by *B. cinerea* and *P. expansum* in tomato fruit [20]. In a recent study, chitosan at (2 and 4) mg·mL⁻¹ significantly reduced postharvest decay of tomato fruit caused by *B. cinerea* [21].

However, little is known about the effect of chitosan coating on weight loss in tomatoes, papaya and peaches. In other works,

chitosan applications did not influence the percentage weight loss during the storage of papaya fruit [26]. Other authors reported that chitosan coating caused an increment in weight loss of peach fruit [27]. On the other hand, chitosan coating has been effective in reducing weight loss in banana and mango [28], longan fruit [29] and strawberries [30]. Taken all together, the effect of chitosan on controlling postharvest diseases shows variations depending on the fungi and kind of fruit.

5. Conclusion

The results obtained in our study demonstrated the antifungal effect of chitosan on three isolates of *R. stolonifer*. In *in vitro* studies, we observed that chitosan at 2 mg·mL⁻¹ affected the mycelial growth, sporulation and germination of peach, papaya and tomato isolates. These results were corroborated in *in situ* assays. Compared with the non-treated control, chitosan was effective in reducing the degree of infection and the severity index of the isolates studied. The results of the present study suggest that chitosan at levels of 2 mg·mL⁻¹ is a good alternative for the control of *Rhizopus* decay in peach, papaya and tomato fruit and could be considered as a potential agent for natural alternatives to control postharvest diseases.

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Efecto del quitosano en tres aislados de *Rhizopus stolonifer* obtenidos de durazno, papaya y tomate.

Resumen — Introducción. *Rhizopus stolonifer* es el agente causal de la enfermedad pudrición de *Rhizopus* en frutos y vegetales. **Materiales y métodos.** El efecto del quitosano fue evaluado *in vitro* en el crecimiento micelial, esporulación, características morfológicas y germinación de esporas de tres aislados de *R. stolonifer* (de durazno, papaya y tomate). El efecto del quitosano para controlar las pudriciones de *Rhizopus* en frutos de durazno, papaya y tomate *in situ* en comparación con el fungicida sintético diclorán fue también estudiado. **Resultados y discusión.** Nuestros resultados mostraron que el crecimiento micelial y la esporulación de los tres aislados fueron marcadamente inhibidos con todas las concentraciones de quitosano probadas. Los principales índices antifúngicos y reducción de la esporulación fueron observados con quitosano a 2 mg·mL⁻¹. En nuestro estudio, las características morfológicas de las esporas de *R. stolonifer* mostraron diferente comportamiento dependiendo de los aislados evaluados. En general, el mayor efecto en la germinación fue observado a la concentración de quitosano de 2 mg·mL⁻¹. Nuestros resultados demostraron que el quitosano fue eficaz en reducir el porcentaje de infección y el índice de severidad en frutos de durazno, papaya y tomate comparados con los del control no tratado. El quitosano no fue más eficaz que el diclorán para reducir el porcentaje de infección. Los resultados de este estudio sugieren que el quitosano (2 mg·mL⁻¹) es una buena alternativa para el control de las pudriciones de *Rhizopus* en frutos de durazno, papaya y tomate; podría ser considerado como agente potencial de alternativas naturales para controlar enfermedades postcosecha.

México / *Prunus persica* / *Carica papaya* / *Lycopersicon esculentum* / frutas / enfermedades postcosecha / enfermedades fungosas / *Rhizopus stolonifer* / quitosano / propiedades antifúngicas