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Chitosan and GRAS substances application in the control of *Geotrichum candidum* isolated from tomato fruits (*Lycopersicum esculentum L.*) in the state of Nayarit, Mexico: *in vitro* tests

Aplicación de quitosano y sustancias GRAS en el control de *Geotrichum candidum* aislado de frutos de jitomate (*Lycopersicum esculentum L.*) en el estado de Nayarit, México: Pruebas *in vitro*

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Abstract

Geotrichum candidum causal agent of tomato sour rot, has the potential to infect a wide variety of fruits and vegetables. In this work, the *In vitro* antifungal activity of Chitosan (Chi), Potassium Sorbate (PS), Sodium Bicarbonate (SB), and Sodium Carbonate (SC) and their mixtures were evaluated. The PS and SC treatments showed a 100% inhibition of mycelial growth (MG), germination, and sporulation. Regarding SB, 1.5 and 2.0% concentrations inhibited 87.5 and 88.5% of MG and affected germination and sporulation. Chi at 1.0, 1.5, and 2.0% inhibited MG by 63.1, 62.4, and 42.1%, respectively, affecting germination and sporulation. The combination of chitosan at 0.5% + PS 0.5% and Chi 0.5% + SC 0.5% demonstrated 99.3 and 71.4%, inhibition of MG, and affected germination and sporulation. Chi 0.5% + SB 1.5% showed no effect on MG, and at 0.5 and 1.0%, the growth rate of the mycelium increased, as well as sporulation and germination. In conclusion, chitosan and salts are an option for the use of synthetic fungicides in the control of pathogenic fungi in postharvest, with low toxicity, and are friendly to the environment.

Keywords: *Lycopersicum esculentum* L, antimicrobial activity, chitosan, GRAS substances, *Geotrichum candidum*.

Resumen

Geotrichum candidum agente causal de la pudrición ácida del tomate, tiene el potencial de infectar una amplia variedad de frutas y hortalizas. En este trabajo, se evaluó la actividad antifúngica *in vitro* del Quitosano (Q), Sorbato de Potasio (SP), Bicarbonato de Sodio (BS) y Carbonato de Sodio (CS), y sus mezclas. El SP y CS mostraron una inhibición del 100% del crecimiento micelial (CM), germinación y esporulación. Concentraciones de BS al 1.5 y 2.0% inhibió el 87.5 y 88.5 % del CM y efecto sobre la germinación y esporulación. Q al 1.0, 1.5 y 2.0% inhibió el CM en 63.1, 62.4 y 42.1% respectivamente. Así como, efecto sobre la germinación y esporulación. La combinación Q 0.5%+SP 0.5% y Q 0.5% + CS 0.5% mostraron un 99.3 y 71.4%, de inhibición del CM, y efecto sobre la germinación y esporulación. Q 0.5% + BS 1.5% no mostró efecto sobre el CM, y al 0.5 y 1.0%, la tasa de crecimiento del micelio aumentó, así como, la esporulación y germinación. En conclusión, el quitosano y las sales son una opción al uso de los fungicidas sintéticos, en el control de hongos patógenos en postcosecha, de baja toxicidad y amigables con el medio ambiente.

Palabras clave: *Lycopersicum esculentum* L, actividad antimicrobiana, quitosano, sustancias GRAS, *Geotrichum candidum*.

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1 Introduction

Geotrichum candidum was first described by Pritchard and Porte in 1922 and is a yeast-like pathogen that has been known to be responsible for acid rot in tomato fruits for more than 30 years (Moline *et al*., 1984). A characteristic of this particular fungus is that it grows optimally at 25-30°C. However, it can grow from 10 °C at pH 4-12 under a minimum water activity of 0.95 aw (Plaza *et al*., 2003; Talibi *et al*., 2015). Generally, its incidence is observed mainly in the postharvest stage and in fruits that present some mechanical or insect damage (Ruiz-Martínez *et al*., 2012). In addition, it can infect fruits stored under refrigeration, especially those that show cold damage (Oladiran and Iwu, 1993; Kader, 2011). In this sense, *G. candidum* can develop on a wide variety of fruits and vegetables, mainly those that have a high percentage of water. Such is the case of tomato fruits, which have more than 90% water in their composition. This makes them susceptible to attack by a wide variety of pathogenic fungi, including *G. candidum* (Geetha and Indhu, 2020). However, its incidence is striking in places and fruits where it had not previously occurred, such as in China, in strawberries (Ma *et al*., 2018); in Thailand, in longkong fruit (Chantarasiri *et al*., 2021); in Pakistan, on carrots (Hameed *et al*., 2019); and in Korea and the US, in melon, cucumber, potato and tomato (Bourret *et al*., 2013; Kim *et al*., 2011; Duellman *et al*., 2021). Likewise, the presence of *G. candidum* in tomato fruits was reported for the first time in Greece, where the efficacy of two fungicides, fludioxonil, and propiconazole, were evaluated. It was observed that the latter was the one that showed the greatest inhibitory effect on the growth of the pathogen (Thomidis *et al*., 2021). In conclusion, its incidence in crops and latitudes is increasing, probably due to its readjustment to climate change, for which, this fungus can threaten tomato fruits in their postharvest stage. In the particular case of Mexico, the tomato fruit has a high commercial value, placing it in the first place of export worldwide, in addition to generating an economic benefit (SIAP, 2021). According to the above, it is essential to study *G. candidum* since there are few reports of its incidence and effect on Mexican tomatoes. Some *in vivo* studies mention the use of antagonistic yeasts, which did not obtain positive results in controlling the pathogen (Robledo-Leal *et al*., 2016). They have also used chitosan (Chi) coatings with essential oils of oregano and beeswax added, where the results showed promising alternatives in the control of *G. candidum* (Rives-Castillo *et al*., 2018). For this reason, there is an exhaustive search for alternative systems to the use of synthetic fungicides for the postharvest control of tomato fruits (Rodriguez-Guzmán *et al*., 2021). In this work, he proposes the use of chitosan and salts as an alternative method to the use of fungicides, which have previously reported their antifungal capacity. For its part, Chi, a natural compound and biocompatible with other antifungal substances, has shown the potential to control diseases in tomato fruits during storage (Parvin *et al*., 2018; Salas-Méndez *et al*., 2019; Peralta-Ruiz *et al*., 2020). On the other hand, the use of antimicrobial substances such as salts generally recognized as safe (GRAS) have been effective against various pathogens such as *Aspergillus niger*, *Rhizopus stolonifer*, *Colletotrichum gloeosporioides*, *Penicillium digitatum*, *P. italicum*, *Botrytis cinerea*, and *Geotrichum candidum* L., showing desirable characteristics such as availability, easy handling and economic accessibility (Bhalerao *et al*., 2019; Youssef and Hussien, 2020). The objectives of this study were 1) to isolate and identify the fungus *Geotrichum candidum*, 2) to evaluate the antifungal effect of chitosan, potassium sorbate, sodium carbonate and bicarbonate, 3) to determine the interactions of chitosan with salts, and 4) to evaluate the effect of treatments alone or in combination on the morphology of the pathogen.

2 Materials and methods

2.1 Isolation and identification of the fungus

Tomato fruits were obtained in the municipality of Compostela, Nayarit, México. Of the fruits that presented symptoms of an infection, the tissue sections (1 × 1 cm) were 50% of healthy tissue and 50% of infected tissue. They were immersed in a 2% (v/v) sodium hypochlorite (NaClO) solution, rinsed with distilled water, and then placed on sterile filter paper to remove moisture. Tissue sections were placed in Petri dishes containing potato dextrose agar (PDA) medium and incubated at 26 ± 2°C for a period of 24 to 72 hours. For purification, mycelium was taken from the most distal part of the central area of the colony and planted in PDA until pure cultures of a single type of fungus were obtained (Salvador *et*

al., 1999). Subsequently, a pathogenicity test was performed with Koch's postulates to eliminate the possibility of saprophytic fungi from phytopathogens. Healthy fruits were wounded in the equatorial area to artificially infect with 20 μL of spore suspension (1 $\times$ 10^6 spores/mL) using a syringe. Next, they were placed in a humidity chamber at 25 ± 2 °C with a relative humidity of 90-95%. For re-isolation, sections (1 $\times$ 1 cm) of 50% infected and 50% healthy tissue were made from diseased fruits. The tissue samples were disinfected with 2% (NaClO), rinsed with sterile distilled water, placed on PDA plates, and incubated at 28 °C for 48 hours. Control fruits were inoculated with sterile distilled water. The identification at the genus level was based on the microscopic morphology of the fungus, placing mycelia on a slide, adding a drop of methylene blue, and a coverslip. The isolation was observed using a Motic BA300 optical microscope (Scientific Instrument Company, Inc., Hamilton, CA, USA). The genus was determined with the help of dichotomous taxonomic keys. For identification at the species level, Petri dishes of the pathogen under study were sent to the Laboratorio de Diagnóstico Integral Fitosanitario (LADIFIT), Campus Montecillos Postgraduate College, municipality of Texcoco, State of Mexico.

2.2 *Preparation of chitosan, salts, and their combinations*

High-density commercial chitosan solutions (molecular weight = 45.7×10^3 g.Mol), degree of deacetylation 89.72%, Zhejiang Golden-Shell Pharmaceutical Co., Ltd.) were prepared at concentrations of 0.5, 1.0, 1.5 and 2.0% in distilled water (w/v) with 1% acetic acid. The solutions were subjected to constant stirring for 24 hours and were adjusted to a pH of 5.6 with 1N NaOH. Finally, to solution of 100 mL the chitosan 0.1 mL of Tween 80 (El Ghaouth *et al*., 1991) was added.

Solutions of potassium sorbate (PS) $C_6H_7KO_2$, sodium carbonate (SC) Na_2CO_3, and sodium bicarbonate (SB) $NaHCO_3$ (Jalmek Ciencia) were prepared at concentrations of 0.5, 1.0, 1.5, and 2.0% (p/v) with distilled water, followed by constant stirring for 30 min at room temperature.

The interactions were prepared from concentrations of Chi 0.5% + SB 0.5, 1.0 and 1.5%, Chi 0.5% + SC 0.5%, and Chi 0.5% + PS 0.5%. Subsequently, they were poured into Petri dishes with PDA agar.

2.3 *Individual tests: mycelial growth, sporulation, and germination*

To evaluate the effect of treatments on mycelial growth, the mycelia of the pathogen were taken and placed in the center of the Petri dish with a PDA medium with the different treatments and their combinations (including the control). The already inoculated boxes were incubated at a temperature of 26 ± 2 °C. The mycelial diameter was measured every 24 h for seven days with the help of a Vernier. To calculate the percentage of mycelial growth inhibition (%MGI), the following equation was used:

$$\%MGI = \frac{dc - dt}{dc} \times 100\% \quad (1)$$

where dc is the diameter of the control colony, and dt is the diameter of the colony with treatment.

Sporulation was evaluated by preparing a spore suspension from the Petri dishes used for mycelial growth. Subsequently, 10 mL of sterile distilled water was placed and the surface was scraped with a glass loop. Finally, it was filtered and deposited in test tubes. For spore counting, 10 μL were placed on the Neubauer chamber. Per treatment, 100 observations were made through a Motic BA300 optical microscope. The results obtained were expressed as the number of spores/mL.

Spore germination was evaluated as follows. From a spore suspension (1 $\times$ 10^6 spores/mL), aliquots of 100 μL were taken and added to Petri dishes with 10 ml of PDA with the different treatments. Once added to the PDA medium, they were incubated at 26 ± 2°C for 4 h. Subsequently, they were placed under the microscope to observe the geminate spores. Spores were considered germinated when the length of the germ tube was twice its diameter.

2.4 *Evaluation of interactions*

To explain the results obtained, considering the potential interactions of the chemical compounds together with chitosan, molecular modeling based on quantum mechanics was carried out, using the Discovery Biovia visualization software (version 21.1.0.0). The molecules were subjected to energy minimization protocols and the Forcefield algorithm was applied, individually and later in the respective combinations, considering chitosan as a single molecule together with a group of molecules of each of the evaluated compounds. The evaluation of the interactions of the treatments was through the Abbott

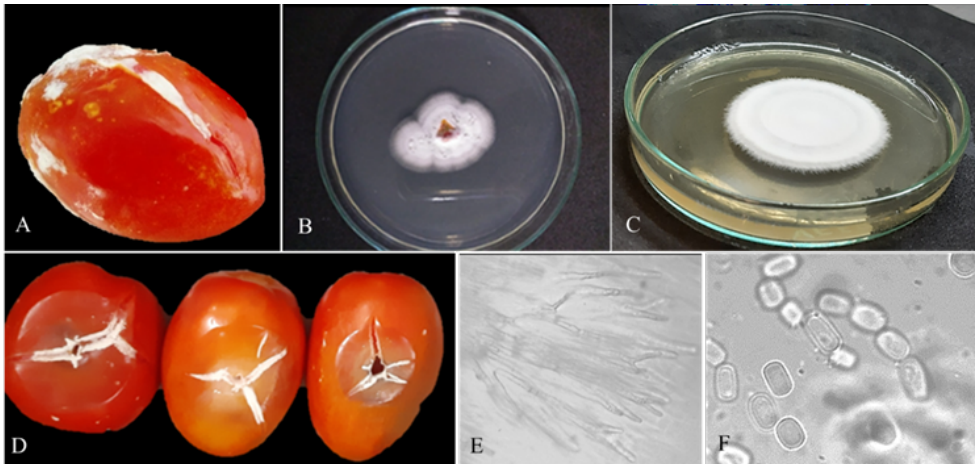


Figure 1.- A) Symptoms of tomato sour rot caused by *Geotrichum candidum*, natural infection. B) Isolation of the pathogen from tissue C) Purification of the fungus and incubation in a petri dish with PDA for 3 days at (26 ±2°C) D) Fruits inoculated with *G. candidum*. E) Dichotomous ramifications of hyaline hyphae. F) Chained arthroconidia derived from hyphae of the isolate at 100x.

method, with some modifications (De Oliveira *et al*., 2017), taking into account the concentration, the individual, and the combined effect of the treatments. The expected mycelial growth inhibition (MGI%exp) was obtained from the following formula: where MGIChi%obs and MGIS%obs are the individual values of MGI% caused by Chi and Salts alone at the mention concentrations.

$$MGI\%exp = (MGIChi\%obs + MGIS\%obs) - (MGIChi\%obs \times MGIS\%obs)/100 \quad (2)$$

while assigning a synergistic effect for IA ≥1.5, an additive effect for IA ≥0.5 to 1.5, and an antagonistic effect for IA ≤0.5.

$$\text{Abbott Index (IA)} = \frac{ICM\%obs}{ICM\%exp} \quad (3)$$

2.5 Scanning electron microscopy

For the Scanning electron microscopy discs of (8 mm in diameter) were cut from Petri dishes with the treatments (pathogen + PDA) and placed in glass vials with 3 mL of 2.5% glutaraldehyde for 20 h at 4 °C. Discs were rinsed with tribasic sodium phosphate for 5 min (three times). The samples (disks) were dehydrated with ethanol at concentrations (30-90% v/v) for 40 min at each concentration and three times at 100% for 20 min. The samples were dried in a glass desiccator (Duran ball cap). Finally, the samples were mounted on slides and covered with gold using a metal ionizer for 4 minutes and were examine using a scanning electron microscope (SEC-CO LTD, SNE-3200 CA, USA) operating at 20 kV.

2.6 Statistical analysis

The analysis was performed using a completely randomized design with five Petri dishes per treatment. Each experiment was repeated three times, using a completely randomized block design. The data were subjected to analysis of variance (ANOVA), and a Tukey test ($p < 0.05$) was used to identify significant differences between the means with the statistical software STATISTICA version 12.

3 Results and discussion

3.1 Isolation and identification of the fungus

In the tomato fruits that were the object of study, the growth and development of an infection of a white color and surfaces soaked in water were observed, in addition, the appearance of softening and sinking of the fruit tissue, characteristic symptoms of the infection caused by the *Geotrichum candidum* fungus (Figure 1A). After the infected tissue from these fruits was placed in a Petri dish with PDA, the growth of soft white mycelia was observed (Figure 1B). From there, a sample of mycelia was taken and placed in a Petri dish with PDA and incubated at 26 ± 2°C. After

three days, it showed a growth of approximately 30 mm of a white, soft, non-shiny mycelium with a sweet smell (Figure 1C). Then, using the pathogenicity test, it was confirmed that the causal agent of the infection was due to some species of *G. candidum*. The results showed that after approximately five days, symptoms of infection were observed on the fruit, presenting acid rot lesions with a white coloration (Figure 1D).

Microscopic identification revealed the presence of hyaline mycelia, and septate, mainly expanded by dichotomous branches (Figure 1E). The arthroconidia (Figure 1F), were chained, hyaline, and presented cylindrical rectangular shapes that were easy to detach. Therefore, the macro and microscopic characteristics mentioned above and according to the information from articles and taxonomic keys placed the fungus in the genus *Geotrichum* (de Hoog and Smith, 2004; Horita and Hatta, 2016). For its identification to the species level (samples sent to the LADIFIT), the pathogen under study were identified with 99% coverage and 100% identity (accession number MT316348_1) as *Geotrichum candidum*.

3.2 Individual tests: mycelial growth, sporulation, and germination of G. candidum

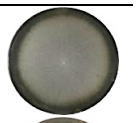
3.2.1 Mycelial growth

Table 1 shows the effect of the substances evaluated individually on the MG of the fungus *G. candidum*. Our results coincide with those reported by some authors such as Türkkan (2019), where salts such as PS and SC at 2.0% inhibited MG by 100%, and SB at 2.0 % inhibited the MG of the fungus *G. candidum* by 90.60%. Likewise, when potassium sorbate has been used in low concentrations from 0.05 to 0.5% on fungi such as *Fusarium oxysporum* f. sp. *radicis-cucumerinum* found 100% inhibition at 96 hours after application (Mirzadeh Abgarmi *et al*., 2021). In the case of (Guimarães *et al*., 2019), he used concentrations from 0.2% to 2.0% and obtained 100% inhibition by increasing the concentrations on *Lasiodiplodia theobromae* on days 3, 5 and 7 after application. On the other hand, Martínez-Blay *et al*., (2020) reported that when applying concentrations 0.2, 1.0 and 2.0% of the salts, they obtained a greater inhibition on *Colletotrichum gloeosporioides* when increasing the concentrations, in addition, the effect was decreasing as the days passed. 3, 5 and 7.

In this sense, in fungi such as *Colletotrichum* sp. potassium sorbate and sodium bicarbonate salts were used in concentrations of 1.0 to 0.04% and 1.0 to 0.2%, respectively. The results showed to be more effective when increasing the concentration of the salts. According to the results, the concentration of the salts has to do with their antifungal activity, however, only for some pathogenic fungi. Therefore, salts have several modes of action. The main antifungal effect attributed to them is the alteration of the pH caused by some compounds present in the salts. However, for the particular case of our study, it has been reported that the MG of *G. candidum* is not affected by the modification of the pH in ranges of 4.0 and 12.0. Therefore, the fungal inhibition obtained in salt-modified media cannot be solely due to a direct effect of pH on pathogen growth. Therefore, it is suggested that both the sodium and potassium cations have a participation in the effect of the differences in toxicity for the strains of the different fungi. In general, the mechanisms can be related to the alteration of the integrity and permeability of the fungal cell membranes, alterations in the transport of nutrients that eventually cause cell inactivation and death, as well as the reduction of cell turgor pressure with collapse and contraction of conidia and/or hyphae (Talibi *et al*., 2015; Gálvez-Marroquín *et al*., 2022). In this sense, the use of salts on the development of fungi such as *P. digitatum*, *B. cinerea*, *A. alternata*, *Monilinia fructicola*, and *G. candidum* (Alaoui *et al*., 2017; Palou, 2018). In addition, when used at high concentrations (4.0%), sodium salts affect MG, inhibiting fungi such as *Fusarium semitectum*, *G. candidum*, *Ulocladium chartarum*, and *Aspergillus niger* in a range of 77.7 to 100% (Tawfik *et al*., 2021).

Regarding Chi, table 1 shows the results obtained on MG. At 0.5%, it only inhibited 12.95%, followed by the 2.0% concentration with 42.12%. However, the concentrations of 1.0 and 1.5% were the ones that showed the highest percentage of inhibition at 63.19 and 62.49%, respectively. In addition, the effect of the treatment on the growth of *Geotrichum candidum* is appreciated. A mycelium with a discoloration and loss of radial growth with respect to the control. Also, it can be seen that increasing the concentration increases the inhibition effect. Which has certain similarities with what was reported by other authors such as (Xing *et al*., 2018) who carried out a study applying chitosan at 0.5%, managing to inhibit the development of the MG of *Ceratocystis fimbriata*, where alterations in the morphology of the *Ceratocystis fimbriata* were also observed the hyphae. On the other hand, (Zivkovic *et al*., 2018) applied medium molecular weight chitosan at concentrations of 0.1, 0.2 and 0.3%, the MG of fungi

Table 1.- Effect of chitosan and salts on mycelial growth of Geotrichum candidum at 7 days.

Treatments	Concentration (%)			
	0.5	1.0	1.5	2.0
Control				
Potassium sorbate				
Sodium bicarbonate				
Sodium Carbonate				
Chitosan				

such as *A. alternata* and *C. gloeosporioides* was limited, showing a greater effect when increasing the concentration. In addition, *A. alternata* presented a higher sensitivity than *C. gloeosporioides*. Also, (Vásquez *et al*., 2021) applied low and medium molecular weight chitosan at 0.1% on *Colletotrichum alatae*, the results show a higher percentage of inhibition with low molecular weight chitosan-based treatments. Similarly (Hua *et al*., 2019) applied chitosan of low and medium molecular weight on *B. cinerea*, their results showed a greater effect on the development of the pathogen with chitosan of low molecular weight. Also, (Karpova *et al*., 2021) applied low molecular weight chitosan at a concentration of 0.2% on *B. cinerea* showing an inhibition percentage of 60%. In the case of (Ramos-Guerrero *et al*., 2020) they used concentrations of 0.1, 0.5, 1.0 and 1.5% of chitosan, on the MG of *C. gloeosporioides* isolated from soursop fruits (*Annona muricata*). Observing a greater inhibition when increasing the concentration on day 12. The main effect attributed to chitosan is its cationic character, where the positively charged amino groups interact with the negative charges of the fungal cell wall, causing alterations in the permeability of the fungal cell wall. plasma membrane, affecting its functionality, such as the entry of vital macromolecules for its development (Chávez-Magdaleno *et al*., 2018; Ke *et al*., 2021). In this sense, it has been reported that the activity of chitosan may depend on its physical-chemical characteristics such as molecular weight, degree of deacetylation, concentration, and source. Likewise, some fungi have chitin in their cell wall, which makes them less susceptible. Finally, it is known that fungi have a variety of evasion or defense mechanisms against conditions that are not favorable for their development (Varlamov *et al*., 2020).

3.2.2 Sporulation

In the case of sodium and potassium salts, they showed 100% inhibition of the sporulation of the fungus *Geotrichum candidum*. Concentrations of only 0.5 and 1.0% SB showed 59.1 and 56.8% inhibition, respectively (Table 2). Studies have reported the use of salts such as sodium carbonate and bicarbonate in the control of *Fusarium oxysporum* f. sp cepae (Türkkan and Erper, 2014), as well as fungi such as *Monilinia fructicola*, which in concentrations of 2.0% affect the development of the fungus (Palou, 2018). Research shows that salts such as sodium chloride and potassium chloride at high concentrations inhibit sporulation. Consequently, sodium and potassium ions can decrease the osmotic pressure of the cells, inhibiting sporulation (Gutiérrez Carranza *et al*., 2018). The effect on the sporulation of *G. candidum* shows that all chitosan treatments showed significant differences compared to the control.

Table 2.- Effect of chitosan and salts on the germination and sporulation of *Geotrichum candidum*.

Treatments	Concentration (%)	pH	% Inhibition	
			Germination	Sporulation
Control	-	6.2	0.0F*	0.0F*
Potassium sorbate	0.5	8. 1	100±0A	100±0A
	1.0	8.3	100±0A	100±0A
	1.5	8.4	100±0A	100±0A
	2.0	8.5	100±0A	100±A
Sodium bicarbonate	0.5	8.9	77.86±1.85E	59.1±2.28B
	1.0	9.2	94.53±0.72D	56.82±1.86B
	1.5	9.8	98.98±0.27B	99.78±0.16A
	2.0	10.0	98.56±0.28BC	99.44±0.06A
Sodium carbonate	0.5	11.3	97.63±0.31C	99.370.09±A
	1.0	12.0	100±0A	100±0A
	1.5	12.1	100±0A	100±0A
	2.0	12.5	100±0A	100±0A
Chitosan	0.5	5.6	100±A	22.73±3.40D
	1.0		100±0A	27.28±3.36D
	1.5		100±0A	47.73±4.56C
	2.0		100±0A	43.19±2.28C

*Similar letters in columns are not significantly different according to Tukey's HSD ($P \leq 0.05$).

Of the chitosan treatments, 1.5 and 2.0% showed more effect on the sporulation of *Geotrichum candidum*, inhibiting 47.73 and 43.19%, respectively. There are reports that chitosan at a concentration of 0.125% inhibited the development of the fungus *A. alternata*. The results showed mycelia with distortions and dehydrated zones. In addition, spores with distorted shapes (Guo *et al*., 2020). Consequently, although the chitosan concentration was higher, the inhibition percentage was not higher on mycelial growth, suggesting that other aspects also influence its antifungal activity. In addition, the particular characteristics of the fungus under study. These results can be explained in two ways: one, the fungus as a defense mechanism can metabolize substances to use them as secondary nutrients; and the second, in a toxic environment, the fungus, as a survival mechanism, could be increasing the reproduction of spores. (Martinez-Moreno *et al*., 2021).

3.2.3 Germination

The results obtained from the salts all showed significant differences from the control. However, they also presented differences between them, such as for PS; all its concentrations inhibited germination by 100%. In addition, SC at 1.0, 1.5, and 2.0% concentrations inhibited germination 100% and inhibited germination 97.6% with the concentration of 0.5%. SB at 0.5, 1.0, 1.5, and 2.0% showed inhibitions from 77.86, 94.53, 98.98, and 98.56%, respectively. Similar results were obtained by (Türkkan *et al*., 2017), where sodium carbonate at 10 mM and sodium bicarbonate at 25 mM inhibited the germination of *B. cinerea* by 98.7 and 78%, respectively. For their part, (Lai *et al*., 2015) reported that applying 0.6% sodium bicarbonate managed to inhibit more than 80% of the germination of *Penicillium expansum* spores after 10 hours of incubation. Also, sodium carbonate at concentrations of 2.0% inhibits 100% of the germination of fungi such as *Fusarium oxysporum* f. sp. *melongenae* (Yildirim *et al*., 2022). Similarly, there are reports of the use of salts in the control of phytopathogenic fungi such as M. fructicola, *B. cinerea*, *G. candidum*, *A. alternata*, *Penicillium expansum*, *Mucor piriformis*, and *R. stolonifera* (Palou *et al*., 2009; Abbas *et al*., 2019). According to the above, it is suggested that some chemical substances, such as salts, can be diffused in the cytoplasm, where they bind to specific receptors that affect membrane permeability and enzymatic synthesis, affecting the metabolic processes of the fungus (Leger *et al*., 1991: Venditti *et al*., 2018).

Table 3. Effect of the combined treatments on the development of *G. candidum*.

Treatments	Effect	% Inhibition		
		Mycelial growth	Germination	Sporulation
CONTROL	-	20.74±3.27C*	0±0E*	26.33±1.78BC*
PS 0.5% + Chi 0.5 %	Additive	99.31±1.05A	100±0A	100±0A
SC 0.5% + Chi 0.5 %	Additive	71.42±1.29B	89.89±0.76B	35.6±4.12BC
SB 0.5% + Chi 0.5 %	Antagonist	0±0D	5.18±0.61D	0±0D
SB 1.0% + Chi 0.5 %	Antagonist	5.44±3.07D	7.72±1.01D	6.6±2.06D
SB 1.5% + Chi 0.5 %	Antagonist	17.34±2.49C	30.08±1.21C	23.7±3.57C

Note: Chitosan (Chi), Potassium sorbate (SP), Sodium carbonate (SC) and Sodium bicarbonate (SB).
*Similar letters in columns are not significantly different according to Tukey's HSD ($P \leq 0.05$).

In the case of the chitosan-based treatment, the results shown in table 2. They present similarities with what was reported by Gutiérrez Martínez *et al*. (2017), where chitosan in concentrations of 1.0 and 1.5% (w/v) showed greater inhibition in the germination of *Colletotrichum* sp. Also, with another study where chitosan was applied at a concentration of 0.5%, a 100% inhibition was observed in the germination of fungal spores such as *Alternaria kikuchiana* and *Physalospora piricola* (Meng *et al*., 2010). Similarly, chitosan at concentrations of 1.0, 1.5 and 2.0% inhibited 100% germination of *Colletotrichum* sp. (Berumen-Valera *et al*., 2015). In addition, when chitosan is applied at concentrations of 0.025 and 0.05% on fungi such as *Phytophthora infestans*, an effect was observed in the reduction of their growth (Zheng *et al*., 2021). One possible cause of this behavior is thought to be that chitosan may neutralize the electrostatic charge on the surface or remove the mucosal layer of the spore, altering the process of substrate recognition and signal transduction for germ tube activation (Santos Montero *et al*., 2017; Castro Marín *et al*., 2021).

3.3 Evaluation of interactions

Table 3 shows the effect of the interactions on the development of *G. candidum*. The interaction Chi 0.5% + PS 0.5% had significant differences between the control and the treatments, inhibiting mycelial growth by 99.31% as well as sporulation and germination by 100%. Therefore, according to the Abbott index, its effect was additive. (Coronado-Partida *et al*., 2021) reported that applying PS at 1.0% inhibited the mycelial growth of *Rhizopus stolonifer* by 70%, and 100% inhibition at 1.5% concentration. However, with a low concentration of chitosan at 0.1% + SP 1.0% combined, they completely inhibited mycelial growth, showing synergism. In addition, when chitosan was applied at low concentrations (0.05%) and PS (1.0%) was added, it showed 99.5% inhibition of the mycelial growth of fungi such as *Penicillium citrinum* (Gonzáles-Estrada *et al*., 2020). A possible explanation of the chitosan interaction with potassium sorbate. Figure 2A shows a structural arrangement of the sorbate, leaving potassium free, which could be available to interact with the components of the medium and generate some disruption of the mechanisms of microorganism growth. There is no interaction of sorbate with the amino groups of chitosan, which does not significantly affect the antifungal activity of chitosan. In this sense (Coma *et al*., 2002; No *et al*., 2007) suggest that the antibacterial action of chitosan could be limited by interactions with SP when there is no availability of groups (NH_3^+) of chitosan in position to interact with the fungal cell membrane.

The Chi 0.5% + SC 0.5% combination showed significant differences from the control and BS combinations, reducing mycelial growth by 71.42%, sporulation by 35.6%, and germination by 89.89%. For this combination, the Abbott effect index was also additive (Table 3). In Figure 2B, the structure of chitosan and its possible interaction with sodium carbonate molecules are shown, in which the H^+ donor zone is light gray, and the H^+ acceptor zone is dark gray. In the same figure, once the chitosan molecule with the carbonate molecules is present in an aqueous medium, the sodium in the carbonate undergoes dissociation, leaving the sodium free, which could be available to interact with other components such as the wall of the hyphae or some other components present in the medium. In addition, it can be seen that in the structure carbon-carbon interactions typical of the torsion that is generated in the chitosan molecule predominate but without generating a steric hindrance

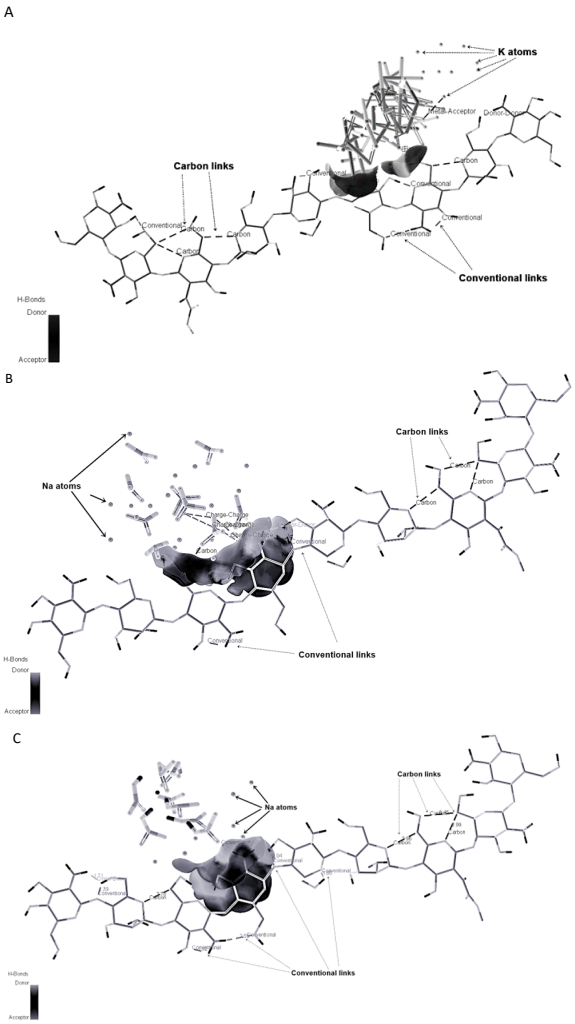


Figure 2. Possible structural interaction of chitosan with A) Potassium sorbate, B) Sodium carbonate, and C) Sodium bicarbonate. Source: self-made.

that does not allow the interaction of the amino groups of chitosan, which are known to be responsible for the antifungal activity.

Regarding the combinations of Chi 0.5% + SB 0.5, 1.0, and 1.5%, no significant differences were observed to the control. According to the Abbott index, the effect was antagonistic (Table 3), stimulating the growth of the pathogen. Figure 2C shows the interaction between chitosan and sodium bicarbonate, where it can also be seen that the bicarbonate molecules come to have a dissociation leaving the sodium molecules free. However, it is understood that interactions can occur between the H^+ of the amino group with the ^-OH present in the bicarbonate, due to the electrostatic charge of said molecules. This could explain why the treatments in which bicarbonate was used in combination with chitosan stimulated the growth of the fungus *G. candidum*. In summary, it is necessary to carry out studies on how the components of chitosan interact and their effect on the integrity of the pathogen, reviewing aspects such as the degree of deacetylation of chitosan, the concentration of the preservative, the pH of the medium, and the fungus under study (Younes *et al*., 2014).

3.4 Scanning electron microscopy

For the micrographs, the most effective treatments were selected. The control sample (Figure 3A) presented mycelia with normal growth, a firm structure, without deformations, uniform, and a turgid appearance. The 0.5% PS treatment showed complete inhibition of the pathogen (Figure 3B). Regarding SC 0.5%, reduced, abnormal, deformed, and collapsed mycelia were observed (Figure 3C). In the case of 1.5% sodium bicarbonate (Figure 3D), it shows abnormal mycelia, distorted, disorganized, and with white pigmentation with a dehydrated appearance. (Rayón-Díaz *et al*., 2021) reported that salts, such as 0.5% sodium silicate, affect the hyphae of fungi such as *C. gloeosporioides*, altering their permeability due to damage to the cell membrane caused by dehydration.

In the treatments of Chi 1.5% (Figure 3E), Chi 0.5% + SC 0.5% (Figure 3F), Chi 0.5% + PS 0.5% (Figure 3G), collapsed, disorganized mycelia were observed, thin and with distortions. Previous studies (Bautista-Baños *et al*., 2012) reported that chitosan-based treatments on *Fusarium oxysporum* f. sp. gladioli presented a mycelium with an intense and distorted dehydrated appearance. In addition, when beeswax and lime essential oil were combined, distorted mycelia were observed on *R. stolonifer*, as well as the absence of sporangia. In the case of fungi such as *B. cinerea*, damaged and thin hyphae were observed, with a granular and corrugated surface (Silva Júnior *et al*., 2014). In addition, (Rodriguez Pedroso *et al*., 2016) reported that in the case of Bipolaris oryzae, after applying chitosan at 3000mg/L, distorted hyphae were observed in the periphery of the mycelia. For their part, (Ramos-Guerrero *et al*., 2018) reported that chitosan at 1.0% alone or in combination with methyl jasmonate/salicylic acid showed a reduction in the diameter of the hyphae, with a distorted appearance, formation of nodules, and thinning of the hyphae in the cell wall of the fungus

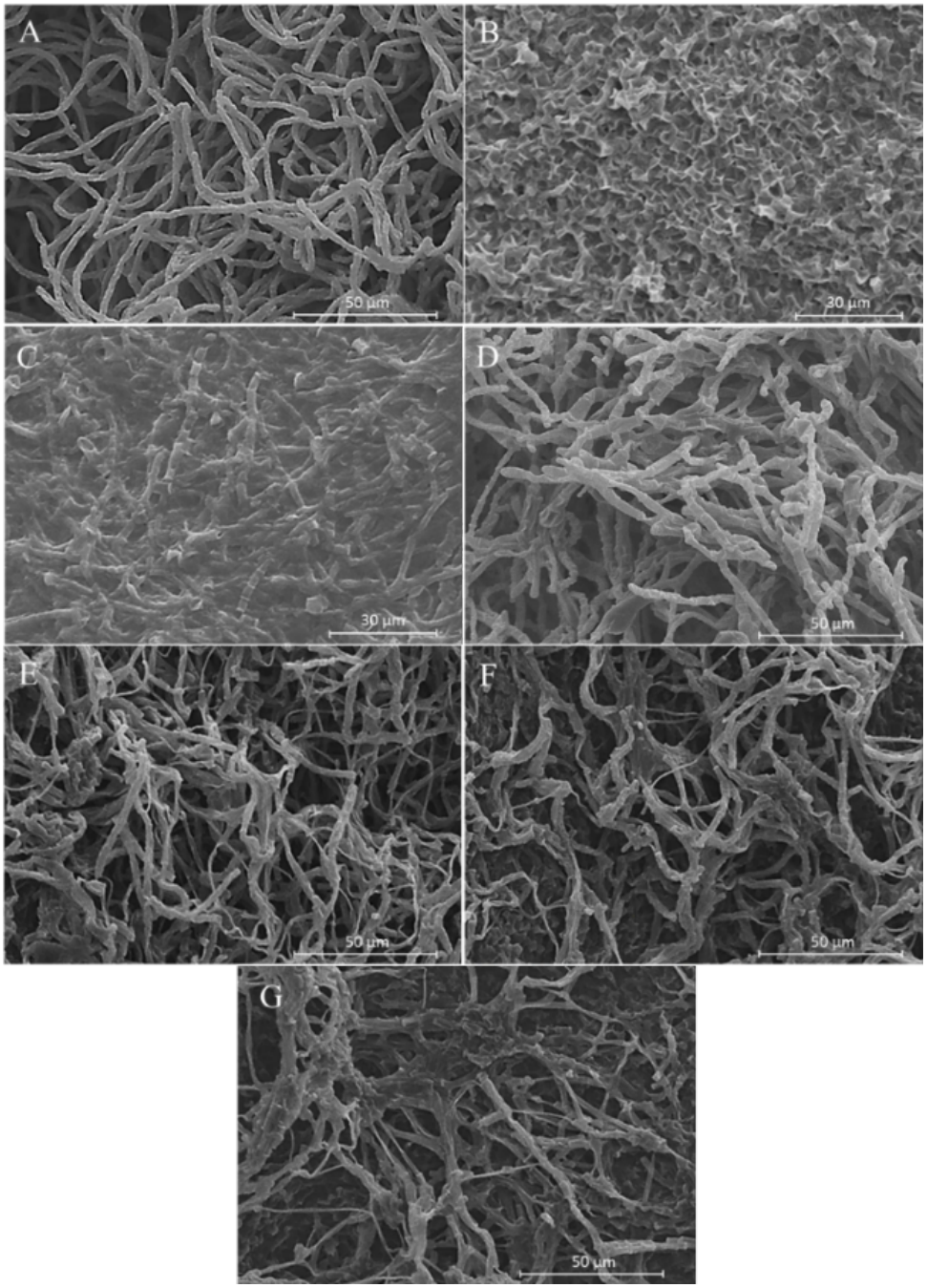


Figure. 3.- Micrographs of *Geotrichum candidum* isolated from the different applied treatments: Control, Potassium Sorbate (SP), Sodium Carbonate (CS), Sodium Bicarbonate (BS) and Chitosan (Chi). A) Control, B) PS 0.5%, C) SC 0.5%, D) SB 1.5%, E) Chi 1.5%, F) Chi 0.5% + SC 0.5%, G) Chi 0.5% + PS 0.5%.

C. gloeosporioides. Likewise, in R. stolonifer, when chitosan was applied at 0.5% alone or in combination with methyl jasmonate/salicylic acid, the mycelia collapsed with a dehydrated appearance and distortions. Also, recent studies have reported the effect of chitosan at 1.0% on the integrity of fungi such as *Colletotrichum siamense*, showing disorganized and collapsed mycelium (Herrera-González *et al*., 2022). In summary, the application of chitosan causes morphological changes on the

surface of the hyphae in a wide variety of fungi that occur in postharvest stages, such as *Alternaria alternata*, *Colletotrichum gloeosporioides*, *Penicillium expansum*, *R. stolonifer*, and *B. cinerea*. Thanks to scanning electron microscopy (SEM) tests, it was possible to observe these changes, since depending on the concentration of chitosan, they can be from an excess in the branching of the hyphae to greater vacuolation. In addition, they show damaged, amorphous hyphae, with distortions on the surface, corrugation, swelling, and the total exit of the components of the cytoplasm in addition to presenting aggregations, and size reduction, and a dehydrated appearance (Bautista-Baños *et al*., 2016).

Conclusions

In conclusion, chitosan and GRAS substances had an antifungal effect on the growth of *Geotrichum candidum*, in addition, in their mixtures of Chi+PS and Chi+SC, an additive effect. Therefore, their combination can be an interesting alternative in the control of pathogens as it has various modes of action. Likewise, it meets the expectations of consumers by not presenting damage to the environment and human health. However, it is reasonable that more studies be carried out at the *in vitro* and *in vivo* level.

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