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In vitro and *in vivo* antifungal activity of chitosan and identification of potentially toxigenic fungi in stored maize of Nayarit, Mexico

Actividad antifúngica *in vitro* e *in vivo* de quitosano e identificación de hongos potencialmente toxigénicos en maíz almacenado del estado de Nayarit, México

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Abstract

Maize is the main food in Mexico since it constitutes the food base of millions of Mexicans. However, production is affected by the presence of mycotoxin-producing fungi, such as *Aspergillus*, *Penicillium*, and *Fusarium*. In order to prevent the growth of these pathogens, the efficacy of high molecular weight commercial chitosan was evaluated to prolong the conservation and quality of the grain during storage. The maize kernels were provided from the state of Nayarit, Mexico. The fungi *A. niger*, *P. funiculosum*, and *F. verticillioides* were isolated and identified morphologically and molecularly. The *In vitro* chitosan concentrations evaluated were 0.5, 1.0, 1.5, and 2.0%. The highest concentration inhibited mycelial growth by 74.97, 93.19 and 89.79% for *A. niger*, *P. funiculosum*, and *F. verticillioides*, respectively. The results demonstrated that commercial chitosan with a high molecular weight can effectively inhibit the growth of mycotoxin-producing fungus in preserved maize kernels.

Keywords: Maize, stored fungi, *Aspergillus niger*, *Penicillium funiculosum*, *Fusarium verticillioides*, chitosan.

Resumen

El maíz es el principal alimento en México, ya que constituye la base alimentaria de millones de mexicanos, sin embargo, la producción se ve afectada por la presencia de hongos productores de micotoxinas, como son *Aspergillus*, *Penicillium* y *Fusarium*. Con el propósito de evitar el crecimiento de estos patógenos, se evaluó la eficacia del quitosano comercial de alto peso molecular, para prolongar la conservación y calidad del grano durante su almacenamiento. Los granos de maíz fueron proporcionados del estado de Nayarit, México. Se aislaron e identificaron de manera morfológica y molecularmente a los hongos *A. niger*, *P. funiculosum* y *F. verticillioides*. Las concentraciones de quitosano evaluadas "*in vitro*" fueron 0.5, 1.0, 1.5 y 2.0% de quitosano. La concentración más alta inhibió el crecimiento micelial en un 74.97, 93.19 y 89.79% para *A. niger*, *P. funiculosum* y *F. verticillioides*, respectivamente. Los resultados mostraron que el quitosano comercial de alto peso molecular puede ser un tratamiento efectivo para controlar el crecimiento de los hongos productores de micotoxinas establecidos en los granos de maíz almacenados.

Palabras clave: Maíz, Hongos de almacenamiento, *Aspergillus niger*, *Penicillium funiculosum*, *Fusarium verticillioides*, quitosano.

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1 Introduction

Maize (*Zea mays* L.) is a plant native to Mexico that is used for human and animal nutrition worldwide. Due to its planted area (7 million hectares) and annual production, it is the most significant food in our country (approximately 27 million tons) (FAOSTAT, 2022; SIAP, 2022). Although Mexico is among the main producers of maize, improper handling during harvest, transportation, and storage favors the proliferation of different microorganisms, mainly fungi, which cause large postharvest losses (Deng *et al*., 2020; Odjo *et al*., 2022). The main genera of phytopathogenic fungi reported in maize are *Aspergillus* spp., *Penicillium* spp., and *Fusarium* spp. (Qi *et al*., 2023; Odjo *et al*., 2022); contamination by these fungi causes physiological damage such as germination inhibition, color changes, and undesirable odors. On the other hand, they represent a potential health risk due to the production of mycotoxins. (Erasto *et al*., 2023; González-Jartín *et al*., 2022; Kolawole *et al*., 2021; Ravichandra, 2022). Mycotoxins are secondary metabolites with varied toxic capacities; chronic exposure to them has been related to hepatotoxicity, nephrotoxicity, neurotoxicity, genotoxicity, teratogenicity, and immunomodulation (Abrehamed *et al*., 2023 IARC, 1993). Therefore, combating these pathogens helps reduce contamination and the risk of mycotoxins in maize.

Control systems to combat the proliferation of mycotoxigenic fungi involve physical methods (thermal treatments, ventilation), chemical products (fungicides), and biological systems (bacterial species, yeasts, atoxigenic strains) (Moumni *et al*., 2023; Orzali *et al*., 2023; Sirohi *et al*., 2021). However, none of these methods has been able to be effective to control or reduce postharvest losses. Additionally, the use of fungicides has been withdrawn from the market and even prohibited in many countries due to the toxicity caused in humans, harmful to the environment, and damage to the quality of the product (Sirohi *et al*., 2021; Wan *et al*., 2021). There is growing interest in the use of antifungal compounds obtained from natural resources, such as chitosan, mainly obtained from shrimp exoskeletons (Mukarram *et al*., 2023; Orzali *et al*., 2023). Several studies have shown that chitosan has direct antimicrobial properties, and film-forming activity, and triggers the defense mechanisms of the plant (Gutiérrez-Martínez *et al*., 2020; Herrera-Gonzaléz *et al*., 2022; Rodríguez-Guzmán *et al*., 2022; Ramos-Bell *et al*., 2022; Rayón-Díaz *et al*., 2021; Saberi *et al*., 2024). The objective of this study was to identify the species of pathogenic fungi with the potential to produce mycotoxins through morphological and molecular characteristics, as well as to evaluate the *in vitro* effectiveness of non-contaminating technologies, such as the application of high-weight commercial chitosan at different concentrations in the control of these pathogens.

2 Materials and methods

2.1 Sample collection

The maize samples were provided by a regional food store located in Acaponeta, Nayarit, and collected in the first-summer cycle of 2019, without having been stored for more than two months. The maize grains were healthy, without any infection or physical damage. The moisture content of the samples was 11.87 ± 0.56 % AOAC, (2005) and the germination percentage was 85 ± 7 % (Warham *et al*., 2003).

2.2 Isolation and purification of potentially toxigenic fungi present in maize grain

The grains (n=150) were sterilized on their surface with 2% sodium hypochlorite for 1 min, rinsed with sterile water, and placed on sterile filter paper to eliminate excess moisture. Subsequently, the grains were sown on potato dextrose agar (PDA) and incubated at 25 °C ± 2 °C for a period of 72 h in the dark (Mohamed *et al*., 2020). Once the presence of mycelium was detected, purification was carried out from the colonies that appeared on the periphery of the grain. The isolated spores were replanted in maize medium (Warham *et al*., 2003) and incubated at 25 ± 2 °C for 6 days. This procedure was repeated until only one type of morphology per plate was obtained.

2.3 Morphological characterization

The identification of the fungus was based on the analysis of the macroscopic and microscopic characteristics of the colony. For microscopic analysis, preparations stained with methylene blue were made. Mycelium and spores were observed in an optical microscope (Motic BA300) with 40X and 100X objectives. External traits, reproductive structures, and conidia were examined and taxonomic keys were employed to determine the genus. (Mohamed *et al*., 2020; Pitt and Hocking, 2009).

2.4 Molecular characterization

Identification was carried out by Polymerase Chain Reaction (PCR) and sequencing, which had been previously standardized at the Colegio de Postgraduados (COLPOS) in the state of Mexico (Fuentes-Aragón *et al*., 2020; Juárez-Vázquez *et al*.,

2019). The internal transcribed spacer (ITS) region of the genomic rDNA was used with the primers ITS1 and ITS4 for *Aspergillus* sp. and *Penicillium* sp.; and the translation elongation factor-1alpha (TEF-1α) gene with primers EF1 and EF2 for *Fusarium* spp. (Lücking *et al*., 2020).

2.5 Chitosan preparation

Commercial chitosan (47.5 kDa, 90% deacetylation, Golden-Shell Co., China) was used at 0.5, 1.0, 1.5, and 2.0% (w/v) in distilled water acidified with 10% vinegar. The control consisted of an acidified water treatment. The solutions were stirred for 24 h at room temperature. The pH was then adjusted to 5.6 with NaOH (1 N). Finally, 0.1% Tween 80 was added and the solution was sterilized using an autoclave (Ramos-Bell *et al*., 2022). Once the chitosan solutions were sterilized, they were poured into Petri dishes containing PDA medium.

2.6 In vitro test

2.6.1 Mycelial inhibition

An 8 mm disc from the margin of the fungal colonies (4 days of incubation) was placed in the center of a Petri dish (90 mm in diameter) with PDA medium and the different chitosan treatments (0, 0.5, 1.0, 1.5 and 2.0%) and then incubated at 25 °C ± 2 °C. The development of mycelial growth was recorded daily and the results were expressed as percentage inhibition (Mohamed *et al*., 2020). The ImageJ 1.52p software (Image Processing and Analysis in Java, 2019) was used to calculate the area (mm^2) and the perimeter of the colonies (mm).

2.6.2 Sporulation

The spore suspension was prepared with 10 mL of sterile water and 0.1 mL of tween 80 in each Petri dish, scraped with a glass loop, and allowed to settle for 5 min, then filtered through sterile gauze on a glass funnel and suspensions were deposited in test tubes (Godana *et al*., 2020). 1:10 dilutions of the three fungi were made. Quantification was performed in a Neubauer chamber with the help of an optical microscope (Motic BA300) with the 40X objective. The results were expressed in the number of spores/mL.

2.6.3 Spore germination

Discs of PDA medium (20 mm in diameter) were prepared with different concentrations of chitosan (0, 0.5, 1.0, 1.5, and 2.0%). These discs were inoculated with 20 μL of the spore suspensions at a concentration of 10^6, placed on slides, and incubated at 25 °C ± 2 °C (Ramos-Bell *et al*., 2022). Germinated spores were counted in a Neubauer chamber under an optical microscope (Motic BA300) with a 40X objective every 1 h. The spores were considered germinated when the length of the germ tube was twice its diameter.

2.7 In vivo test

2.7.1 Application of chitosan to maize grains

110 previously disinfected grains were used, to which the 2% chitosan treatment (most effective) was applied by immersion, submerging the grain for 5 minutes. The grains were then allowed to air-dry at room temperature for three hours to remove the moisture excess (Ventura-Aguilar *et al*., 2022). The grains immersed in sterile distilled water were used as controls.

2.7.2 Inoculation of spores to the maize grain

Once the grains were dry, they were placed in sterile Petri dishes (5 grains per box) and the spore suspensions of each pathogen were sprayed at a concentration of 10^6 using a manual atomizer until draining and stored at 12 and 25 ℃ ± 2 °C.

2.7.3 Percentage of incidence and severity

These parameters were evaluated in grains contaminated with mycelial growth. The incidence was evaluated using the following equation (Mohamed *et al*., 2020):

$$\text{corn kernel infection (\%)} = \frac{\text{number of contaminated grains}}{\text{number of total grains}} \times 100$$

The severity was determined utilizing a diagrammatic scale (Pabón-Baquero *et al*., 2015), which is represented with an image of the infected grain divided into 5 parts, where 1= 0-20%, 2= 21-40%, 3= 41-60%, 4= 61-80%, 5= 81-100% (figure 1).

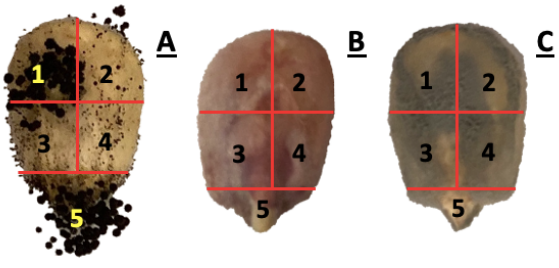

Figure 1. Diagrammatic scale (Pabón-Baquero *et al*., 2015). Maize grain is divided into 5 parts with the growth of (a) *Aspergillus niger*, (b) *Penicillium funiculosum*, and (c) *Fusarium verticillioides*.

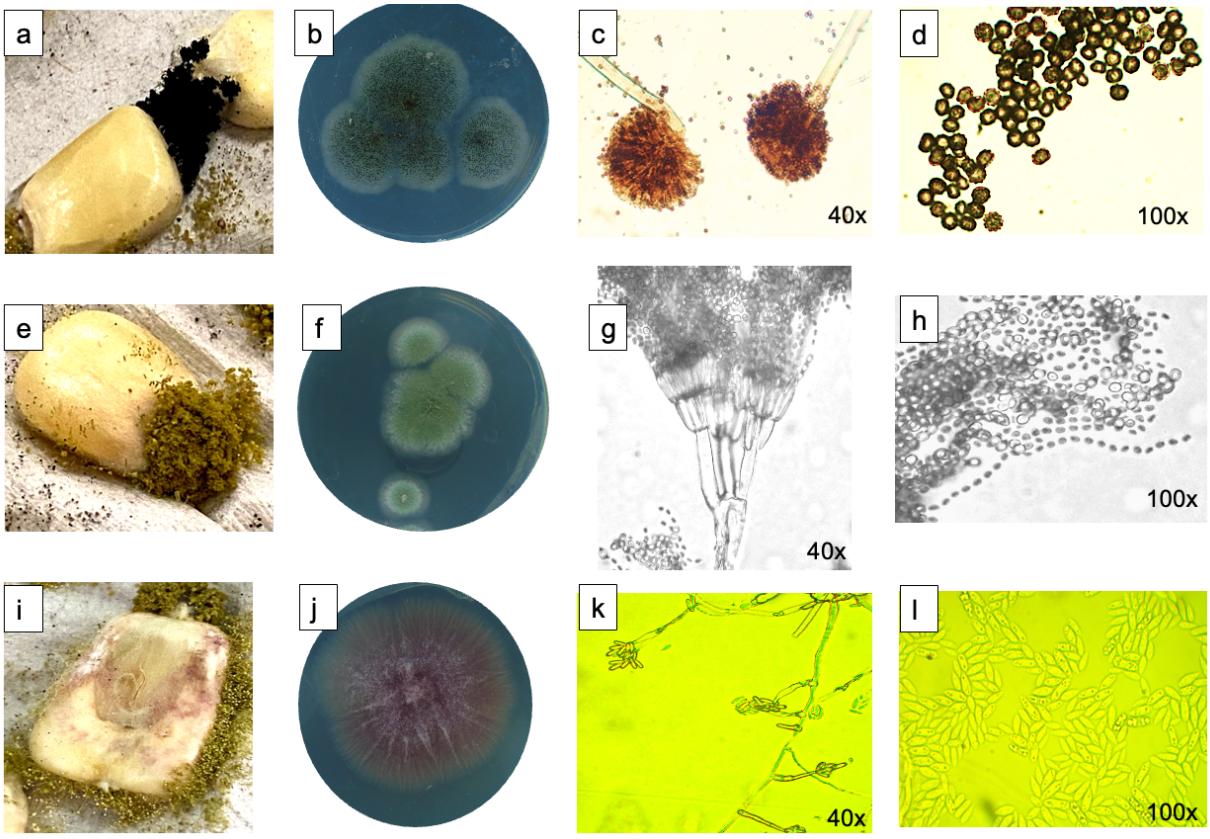

Figure 2. Presence of pathogens. (a, e, i) Fungal pathogens developed in maize kernels. (b, f, j) Strains isolated in the PDA medium. (b, c, d) Morphological characteristics of *Aspergillus niger*. (f, g, h) Morphological characteristics of *Penicillium funiculosum*. (j, k, l) Morphological characteristics of *Fusarium verticillioides.*

2.8 Statistical analysis

For *in vitro* tests, a completely randomized block design was applied. The experiment was performed in triplicate in two independent experiments. In the case of the *in vivo* tests, a 2^2 factorial design was used. The experiment was performed in triplicate in three independent experiments. The results were analyzed using an analysis of variance (ANOVA) and the Tukey test ($P < 0.05$) for the comparison of means with the statistical package STATISTICA v12.0 (StatSoft Inc., 2013).

3 Results and discussion

3.1 Morphological characteristics

Three strains of potentially toxigenic fungi were purified and morphologically characterized (figure 2a, e, i). The three different isolates were recorded as A1, P1, and F1 (figure 2b, f, j, respectively).

Based on the infection found in the maize grain, and the macroscopic characteristics in the PDA medium, the A1, P1, and F1 strains may belong to the genera *Aspergillus* spp., *Penicillium* spp., and *Fusarium* spp., respectively (Pitt and Hocking, 2009; Warham *et al*., 2003). In Mexico, the presence of potentially toxigenic fungi has a high frequency in stored maize grain, the genus *Aspergillus* spp. is one of the fungi that occur most frequently followed by the genus *Penicillium* spp., while in the field the highest incidence is of the genus *Fusarium* spp. (Akonda *et al*., 2016; Erasto *et al*., 2023; González-Jartín *et al*., 2022; Gulbis *et al*., 2016; Muhammad *et al*., 2019; Odjo *et al*., 2022; Pfliegler *et al*., 2020; Qi *et al*., 2023).

3.1.1 Morphological characteristics of Aspergillus niger

The isolated A1 colonies were identified as *A. niger* and based on the morphological characteristics described by several authors, they can be classified in the *Nigri* section (Pitt *et al*., 2012; Warham *et al*., 2003). In 4 days at 25 ± 2 ºC, abundant white superficial mycelium and a black layer that covered the Petri dish's surface were detected (figure 2b). The conidiophores presented globose conidial heads, brown stipes, without septa, and spherical vesicles carrying metulas and phialides compacted on the surface (figure 2c), their conidia were globose brown with irregular crests (figure 2d).

A very important property to differentiate *Aspergillus* species from *Penicillium* is that *Aspergillus* stipes are usually formed from a short cell called the foot cell within a fertile hypha and are not septate, so vesicles, stipes, and cell of the foot form a single very large cell (Pitt and Hocking, 2009; Wadzani *et al*., 2019).

3.1.2 Morphological characteristics of Penicillium funiculosum

P1 colonies identified as *P. funiculosum* showed blue-green mycelium with white edges and a powdery texture. Their maximum growth rate was after 8 days of incubation at 25±2 ºC (figure 2f).

The conidiophores were observed to have a hyaline and septate stipe, ending in a terverticillate penicillus (figure 2g), with a series of typical hyaline phialide ramifications, which produce long chains of small conidia (figure 2h). The morphology of *P. funiculosum* is similar to that described by Pitt *et al*. (2012) and Yadav *et al*., (2018).

3.1.3 Morphological characteristics of Fusarium verticillioides

Colony isolated as F1 identified as *F. verticillioides*, presenting mycelium of pink-violet color with raised white cottony texture and filamentous shape that covered the Petri dish in 9 days (figure 2j). In the microscopic structures, monophialides were observed with mass microconidia giving the appearance of false heads (figure 2k) and abundant unicellular hyaline microconidia and very few bicellular ones (figure 2l). The described morphology of *F. verticillioides* agrees with those reported by several authors (Pitt and Hocking, 2009; Torre-Hernández *et al*., 2014; Warham *et al*., 2003).

3.2 Molecular identification

The sequencing of the amplification products with ITS1-ITS4 and EF1-EF2, allowed the identification of the three isolated strains at the species level. The ITS has been widely used for the molecular identification of filamentous fungi. Phylogenetic approaches based on multiple sequence alignment are necessary to avoid misidentification of species of the main genera of mycotoxigenic fungi (Lücking *et al*., 2020).

According to the BLAST search, the A1 strain showed 100% coverage and 100% identity with *A. niger*, the P1 strain had 100% coverage and 100% identity with *P. funiculosum*, and the F1 strain had 99% coverage and 99.57% identity with *F. verticillioides*. Mycotoxin-producing pathogenic fungi, such as *A. niger*, *P. funiculosum*, and *F. verticillioides*, are often isolated from cereals, particularly maize, throughout the world (Erasto *et al*., 2023; González-Jartín *et al*., 2022; Muhammad *et al*., 2019; Odebode *et al*., 2020; Odjo *et al*., 2022; Pfliegler *et al*., 2020; Qi *et al*., 2023).

3.3 In vitro tests

3.3.1 Mycelial growth inhibition

Mycelial growth inhibition tests were performed with the three fungi identified. The application of the different concentrations of chitosan showed significant differences ($p < 0.05$) in mycelial growth for the three fungi (table 1). In the case of *A. niger*, the greatest inhibition of the mycelium was observed at 2% concentration. On the other hand, 1% and 1.5% concentrations presented inhibitions of less than 25%; while the lowest concentration and the control did not influence the growth of the fungus. For *F. verticillioides*, mycelial growth was inhibited in all concentrations tested, from the concentration of 1.0%, the inhibition was greater than 60% (table 1). *P. funiculosum* showed more than 80% of mycelial growth inhibition at the lowest chitosan dose, being the most sensitive fungus to chitosan treatments (table 1).

Table 1. Effect of the application of chitosan at different concentrations on the percentage of mycelial inhibition of *Aspergillus niger*[1], *Penicillium funiculosum*[2] and *Fusarium verticillioides*[3].

Treatment (%)	*Aspergillus niger*	*Penicillium funiculosum*	*Fusarium verticillioides*
Control	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
Chitosan 0.5	0.27 ± 0.44^{a}	81.48 ± 9.25^{b}	31.82 ± 6.98^{b}
Chitosan 1.0	24.99 ± 9.05^{b}	82.68 ± 1.57^{b}	60.14 ± 1.56^{c}
Chitosan 1.5	19.40 ± 5.83^{b}	90.58 ± 0.76^{c}	74.99 ± 3.95^{d}
Chitosan 2.0	74.97 ± 2.61^{c}	93.19 ± 3.20^{c}	89.79 ± 2.57^{e}

Mean ± Standard deviation of 3 independent experiments with 2 replicates. The values with the same letter in the column of each treatment are not significantly different ($p > 0.05$). Incubation at 25±2 ºC for 5^{1}, 8^{2}, and 9^{3} days.

Table 2. Effect of chitosan at different concentrations on the sporulation of *Aspergillus niger*[1], *Penicillium funiculosum*[2] and *Fusarium verticillioides*[3].

Treatment (%)	*Aspergillus niger*	*Penicillium funiculosum*	*Fusarium verticillioides*
Control	$9.1\times10^{7}\pm3.8\times10^{6a}$	$5.33\times10^{8}\pm4.93\times10^{7a}$	$1.71\times10^{8}\pm3.41\times10^{7a}$
Chitosan 0.5	$7.9\times10^{7}\pm5.8\times10^{6b}$	$2.66\times10^{8}\pm8.60\times10^{7b}$	$1.40\times10^{8}\times2.39\times10^{7a}$
Chitosan 1.0	$5.7\times10^{7}\pm6.7\times10^{6c}$	$1.26\times10^{8}\pm7.42\times10^{7c}$	$1.48\times10^{8}\pm1.33\times10^{7a}$
Chitosan 1.5	$4.9\times10^{7}\pm6.9\times10^{6c}$	$1.49\times10^{8}\pm7.76\times10^{7c}$	$7.14\times10^{7}\pm4.36\times10^{7b}$
Chitosan 2.0	$1.5\times10^{7}\pm3.3\times10^{6d}$	$5.04\times10^{7}\pm3.16\times10^{7c}$	$7.53\times10^{6}\pm4.19\times10^{6c}$

Mean ± Standard deviation of 3 independent experiments with 2 replicates. The values with the same letter in the column of each treatment are not significantly different ($p > 0.05$). Incubation at 25±2 ºC for 5[1], 8[2], and 9[3] days.

Table 3. Effect of chitosan on the germination of *Aspergillus niger*, *Penicillium funiculosum* and *Fusarium verticillioides*, 9, 12, and 8 h, respectively.

	% Germination		
Treatment	*Aspergillus niger*	*Penicillium funiculosum*	*Fusarium verticillioides*
Control	98.67 ± 1.03^{a}	98.44 ± 0.34^{a}	97.33 ± 1.60^{a}
Chitosan 0.5 %	28.78 ± 1.66^{b}	2.44 ± 1.87^{b}	0.0 ± 0.0^{b}
Chitosan 1.0 %	23.45 ± 2.32^{c}	0.22 ± 0.34^{c}	0.0 ± 0.0^{b}
Chitosan 1.5 %	35.12 ± 2.56^{d}	0.0 ± 0.0^{c}	0.0 ± 0.0^{b}
Chitosan 2.0 %	16.78 ± 3.88^{e}	0.0 ± 0.0^{c}	0.0 ± 0.0^{b}

Mean ± Standard deviation of 3 independent experiments with 3 replicates. The values followed by the same letter in the column of each treatment are not significantly different ($p < 0.05$).

In this study, the application of high molecular weight commercial chitosan with a degree of deacetylation of 90%, showed positive results as a fungistatic, at the highest concentrations (1.5 and 2.0%) in the different fungi evaluated. These results can be compared with those found in other investigations, achieving the inhibition of *A. niger* (Dewi and Nur, 2018; El-araby *et al*., 2022), different species of *Penicillium* (Carvalho *et al*., 2020; Sun *et al*., 2021) and *Fusarium* sp. (Kocięcka and Liberacki, 2021) a chitosan application greater than 1.0% is needed. However, the antifungal effect of chitosan may vary depending on its characteristics, such as the source from which it is extracted, molecular weight, and degree of deacetylation (Mukarram *et al*., 2023; Saberi *et al*., 2024).

Several mechanisms have been proposed for the antifungal action of chitosan, the main mode of action is based on the positive charge of its free amino groups, at acidic pH, conferred by protonation. Therefore, polycationic chitosan can potentially interact with negatively charged fungal cell membrane components (phospholipids, proteins). This electrostatic interaction results in the permeabilization of the plasma membrane, interfering with the normal growth and growth metabolism of fungal structures, mycelium, and spores (Debnath *et al*., 2022; Mukarram *et al*., 2023; Saberi *et al*., 2024).

3.3.2 Sporulation

The highest chitosan concentration showed a significant sporulation reduction ($p < 0.05$) for *A. niger*, *P. funiculosum*, and *F. verticillioides* (table 2). The most sensitive fungus was *F. verticillioides*, followed by *P. funiculosum*, and finally *A. niger*.

The negative effect of chitosan on spore production has been related to irreversible damage to membrane permeabilization, causing irregularities at an intracellular and extracellular levels. Damage to the structure of surface proteins (G proteins), for instance, renders them incapable of responding to extracellular signals and transmitting this information intracellularly. Therefore, the signaling cascade and numerous biological processes, such as sporulation, cannot be regulated and at that point, the cell ceases to be actively functional (Baltussen *et al*., 2019; Mukarram *et al*., 2023; Saberi *et al*., 2024). These results demonstrated that chitosan is effective in reducing the production of spores and thereby controlling the dissemination and proliferation of the pathogen.

3.3.3 Germination

Spore germination and germ tube elongation were inhibited in a dependent manner on chitosan concentrations. *F. verticillioides* had a 100% inhibition of germination at all concentrations (table 3).

Table 4. Percentage of incidence and severity for *A. niger*, *P. funiculosum* and *F. verticillioides* in maize grain with 2.0% chitosan treatment stored at two temperatures (12 and 25 ºC).

	Treatment	Incidence (%)		Severity (%)	
		12 ºC	25 ºC	12 ºC	25 ºC
A. niger	Control	0 ± 0^a	100 ± 0^a	0 ± 0^a	100 ± 0^a
	Chitosan 2.0%	0 ± 0^a	100 ± 0^a	0 ± 0^a	80 ± 7.7^b
P. funiculosum	Control	0 ± 0^a	100 ± 0^a	0 ± 0^a	100 ± 0^a
	Chitosan 2.0%	0 ± 0^a	97.78 ± 3.85^a	0 ± 0^a	64.2 ± 23.0^b
F. verticillioides	Control	100 ± 0^a	100 ± 0^a	100 ± 0^a	100 ± 0^a
	Chitosan 2.0%	17.78 ± 3.85^b	15.55 ± 10.18^b	11.56 ± 4.28^b	14.9 ± 8.7^b

Mean ± standard deviation of 3 independent experiments with 3 replicates. Values with different letters within a column in the same fungus represent a significant difference according to Tukey's mean comparison test ($p < 0.05$).

For *P. funiculosum*, an effect was detected at all concentrations, with 100% inhibition beginning at a concentration of 1.5% (table 3). In the case of *A. niger*, concentrations of 0.5% and 1% had an inhibition effect greater than 70%. However, at a concentration of 1.5%, spore germination increased, whereas, at a concentration of 2%, germination decreased until inhibition of greater than 80% was achieved (table 3). The spore germination process is considered the first stage of the development of the fungus with the formation of the germ tube to infect and cause the disease (Baltussen *et al*., 2019; Gálvez-Iriqui *et al*., 2019). Hence, to limit the growth of these pathogens, the treatment employed should primarily impede this process.

Chitosan has the ability to chelate, and bind to metals, such as some important enzyme cofactors in the growth and development of fungi that are necessary for processes related to spore germination. By depriving the fungus of metallic cofactors, various enzymatic reactions are altered and this is reflected in physiological and morphological changes in the cell (Debnath *et al*., 2022; Mukarram *et al*., 2023; Saberi *et al*., 2024). The results of this study coincide with those reported by various authors for the three different fungi (Carvalho *et al*., 2020; Kocięcka and Liberacki, 2021; Segura-Palacios *et al*., 2021; Sun *et al*., 2021).

3.4 *Effects of chitosan on infected maize grain*

The application of 2.0% chitosan as a seed coating for the control of *A. niger*, *P. funiculosum*, and *F. verticillioides* showed significant differences ($p < 0.05$) compared to the control samples (table 4).

3.4.1 *Aspergillus niger*

A. niger began to develop at 72 h in the maize grain without chitosan (control) at a temperature of 25 ºC, the white mycelium covered the grains and at 6 days the surface was covered with black spores (figure 3a). There were no significant changes between the

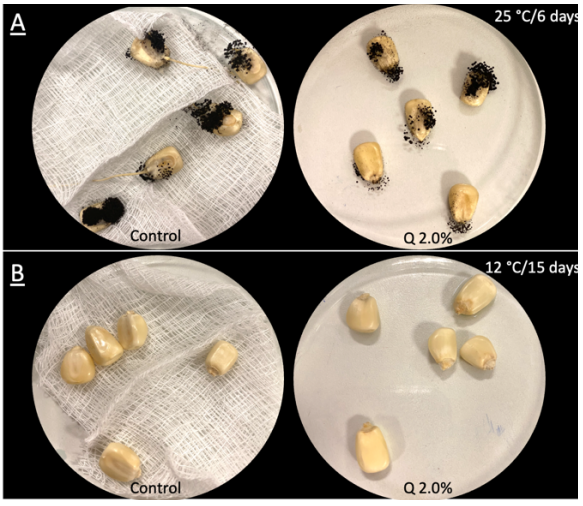

Figure 3. Growth of *Aspergillus niger* in maize kernels treated with 2.0% chitosan. (a) grains stored at 25 ºC and (b) at 12 ºC.

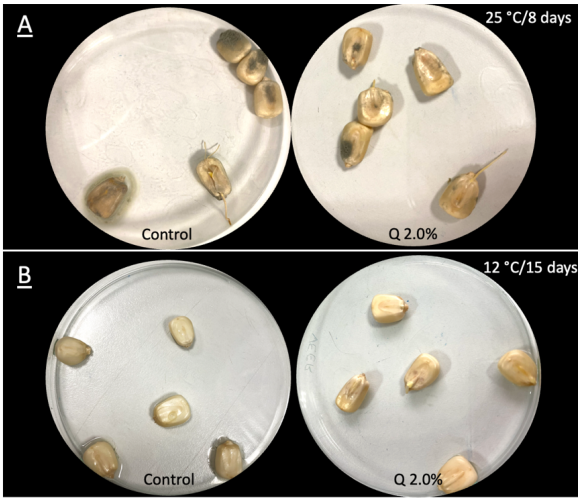

Figure 4. Growth of *Penicillium funiculosum* in maize grains treated with 2.0% chitosan. (a) Grains stored at 25 ºC and (b) at 12 ºC.

coated grain and the control at 25 ºC. However, in the variable of severity, some effects were observed in the treated grain (table 4). There was no development of the fungus in the grains coated with chitosan and the control until 15 days of storage at 12 ºC (figure 3b). *A. niger* grows optimally at a temperature of 35-37 ºC and a minimum of 8 ºC (Pitt and Hocking, 2009). Therefore, the use of low temperatures is an important factor to control its development.

The application of chitosan was not so promising for the control of this fungus, Segura-Palacios *et al*., (2021) applied chitosan with other compounds to seeds, achieving a reduction of around 100%. In this sense, we can infer that the application of chitosan in combination with other treatments could become an effective method to reduce postharvest losses of this fungus.

3.4.2 Penicillium funiculosum

The growth of *P. funiculosum* in control maize grains occurred after 8 days of incubation at a temperature of 25 ºC (figure 4a). The coated grain with chitosan showed no significant differences compared to the control in the incidence. Nevertheless, the severity showed a decrease of 35.78% in the development of *P. funiculosum* (table 4). When using a temperature of 12 ºC, no development of the fungus was observed for the control and the coated grain (figure 4b). This may be due to the fact that the growth temperature of *P. funiculosum* ranges from 8-42 ºC with an optimum of 25 -28 ºC (Elgharably and Nafady, 2021). Like *A. niger*, the temperature is an important factor that can be combined with the application of treatments to control these pathogens to retard or inhibit their growth. Carvalho *et al*., (2020) compared the effectiveness of chitosan in *in vivo* tests to control different species of *Penicillium*, finding different percentages of disease incidence after 14 days of storage depending on the type of species, these results can be compared to those of the present work.

3.4.3 Fusarium verticillioides

Unlike *A. niger* and *P. funiculosum*, temperature is not a factor that interferes with growth for *F. verticillioides*, because this fungus can develop at an optimum temperature of 25 ºC and a minimum of 2 ºC (Pitt *et al*., 2012). *F. verticillioides* at 25 ºC had rapid growth in the control samples (4 days), observing a pink coloration throughout the grain and a slight development of white mycelium, while most of the treated grains did not show growth of the fungus (figure 5a). The results obtained when *F. verticillioides* developed at a relatively low temperature (12 ºC) were the same as at the optimum temperature of 25 ºC, only with a 3-day delay in its development (figure 5b).

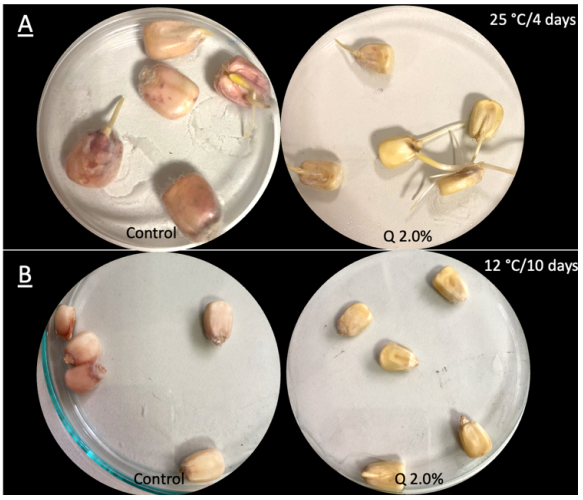

Figure 5. Growth of *Fusarium verticillioides* in maize kernels treated with 2.0% chitosan. (a) Grains stored at 25 ºC and (b) at 12 ºC.

Therefore, the effectiveness in the treated maize was similar in the two temperatures, since the percentages of incidence and severity were less than 20 % (table 4), which confirms that chitosan can be effective to control or inhibit the symptoms of infection without having so much influence the temperature factor.

The chitosan coating acts as a semipermeable film that regulates gas exchange, and reduces water loss and nutrient uptake, thus affecting fungal development. On the other hand, chitosan can induce defense mechanisms in fruits and vegetables such as the synthesis of phenolic compounds, hydrolase antifungal enzymes such as chitinases and glucanases that hydrolyze the main components of the cell wall of fungi, thus inhibiting their growth. In this sense, the development of post-harvest diseases can be controlled or inhibited and the duration of storage for fruits, vegetables, and seeds can be prolonged (Moumni *et al*., 2023; Orzali *et al*., 2023).

The conditions evaluated in the present work can be applied under storage conditions in silos. The chitosan coating and a low temperature, in the case of *F. verticillioides*, can control the development of these pathogens for up to 15 days of storage and reduce the deterioration of the grains. Chitosan did not have a significant effect on the incidence of the disease caused by *A. niger* and *P. funiculosum* in the grain, however it did reduce the severity at room temperature. In this regard, other investigations have concluded that the antifungal activity of chitosan as a coating can control *A. niger* (El-araby *et al*., 2022), *P. funiculosum* (Carvalho *et al*., 2020) and *F. verticillioides* (Mohamed *et al*., 2020).

Conclusions

It was possible to isolate and identify fungi such as *A. niger*, *P. funiculosum*, and *F. verticillioides* from the maize grain for human consumption. These fungi are classified as potential mycotoxins-producing organisms that cause adverse health effects. Therefore, chitosan treatment at high concentrations (2.0%) proved to be a safe alternative for the control of some pathogens such as *F. verticillioides*, which has implications for the preservation of stored grains and the reduction of mycotoxin exposure. However, it is necessary to evaluate chitosan with the addition of a non-contaminating component that allows more effective control of resistant fungi, such as *A. niger*.

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