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Identification of a *Colletotrichum* species from mango fruit and its *in vitro* control by GRAS compounds

Identificación de especies de *Colletotrichum* aisladas de fruto de mango y su efecto de control por la aplicación de compuestos GRAS

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Abstract

The fungus *Colletotrichum* sp. is the causal agent of anthracnose in mango fruit and leads to substantial postharvest losses (>30 %). Efficient control agents continue to be investigated to reduce the incidence of this pathogen, preferably of a biological-organic nature. The objective of this work was to evaluate the *in vitro* application of commercial-grade generally recognized as safe (GRAS) compounds as chitosan, hydrogen peroxide, acetic acid, and peracetic acid, in the control of *Colletotrichum* species isolated from mango fruit. The application of commercial chitosan (1.5 % and 2.0 %) confirmed its antifungal effect, with an average inhibition of 34 %, no significant difference was shown with respect to reactive grade chitosan. Hydrogen peroxide, acetic acid, and peracetic acid completely inhibited the development of *C. asianum* at concentrations > 1.0 %. Each GRAS agent caused morphological damage to the spores, including loss of turgidity, intracellular disorder, effusion of liquid from the cytoplasm, and total loss of integrity. Scanning electron microscopy confirmed the damage to the mycelia, with collapsed and dehydrated structures due to exposure to the control agents. Overall, commercial GRAS agents showed an *in vitro* control effect on the growth of *C. asianum* at different stages of their development.

Keywords: *Mangifera indica*, postharvest fungi, chitosan, hydrogen peroxide, acetic acid, peracetic acid.

Resumen

Colletotrichum sp. causa la antracnosis en frutos de mango provocando importantes pérdidas poscosecha (>30 %). Para reducir la incidencia de la enfermedad se continúan investigando agentes de control eficientes preferentemente de carácter biológico-orgánico. El objetivo del presente trabajo fue evaluar la aplicación *in vitro* de compuestos de grado comercial Generalmente Reconocidos como Seguros (GRAS) como quitosano, peróxido de hidrógeno, ácido acético y ácido peracético, en el control de especies de *Colletotrichum* aisladas de frutos de mango. La aplicación de quitosano comercial (1.5 % y 2.0 %) confirmó su efecto antifúngico, con una inhibición promedio del 34 %, no se presentó diferencia significativa con respecto al quitosano grado reactivo. El peróxido de hidrógeno, el ácido acético y el ácido peracético inhibieron por completo el desarrollo de *C. asianum* en concentraciones > 1.0 %. La exposición a los agentes GRAS generó daño morfológico en las esporas como pérdida de turgencia, desorden intracelular, efusión de líquido del citoplasma y pérdida de integridad. La microscopía electrónica de barrido confirmó el daño en el micelio, observadose estructuras colapsadas y deshidratadas. De acuerdo a los resultados, los agentes GRAS comerciales mostraron un efecto de control *in vitro* sobre el crecimiento de *C. asianum* en sus diferentes etapas de su desarrollo.

Palabras clave: *Mangifera indica*, antracnosis, quitosano, peróxido de hidrógeno, ácido acético, ácido peracético.

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1 Introduction

The attacks of phytopathogens cause substantial postharvest losses of mango, a tropical fruit appreciated for its sweet flavor and attractive color (Zakawa *et al*., 2020). In general, the fungus *Colletotrichum* sp. causes anthracnose in mango fruit during the postharvest period. Moreover, the incidence of anthracnose limits the commercialization of the fruit, and its inadequate control causes losses of up to 30 %, Overall, the disease symptoms are dark brown-black spots on the fruit cuticle (Nelson, 2008).

Mexico is one of the main exporters of mango fruit (SIAP, 2019). The export process of the mango requires fruit free of damage and infections by pathogens. Several species of the *Colletotrichum* complex have been reported to infect Mexican fruits, including *C. asianum*, *C. siamense*, *C. gloeosporioides*, *C. acutatum*, *C. tropicale*, and *C. fruticola* (Tovar-Pedraza *et al*., 2019; Ramírez-Benítez *et al*., 2021; Valenzuela-Ortiz *et al*., 2022). The use of postharvest alternatives has reduced the incidence of *Colletotrichum* in mango fruit (Montecalvo *et al*., 2019; Mustari *et al*., 2020). However, the control effect of each postharvest alternative is different for each species of *Colletotrichum*, in this sense its precise identification provides information to understand the effective control measures (Valenzuela-Ortiz *et al*., 2022).

In recent years, to protect human health and reduce environmental pollution, biological-organic control agents have been tested (Karunanayake *et al*., 2020; Shah and Hashmi, 2020; Rayón-Díaz *et al*., 2021; Herrera-González *et al*., 2022). This group includes the chitosan biopolymer, a natural, biodegradable, and non-toxic compound obtain from chitin that is generally recognized as safe (GRAS) by the Food and Drug Administration (Palou *et al*., 2016; Berumen-Guerrero *et al*., 2020). This biopolymer has been shown to have an antifungal effect against pathogens isolated from mango fruit in *in vitro* (Coronado-Partida *et al*., 2017; Gálvez-Marroquín *et al*., 2022; Xing *et al*., 2021). However, most research reports have evaluated the antifungal effect of reagent chitosan (RC). This product has a high degree of purity and a high cost, which makes it inaccessible for large-scale applications. Different manufacturing companies produce commercial-grade chitosan (CC), with more affordable price and available in greater quantities. However, CC needs to be evaluated to confirm its efficacy as an antifungal agent against different pathogens such as *Colletotrichum* to be considered for fruit application.

Hydrogen peroxide (HP), acetic acid (AA), and peracetic acid (PA) are considered GRAS substances and are used as disinfectant agents in fruit processing (Palou, 2018). Several *in vitro* studies have confirmed the antifungal effect of these compounds (Alawlaqi and Alharbi, 2014; Sehirli *et al*., 2020; Gálvez-Marroquín *et al*., 2022). According to the literature review, few information has been reported on the effect of GRAS compounds on the antifungal against *Colletotrichum* sp. of mango fruit. The objective of this work was then to identify the *Colletotrichum* species isolated from "Tommy Atkins" mango fruit, and to evaluate the *in vitro* antifungal effect of various GRAS compounds on the identified *Colletotrichum* species.

2 Materials and methods

2.1 Isolation of pathogen

Colletotrichum sp. was isolated from "Tommy Atkins" mango fruit, obtained from local markets of Nayarit, Mexico and were transferred to the Biotechnology Laboratory of the ITTepic. The fruit was stored at room temperature (25 °C) and high relative humidity until anthracnose symptoms developed. Infected tissue sections were cut (1 × 1 cm) and washed with a solution of 2.0 % sodium hypochlorite (NaOCl) and then immersed in sterile distilled water for 1 min. The tissue sections were placed on potato dextrose agar (PDA) medium plates at room temperature for 5 d. The isolated fungi were purified by constant reseeding until obtaining a strain with a homogenous morphology.

2.2 Pathogen identification

The morphological identification was realized by evaluating the color, shape, and size of the mycelia of the pure isolate fungi. The structural components (spores, hyphae, and appressorium) were visualized with an optical microscope (40× magnification) and identified according to the keys described by Weir *et al*. (2012).

Molecular identification of *Colletotrichum* sp. was carried out in the Laboratory for Comprehensive Phytosanitary Diagnosis (LADIFIT) of the Postgraduate College. The fungal DNA was

extracted from colonies grown for 10 days on PDA medium, using the protocol described by Harwood, (1996) with some modifications. Polymerase chain reaction (PCR) was used to confirm the identification of the isolated fungus. For the amplification of the internal regions ITS the primers ITS1 and ITS4 were used (White *et al*., 1990). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was amplified using GDF1 and GDR1 primers (Guerber *et al*., 2003). The PCR products obtained were sent to MACROGEN Korea for sequencing. DNA sequences were aligned using the Basic Local Alignment Search Tool (NCBI BLAST) from the NCBI online database.

2.3 Pathogenicity test

"Tommy Atkins" mango fruit without visual damage was washed by immersion in 2.0 % NaOCl solution and water. A spore solution (1×10^6 spores/mL) of *Colletotrichum* sp. was used to inoculate two different points on the fruit surface. The fruit was stored by 8 d under controlled conditions at 25 °C and 80 % relative humidity. The development of predominant black spots on the fruit surface was considered an indicator a positive pathogenicity test of anthracnose, fulfilling Koch's postulates.

2.4 Chemical compounds and control agents

The commercial control agents applied were CC (Zhejiang Golden-Shell Pharmaceutical Co. Ltd.) low molecular weight (45,700 MV, g/ml) and high density, HP (Jaloma), AA (Member's Mark, México), PA (Titan plus). Reactive grade compounds used were RC (Sigma-Aldrich) deacetylation ≥75 %, AA (Meyer), azoxystrobin (Bankit gold), Tween 80 (Sigma Aldrich), potato dextrose broth (PDB) (BD Difco), papa dextrose agar (PDA) (Dibico), and sodium hydroxide (NaOH) (J.T. Baker).

2.5 Preparation of control agents

The commercial agents were prepared at 0.1 %, 0.5 %, 1.0 %, and 1.5 % concentrations; for CC, a 2.0 % concentration was also prepared. Azoxystrobin and RC were used as a positive control and prepared according to the supplier instructions. CC solutions were prepared in commercial-grade AA at 5 %, 10 %, and 15 % (v/v) as a dissolving agent. Each CC solution was agitated constantly for 24 h. Subsequently, the pH was adjusted to 5.6 with 1 N NaOH solution, and then 0.1 mL of Tween 80 was added.

2.6 In vitro application of control agents

In Petri dishes, PDA was combined with each control agent at an 80:20 (v/v) ratio. Mycelial disks (7 mm) of 10-day old *Colletotrichum* sp. were inoculated on the Petri dishes and then incubated at 25 °C for 10 d (El Ghaouth *et al*., 1992).

2.7 In vitro antifungal effect of control agents

The radial diameter of *Colletotrichum* sp. was measured every 24 h until the end of the incubation period. The percentage of inhibition was calculated by using the final growth values; the growth of the control was taken as a reference (Gutiérrez-Martínez *et al*., 2017). A spore solution was obtained by adding sterile water to the mycelia of Petri dishes that had been incubated for 10 d. Twenty microliters of the spore solution was placed in a hemocytometer to obtain the final concentration of spores of each treatment (Cortés-Rivera *et al*., 2019). Germination was realized by the method described by Qin *et al*. (2011) with modifications. Aliquots of *Colletotrichum* sp. were placed in Eppendorf tubes and diluted with PDB to obtain a final concentration of 106 spores/mL. The control agents were added in a proportion of 20 % to the volume of PDB. The Eppendorf tubes were incubated at 25 °C with moderate agitation for 9 h until spore germination. The germ tube development was confirmed by using an optical microscope with a magnification of 40× (Motic Images Plus 3.0).

2.8 Spore viability

The viability of the spores was determined in treatments with the best antifungal effect. Spores exposed to control agents were then replated on PDA medium and incubated at 25 °C for 3 d. The absence of mycelium was considered as a fungicidal effect and the development of mycelium was considered as viable spores and without damage (Qin *et al*., 2011).

2.9 Morphological alterations

Tissue fragments (0.5 × 0.5 mm) from the 10-day-old *C. asianum* was placed on the on a scanning electron microscopy (SEM) samples holder. Then the samples were dehydrated at low temperature (4 °C) by 5 days.

Dehydrated samples were coated with gold by using a JFC-1100 Sputter Coater (JEOL, Tokyo, Japan) (20 nm) and observed under a JEOL JSM-6060LV SEM ® (JEOL, Tokyo, Japan) at an acceleration voltage of 20 kV and visualization at 2,500x (Zambrano-Zaragoza *et al*., 2014).

2.10 Statistical analysis

A completely randomized block design was used. The differences between means of three replicates per treatment were analyzed using a Tukey's test ($p \leq 0.05$ considered significant) in SAS system 9.0.

3 Results

3.1 Pathogen identification

The morphology of *Colletotrichum* isolated from "Tommy Atkins" mango fruit is shown in Figure 1. According to the macroscopic characteristics, a colony with radial growth, predominantly white-gray in color and with a dense layer of orange hyphae and acervuli, was observed. The maximum growth of the colony was seen after 10 d of storage at 25 °C in PDA (Figure 1). The *Colletotrichum* spore was cylindrical with granular content and rounded ends (length: 13-17 μm, width: 4-5.5 μm) visualized at 100× magnification. According to the comparison of the sequences of the *Colletotrichum* isolate with the GenBank database of the National Center for Biotechnology Information (NCBI), USA, the isolated strain presented 100 % identity with the fungus *C. asianum* (Table 1).

The pathogenicity test confirmed that the inoculated fungus *Colletotrichum* caused the characteristic symptoms of anthracnose in "Tommy Atkins" mango which were: oval dark brown-black lesions on the surface, visible on the third day of storage, and necrotic tissue observed by the eighth day (Figure 2).

3.2 In vitro effect of control agent agents

3.2.1 Percentage of inhibition of C. asianum

The *in vitro* application of CC, HP, AA, and PA controlled *C. asianum* isolated from mango fruit (Figure 3). A total inhibition of *C. asianum* was observed with the application of HP4, AA3, AA4, and the fungicide azoxystrobin. For chitosan, the application of RC inhibited the development of the pathogen by 36.3 %, whereas the application of CC4 and CC5 reduced the development by 28.6 % and 38.2 %, respectively. According to the results, CC effectively controlled *C. asianum* and showed no significant differences compared with RC.

3.2.2 Sporulation and germination

Table 2 shows the effects of the tested GRAS agents on the control of *C. asianum* sporulation and germination. CC reduced the spore concentration in a dose-dependent manner, with CC4 and CC5 reducing the sporulation of *C. asianum* by 75 % and 85 %, respectively, compared with the control. Samples treated with HP (1.5 %), AA (1.0 % and 1.5 %), and PA (0.5 %, 1.0 %, and 1.5 %) did not show spore production due to inhibition of the mycelia. A similar effect was demonstrated with the application of azoxystrobin as the positive control (total inhibition of the development of the pathogen).

Spore germination was reduced with the application of each GRAS agent at different concentrations. Germination in CC4 and CC5 treatments was 25% lower than in the control treatment. Treatments of HP, AA, and PA at 1.5% completely inhibited germination.

3.2.3 Spore viability

All CC concentrations showed a fungistatic effect. Even with the inhibition of germ tube development after application of CC4 or CC5, mycelia developed when spores were reseeded in PDA medium (Figure 4A).

Table 1. PCR primers for molecular identification of *C. asianum* isolate from "Tommy Atkins" mango fruit.

Gene	Primer	Primer sequences	GenBank accession numbers	Species
ITS	ITS1	TCCGTAGGTGAACCTGCGG	MN272369.1	*C. asianum*
	ITS4	TCCTCCGCTTATTGATATGC		
GPDH	GDF1	GCCGTCAACGACCCCTTCATTGA	MK376935.1	
	GDR1	GGGTGGAGTCGTACTTGAGCATGT		

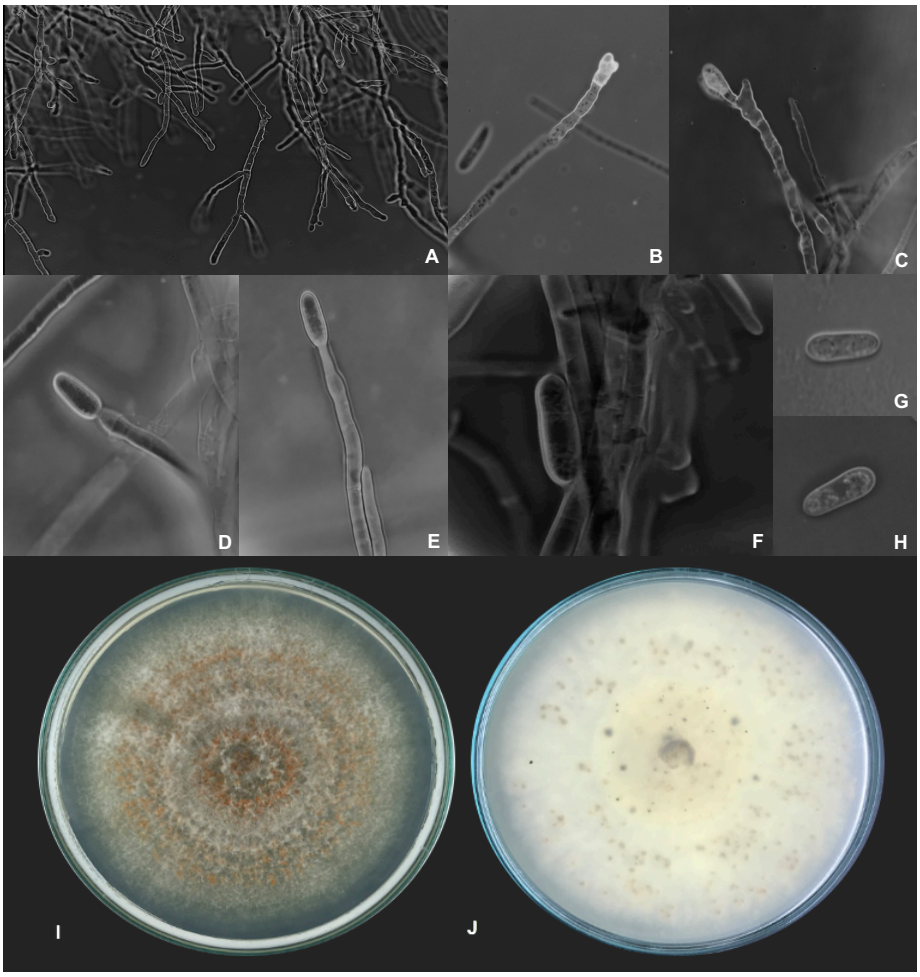

Figure 1. The morphology and cultural characteristic of *C. asianum* isolated from "Tommy Atkins" mango fruit: (A) hyphae; (B and C) appressoria; (D and E) conidiophores; (F and G) spore; (I) the top of a potato dextrose agar plate grown for 10 d at 25 °C, showing the fungal growth; (J) the bottom side of the plate.

Figure 2. Pathogenicity test results of *C. asianum* on "Tommy Atkins" mango fruits after 8 d of storage at 25 °C.

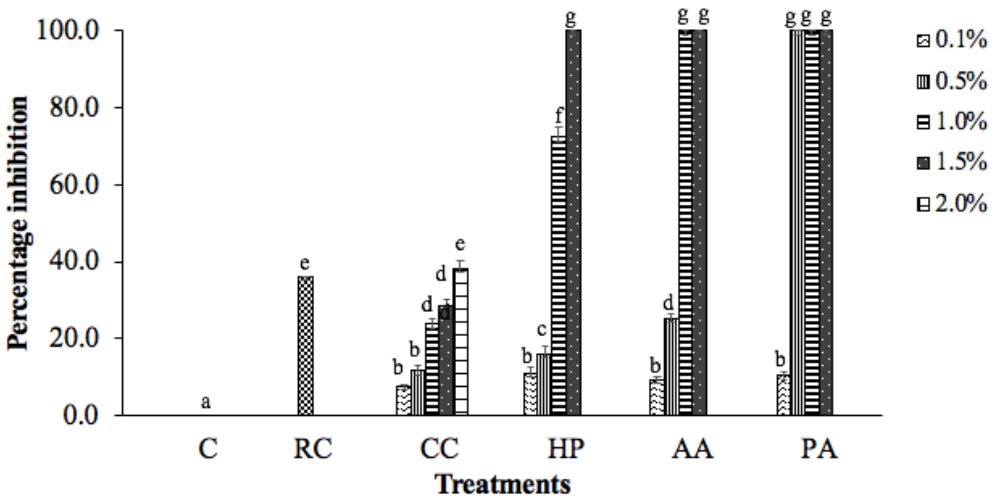

Figure 3. Percentage inhibition of the fungus *C. asianum* with the application of C=control, RC= reactive chitosan, CC = commercial chitosan, HP = hydrogen peroxide, AA = acetic acid and PA = peracetic acid for 10 d at 25 °C on PDA. Different letters indicate a significant difference (Tukey's test, $p \leq 0.05$).

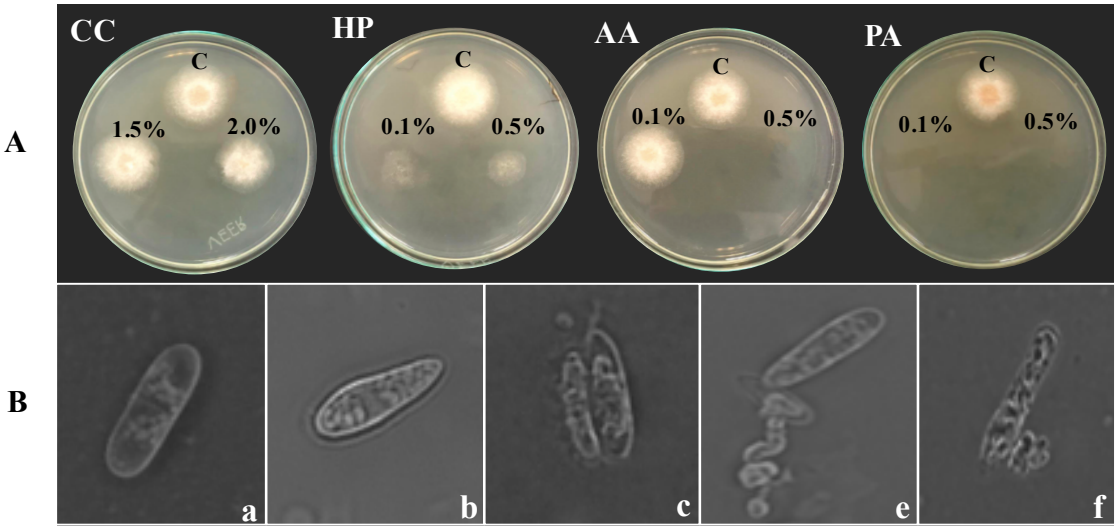

Figure 4. Effect of generally recognized as safe (GRAS) compounds on *C. asianum* spores. A) Spore viability after the application of GRAS compounds, CC = commercial chitosan; HP = hydrogen peroxide; AA = acetic acid; PA = peracetic acid. B) Damage to spore integrity in response to the exposure to the GRAS compounds: (a) integral spores, (b) loss of turgor, (c) intracellular disorder, (e) effusion fluid of cytoplasm, (f) total integrity loss.

HP (0.1 % and 0.5 %) and AA (0.1 %) showed a fungistatic effect. Higher concentrations of these GRAS compounds produced a fungicidal effect, with the absence of mycelial development. Exposure of the spores to any PA concentration did not allow them to reactivate metabolically, no mycelia were observed.

3.2.4 Morphological alterations in spores and mycelia

During the analysis of sporulation and germination, morphological damage was detected in *C. asianum* after direct exposure to the tested GRAS agents.

Table 2. Effect of biological-organic control agents on

the sporulation and germination of *C. asianum*.

Treatments	Sporulation (10^7 spores ml^{-1})	Spore germination (%)
C	6.49e	100i
F	0.0a	0.0a
RGC	1.74b	25.0d
CC1	5.41e	81.0g
CC2	4.03c	59.0f
CC3	3.0c	42.0e
CC4	1.60b	24.0d
CC5	0.92b	15.0c
HP1	3.72c	60.0f
HP2	0.93b	12.0c
HP3	0.48d	8.0b
HP4	0.0a	0.0a
AC1	5.92e	89.0h
AC2	2.60c	39.0e
AC3	0.0a	0.0a
AC4	0.0a	0.0a
PA1	0.35d	8.0b
PA2	0.0a	0.0a
PA3	0.0a	0.0a
PA4	0.0a	0.0a

Values are expressed as the mean (n = 3). Different letters indicate a significant difference (Tukey's test, $p < 0.05$).

This damage included loss of turgidity, intracellular disorder, spillage of liquid from the cytoplasm, and total loss of integrity (Figure 4B). As the GRAS agent concentrations increased, the morphological damage to the spores increased. Exposure to CC4 and CC5 generated spillage and cytoplasmic disorder, and loss of turgidity in the spores, but there was not total damage to the integrity of the spores. The control agents HP, AA, and PA, showed the damage described for CC and loss of total integrity at intermediate concentrations (0.5 % and 1.0 %).

SEM analysis showed the structural damage to *C. asianum* mycelia after exposure to the tested control agents (Figure 5). The control mycelium had hyphae of a homogeneous size and without morphological alterations. There was different morphological damage after the application of CC, HP, or PA. With CC5, amorphous hyphae were observed, Amorphous hyphae and with a biopolymer coating effect were observed. There were dehydrated and collapsed mycelia after treatment with 1.0 % HP or 0.1 % PA. The morphological damage to spores and mycelia could be related to the possible mechanisms of the action exerted by these GRAS compounds to prevent the development of the pathogen.

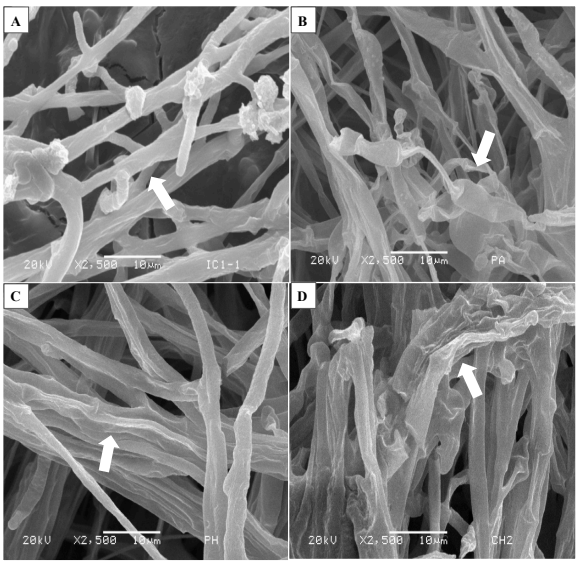

Figure 5. Scanning electron micrographs of *C. asianum* mycelia after 10 d of incubation at 25 °C and with the control agent's application. (A) C= control mycelium, (B) PA= peracetic acid 0.1 %, (C) HP= hydrogen peroxide 1.0 %, (D) CC= commercial chitosan 2.0 %. The images represent 2,500× of magnification. The scale bar is 10 μm.

4 Discussion

The pathogen *C. asianum* has previously been identified in mango fruit. Tovar-Pedraza *et al*. (2020) reported a high incidence of the pathogen in mango fruit from different producing areas of Mexico. According to these authors, in the state of Nayarit, the pathogens *C. asianum* and *C. siamense* are responsible for the development of anthracnose. The present study confirms what these authors reported. The pathogenicity test showed that *C. asianum* promoted the development of anthracnose after a few days of storage on fruit from the same producing area. This is the second report that has identified this pathogen in the producing area of Nayarit. Moreover, *C. asianum* has been reported to have higher virulence compared with other species in this region, including C. alienum, C. fructicola, and *C. tropicale*. Researchers from countries such as Indonesia (Benatar *et al*., 2021), Philippines (Alvarez *et al*., 2020), and Taiwan (Wu *et al*., 2020) have recently reported anthracnose in mango fruit due to the pathogen *C. asianum*.

A potential antifungal control agent should first be evaluated with *in vitro* tests to determine which stages

of pathogen development it affects. The antifungal effect of chitosan has been validated in different *in vitro* studies. Xing *et al*. (2021) applied chitosan at 1.0 % and reported 80 % inhibition of *C. gloeosporioides*. A higher control effect of about 90 % was reported by Gálvez-Marroquín *et al*. (2022) for *Colletotrichum* sp. isolated from "Ataulfo" mango from Chiapas, Mexico. The fungus *Cladosporium oxysporum*, *Penicillium steckii*, and *A. alternata* isolated from mango fruits reduced mycelial growth with the application of 1.0% chitosan (López-Mora *et al*., 2013; Xing *et al*., 2021). In the present study, CC showed a lower percentage of inhibition (compared to previous studies, due to the nature of chitosan, and sensitivity of the pathogen to this biopolymer.

Fungal infection control focuses on modulating sporulation and germination to prevent colonization of the pathogen. CC showed a dose-dependent control effect for both events, a finding consistent with Gutiérrez-Martínez *et al*. (2017), and Coronado-Partida *et al*. (2017). The reduction in the concentration of spores has been related to the ability of the biopolymer to affect the reproductive process of the pathogen. The polycationic nature of chitosan, when interacting with the extracellular components of the pathogen, causes damage at the structural and metabolic level that may be irreversible (Lopez-Moya *et al*., 2019). Inhibition of germination has been related to the decrease in the enzymatic activity of polygalacturonase, an enzyme that is responsible for the rupture of the fungal cell wall to proceed with the formation and development of the germinative tube (Ochoa-Jiménez *et al*., 2015).

Morphological modification of fungal structures has been reported after exposure to chitosan. Exposure of *C. gloeosporioides* to 1.0 % of chitosan caused swelling and deformation of the hyphae (Rayón-Díaz *et al*., 2021). Ramos-Guerrero *et al*. (2018) for the same pathogen observed distorted hyphae, nodule formation, and hyphal diameter reduction. In the present work, the chitosan application showed abnormal, deform, and collapsed hyphae, this effect could prevent the development of the *C. asianum*.

HP, AA, and PA are oxidizing agents. HP is relatively unstable and can easily interact with other molecules. HP has been reported to damage respiration and energy metabolism (ATP), to increase reactive oxygen species (ROS), and to promote autoxidation that inhibits pathogen growth (Qin *et al*., 2011). HP application reduced radial growth, sporulation, and germination of *C. asianum*. A total reduction of mycelium of *Colletotrichum* sp. was observed with the application *in vitro* of HP at 1.0 % (Gálvez-Marroquín *et al*., 2022). Nandi *et al*. (2017) reported an *in vitro* inhibition of > 70 % of the phytopathogens *C. capsica* and *Alternaria alternata* with the application of 3.0 % HP. Similar effects have been reported for the control of phytopathogens such as *Botrytis cinerea* and *Penicillium expansum* with the application of 500 μL L^{-1} and 125 μL L^{-1} HP (Sehirli *et al*., 2020).

AA and PA produced the greatest control of *C. asianum*. Suwapanich *et al*. (2019) confirmed this same effect in *C. gloeosporioides* isolated from mango. In previous studies, application of these acids led to 80-100 % inhibition of the phytopathogens *A. alternata*, *Fusarium solani*, *Rhizoctonia solani*, and *Sclerotium rolfsii* (Abd-AllA *et al*., 2011; Abd-El-Kareem and Abd-El-Latif, 2014; Alawlaqi and Alharbi, 2014). PA is formed by a mixture of HP and AA; the mixture of these oxidants likely increases the antifungal effect of this product. The application PA reduced B. cinerea by 66.6 % (Abd-AllA *et al*., 2011). AA and PA form the hydroxyl radical, one of the most aggressive ROS. This highly unstable ROS attacks molecules and leads to an oxidation chain reaction. At the intracellular level, AA and PA modify the cytoplasmic pH, generating energy waste in the pathogen that leads to cell death. In the present study, the *in vitro* application of A and PA (1.0 % and 1.5 %) generated morphological damage in spores and mycelium, causing the death of the pathogen. This effect is consistent with the study by Kitis, (2004), where *Colletotrichum* sp. isolated from papaya and chili did not develop when in contact with these acids.

Conclusions

C. asianum was identified as the only strain isolated and identified that infect "Tommy Atkins" mango fruits. The individual application of the GRAS agent's hydrogen peroxide (1.5 %), acetic acid (1 and 1.5 %) and peracetic acid (0.5-1.5 %) inhibited the *in vitro* growth of *C. asianum*, causing alterations in the morphology of spores and mycelium. The present results provide relevant information on the *in vitro* antifungal effect of GRAS compounds. These results promote the development of future *in vivo* research to confirm the control effect of these compounds and provide an alternative that could preserve the postharvest quality of mango fruit.

Acknowledgments

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