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ARTÍCULO DE INVESTIGACIÓN / RESEARCH ARTICLE

# CHITOSAN INHIBITS THE *IN VITRO* DEVELOPMENT OF *Colletotrichum* SP. FROM BANANA (*Musa x paradisiaca* L.) FRUITS

# El quitosano inhibe el desarrollo *in vitro* de *Colletotrichum* sp. en frutos del plátano (*Musa x paradisiaca* L.)

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## ABSTRACT

The banano (*Musa x paradisiaca* L.) is a tropical fruit, susceptible to infection by *Colletotrichum* sp. Fungicides are the most typical approach for controlling postharvest infections. Concerns regarding its negative impact on human health and the environment have prompted the quest for alternate remedies. Because of its antimicrobial activity, chitosan is an environmentally friendly alternative. This study aimed to determine the influence of chitosan on the *in vitro* development of *Colletotrichum* sp. isolated from banana fruits. Inhibition mycelial growth, spore concentration and spore germination were evaluated in three chitosan concentrations, 0.5 %, 1.0 % and 1.5 %. Optical, fluorescence, and scanning electron microscopy were used to examine the impact of chitosan on spore growth and morphology. We observed that chitosan solutions inhibited *Colletotrichum* species *in vitro*. Chitosan at 1.5 % significantly decreased the percentage of mycelial growth inhibition and spore concentration in comparison with the control. Compared to the control, a concentration of 1.5 % chitosan considerably decreased the mycelial growth and spore concentration. In addition, a complete inhibition of spore germination and a low mycelium content was observed with 1.0% and 1.5% chitosan, controlling the *in vitro* development of *Colletotrichum* sp. in banana fruits.

**Keywords:** Antimicrobial activity, disease control, fungi, pathogens, postharvest

## RESUMEN

El plátano (*Musa x paradisiaca* L.) es un fruto tropical que puede ser infectado por hongos del género *Colletotrichum* sp. cuyo método habitual de control se realiza mediante el uso de agentes químicos. Sin embargo, la preocupación por los daños que estos agentes pueden causar en la salud humana y el medio ambiente ha llevado a la búsqueda de tratamientos alternativos. Una alternativa respetuosa con el medio ambiente es el uso de quitosano debido a su actividad antimicrobiana. El objetivo de esta investigación fue evaluar el efecto del quitosano en el desarrollo *in vitro* de *Colletotrichum* sp. aislado de frutos de plátano. Se evaluó la inhibición del crecimiento micelial, concentración y germinación de esporas en tres concentraciones de quitosano 0,5 %, 1,0 % y 1,5 %. Se utilizaron microscopios ópticos, de fluorescencia y electrónicos de barrido para observar la alteración en el desarrollo y la morfología de las esporas causado por la aplicación de quitosano. Se observó un efecto positivo de las soluciones de quitosano sobre la inhibición *in vitro* de *Colletotrichum* sp. El quitosano al 1,5 % disminuyó significativamente el crecimiento del micelio y concentración de esporas

en comparación con el control. Asimismo, se observó un inhibición completa de la germinación de esporas y un bajo contenido de micelio con quitosano al 1,0 % y 1,5 % controlándose el desarrollo *in vitro* de *Colletotrichum* sp. en frutos de plátano.

**Palabras clave:** Actividad antimicrobiana, control de enfermedades, hongo, patógenos, poscosecha

## INTRODUCTION

Banano fruits deteriorate due to the rapid ripening at room temperature. Hence, banano fruits are susceptible to pathogen attack during storage because several physiological changes occur in the maturation. *Colletotrichum* sp. represents the most important fungi that affect banano fruit causing anthracnose disease (Kumar *et al*., 2017; Vieira *et al*., 2017). The mechanism of action begins with the germination of conidia and the formation of appressoria penetrating the fruits. Once infected, it remains in a latent form. Additionally, fruit maturity, climatic, transportation and storage conditions contribute to the growth of infections, which causes postharvest quality and quantity losses (Shaw *et al*., 2016; Zakaria, 2021). Synthetic fungicides are the most commonly used method of controlling anthracnose; however, these chemical products are harmful to human health and the environment. Further, *Colletotrichum* species are developing fungicide resistance to methyl benzimidazole carbamates, quinone-outside inhibitors and demethylation inhibitors, causing mutations in the fungi DNA (Cortaga *et al*., 2023). In order to limit the usage of synthetic fungicides in the treatment of postharvest diseases, strategies are sought with the use of natural chemicals. As a result, sustainable strategies for pathogen control, such as the use of inductors, are currently being used. Chitosan (poly-β-(1,4) N-acetyl-d-glucosamine) is a non-toxic polysaccharide derived from crustacean exoskeletons and fungi cell wall. Chitosan has several properties such as antifungal activity and can induce a plant defense response (Xing *et al*., 2015). Several studies have analyzed the *in vitro* effect of chitosan against postharvest fungi, finding a high antifungal activity in the control of *Colletotrichum* sp. and *Rhizopus* (Castañeda-Ramírez *et al*., 2016; Coronado-Partida *et al*., 2017). Furthermore, chitosan has effectively controlled the anthracnose disease in tropical fruits such as mango and soursop (Berumen-Varela *et al*., 2015a; Ramos-Guerrero, *et al*., 2018a). Although the antimicrobial activity of chitosan has been explored, no information regarding the effect of chitosan on the development of *Colletotrichum* sp. in banano fruits can be found.

Taking this into consideration, the purpose of this investigation was to determine the influence of chitosan on the in vitro development of *Colletotrichum* sp.

## MATERIALS AND METHODS

### Isolation and determination of the species of *Colletotrichum* sp.

Physiologically ripe bananos, grade two (light green) according to the Von-Loesecke (1950) maturity scale, were gathered at a local market in Tepic, Nayarit, Mexico. To induce anthracnose, fruits were put in rooms with high relative humidity (90-95 %) and 28 °C. Tissue sections (50 % healthy, 50 % infected) were cut and disinfected with 2 % sodium hypochlorite, washed with sterile distilled water, put in the middle of a petri dishes with potato dextrose agar (PDA) and then incubated at 28°C for five days. Frequent re-isolations were performed to obtain purity strains. Five microcultures were performed on a slide, incubated at 28°C for five days and then observed on a Motic BA300 optical microscope at 40X to identify the pathogen at genera level based on the conidia and mycelium according to the taxonomic keys and previous investigation performed by our research group (Berumen-Varela et al., 2015b).

### Preparation of chitosan solutions

1.5 g of low molecular weight chitosan from Sigma Aldrich (Mw=1.74104 Da, 75-85 % deacetylation degree, FW= 161 20,000 cps) was dissolved in 100 mL of distilled water with 2 mL of acetic acid from Sigma Aldrich to produce a stock solution of chitosan. The solution was constant agitation for 24 h at room temperature. Chitosan solutions of 0.5 %, 1.0 % and 1.5 % were prepared for this investigation. Using 1 N NaOH, the pH of the solutions was adjusted to 5.5, and 0.1 mL of Tween 80 from Sigma Aldrich was added (El Ghaouth *et al*., 1991). Separately sterilized chitosan solutions were combined with PDA and then dispersed on petri dishes.

### *In vitro* assay

The *in vitro* mycelial and radial growth of chitosan on *Colletotrichum* sp. were recorded. A 5 mm diameter disc of 7-day-old cultures of *Colletotrichum* sp. was placed on the petri dishes containing each of the chitosan concentrations. The colony diameter of fungus was measured every day for nine days while petri dishes were incubated at 28 °C. Control treatments contained only the PDA medium. The percentage of mycelial growth inhibition in comparison to the control was recorded. After adding 10 mL of sterile distilled water to the petri dishes, a sterile glass rod was used to rubbed the plates. Mycelium was extracted by filtering the solution through sterile gauze. Then, the spore concentration was determined (number of spores/mL) with 50 µL of the filtered solution using a hemocytometer. 100 observations per treatment were completed with a Motic BA300 optical microscope (Motic Instruments Inc., Canada). Placing 50 L of a *Colletotrichum* sp. spore solution (1x106 spores/mL) on PDA discs with different chitosan concentrations was used to examine spore germination. The discs were examined under an optical microscope every hour for eight hours to look for germinated spores. When the length

of the germinative tube was at least twice the spore diameter, spores were deemed germinated.

### Fluorescence microscopy and scanning electron microscopy (SEM)

A total of 50 L of the previously described spore suspension was injected on discs of each of the chitosan solutions. In the case of fluorescence microscopy, 20 µL of Calcofluor White as fluorescent brightener were added to the discs. Alterations to the structure and fluorescence intensity of the spores were observed on a Leica DM6000B Fluorescence Microscope. On the other hand, for the SEM analysis, discs were directly placed in a carbon plate and then observed in a Zeiss EVO 40 SEM to visualize the structure of the mycelium.

### Statistical analysis

Three plates dishes per treatment and three replicates were used for *in vitro* study, and all assays were conducted twice. To analyze mycelial development and spore concentration, a totally randomized block design was adopted. The data were evaluated using analysis of variance (ANOVA) at a 5 % significance level, and the Tukey test ($p$<0.05) was used to compare means.

## RESULTS

The mycelial and radial growth of the fungi are shown in (Fig. 1a) and (Fig. 1b), respectively. High mycelial growth inhibition after nine days in all the chitosan solutions tested compared with the control treatment was recorded. The highest inhibition was found at 1.0 % and 1.5 % chitosan, reaching final mycelial growth values of 7.5 mm and 3 mm, respectively (Fig. 1a). Besides, we observed a dramatic reduction in the colony diameter after nine days when chitosan solutions of 1.0 % and 1.5 % were used (Fig. 1b). All chitosan solutions presented an effect on the mycelial growth, showing high inhibition values from 49 % to 92.1 % among the chitosan solutions tested (Table 1). In this regard, 1.5 % chitosan showed the highest percentage of mycelial growth inhibition ($p$<0.05).

**Table 1.** Effect of chitosan on the mycelial growth and spore concentration of *Colletotrichum* sp. for nine days.

| Chitosan solutions | Percentage of mycelial growth inhibition (%) | Spore concentration (spores/mL) |
|---|---|---|
| Control | 0 a | 3.85 x $10^7$ a |
| 0.5 % | 49 b | 1.5 x $10^7$ b |
| 1.0 % | 78.94 c | 1.125 x $10^7$ b |
| 1.5 % | 92.1 c | 6.0 x $10^6$ c |

According to Tukey's test, the values with different letters in the same column are statistically different ($p$<0.05).

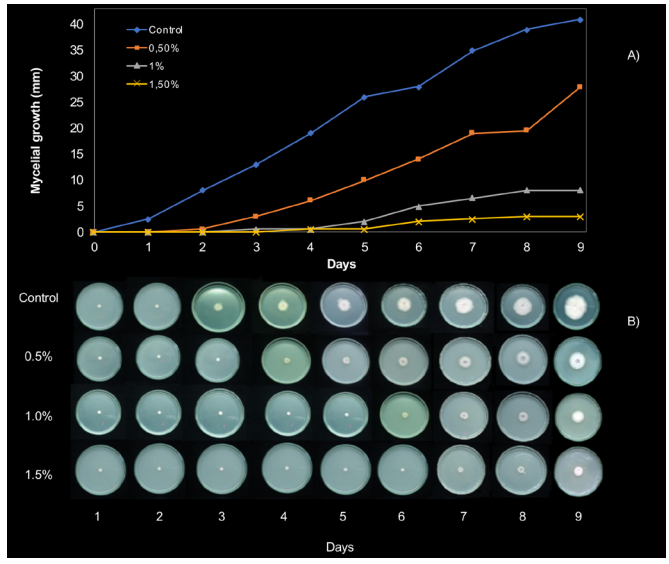


**Figure 1.** *In vitro* effect of chitosan on *Colletotrichum* sp. incubated at 28°C for 9 days. A) Mycelial growth B) Colony radial growth of the petri dishes

On the other hand, (Fig. 2) shows the different types of microscopies used to evaluate the influence of chitosan on the morphology of the spores and mycelium of *Colletotrichum* sp. In this regard, no spore germination was detected in all the chitosan solutions examined (Fig. 2a). Chitosan inhibited the germinative tube of the spore, showing a complete inhibition at 1.0 % and 1.5 %. Additionally, an effect of chitosan on spore concentration was detected, exhibiting a high reduction in the concentration (Table 1). A 2.5-fold, 3.4-fold and 6.4-fold decrease in spore concentration was registered at 0.5 %, 1.0 % and 1.5 % chitosan respectively, compared with the control. Indeed, 1.5 % chitosan displayed the highest diminution of spores ($p$<0.05).

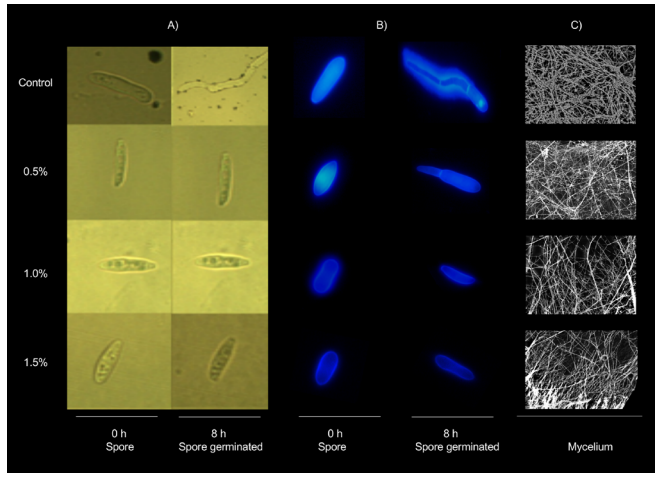


**Figure 2.** Images of spores, spore germinated and mycelium of *Colletotrichum* sp. exposed to different chitosan concentrations. A) Optical microscopy at 40X, B) Fluorescence microscopy at 100X and C) SEM of mycelium at 1200 X.

Furthermore, the chitosan concentration affected the fluorescence of the spores and spore germinated. The control showed higher intensity in spore and spore germinated compared with the chitosan solutions, likewise, when chitosan concentration increased, the fluorescence intensity decreased (Fig. 2b). In (Fig. 2c), is presented the SEM of the mycelium of the different chitosan solutions. In control treatment, a great amount of mycelium without deformation was observed. However, chitosan solutions affected the mycelium amount. In this sense, at 1.0 % and 1.5 % chitosan, a significant reduction in the mycelium concentration and the absence of spores were detected.

## DISCUSSION

*In vitro* studies have shown the influence of chitosan on the inhibition of mycelial development and spore germination in a variety of plant diseases. In this regard, García-Rincón *et al*. (2010) studied the impact of low molecular weight chitosan on mycelial growth inhibition of *Rhizopus stolonifer*, and the greatest antifungal activity was found with chitosan at 2 mg $mL^{-1}$, with an inhibition percentage of 65 %. On tropical fruits, Xiangchun *et al*. (2012) recorded that oligochitosan solutions at 4 and 8 g $L^{-1}$ reduced by 65.4 % and 71.6 % of mycelial growth of *Colletotrichum musae* from banana, respectively. We obtained higher percentage values of inhibition than the previously mentioned. López-Mora *et al*. (2013) evaluated different chitosan concentrations of low molecular weight on *Alternaria alternata* isolated from mango. These authors found 70 % of reduction in the mycelium concentration and the absence of spores using 1.0 % chitosan. According to these results, a direct relationship was observed between the parameters evaluated in the inhibition of *Colletotrichum* sp. and the concentration of chitosan applied.

Further, Berumen-Varela *et al*. (2015a) reported no spore germination of *Colletotrichum* sp. isolated from mango at 1.0 %, 1.5 % and 2.0 % chitosan of low molecular weight. The same authors found a decrease in spore concentration at the same concentrations previously mentioned. These results agree with those obtained in this investigation. In addition, Ramos-Guerrero, *et al*. (2018b) discovered that medium molecular weight chitosan completely inhibited spore germination of *Colletotrichum gloeosporioides* and *Rhizopus stolonifer* isolated from soursop fruits. Moreover, Xoca-Orozco *et al*. (2018) investigated the *in vitro* growth of *Colletotrichum* sp. isolated from avocado fruits using two types of chitosan at various doses. Low and medium molecular weight chitosan at a concentration of 1.0 % significantly inhibited the mycelial growth, concentration and germination spore of *Colletotrichum* sp.

Taking this into consideration, our findings imply that chitosan concentration, as well as molecular weight and percentage of deacetylation, influence fungal inhibition. This can be explained due to the polycationic nature of chitosan, where the positively charged amino groups in the medium can interact with the negatively charged phospholipids in the fungal cell wall. This changes the permeability of the plasma membrane, which changes how the cell works (Ardean *et al*., 2021). Furthermore, Lopez-Moya *et al*. (2019) reported that a relationship exists between the cell wall and membrane because plasma membrane-associated synthase complexes are responsible for the production of glucans and chitin.

On the other hand, according to the chitosan concentration, fluorescence of the spores was affected. A possible explanation to these results, is that the chitosan alters the permeability of the membrane (produce pores) of the fungi cell wall, leading to a low affinity to Calcofluor white. Bautista-Baños *et al*., (2017) reported that chitosan adheres to the membrane cell wall and suppresses the metabolic activity which leads to cell death. Ramos-Guerrero, *et al*. (2018b) observed distorted and collapsed hyphae by SEM at 0.5 % chitosan alone or in combination with other inducers. Xiangchun *et al*. (2012) evaluated oligochitosan concentrations on *Colletotrichum musae* from banano. These authors reported that oligochitosan at 4 $g{\cdot}L^{-1}$ modified the hyphal cell wall and reduced the hyphal diameter of *C. musae*. Further, Rodríguez-Pedroso *et al*. (2016) used SEM to observe changes in the spores of *Bipolaris oryzae*, reporting the deformation of the spores. Moreover, Sánchez-Domínguez *et al*. (2011) observed severe alteration in the spores and hyphae of *Alternaria alternata* isolated from tomato using transmission electron microscopy.

The antifungal activity of chitosan at the level of spores may be attributable to the inhibition of enzyme and/or nutrient production. El Ghaouth *et al*. (1991) mentioned that the chitosan is a chelating agent able to sequester the ions metals required for the enzymatic reactions. According to our results, the chitosan probably degraded the fungi cell wall due to the ability to bind the metals in the cell wall and therefore, inhibit the production of toxins. Nonetheless, the mechanism by which chitosan impacts the spore germination still largely unknown and further studies need to be done to prove the previous statement.

## CONCLUSIONS

Chitosan concentrations of 1.0% and 1.5% inhibited the *in vitro* growth and alter the morphology of *Colletotrichum* sp.

## AUTHOR'S PARTICIPATION

Formal analysis, V.A.O-J.; Conceptualization, G.B-V. and P.G-M; methodology, V.A.O-J and M.A.C-L.; investigation, R.B.M.; resources, P.G-M; data curation, V.A.O-J; writing-original draft preparation, V.A.O-J.; writing-review and editing, G.B-V and P.U.B-R.; supervision, P.U.B.R; funding acquisition, P.G-M and M.A.C-L. All authors have read and agreed to the published version of the manuscript.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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