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Additive effect of alternative treatment to chemical control of *Botrytis cinerea* in blueberries

Efecto aditivo de tratamiento alternativo al control químico de *Botrytis cinerea* en arándanos

S. Ramos-Bell[1], L.G. Hernández-Montiel[2], R.M. Velázquez-Estrada[1], J.A. Sánchez-Burgos[1], P.U. Bautista-Rosales[3], P. Gutiérrez-Martínez[1]*

[1]*Laboratorio Integral de Investigación en Alimentos, Tecnológico Nacional de México/Instituto Tecnológico de Tepic, Avenida Tecnológico # 2595, Col. Lagos del Country, 63175 Tepic, Nayarit, México.*
[2]*Centro de Investigaciones Biológicas del Noroeste, S.C., La Paz, Baja California Sur, 11 23096, México.*
[3]*Centro de Tecnología de Alimentos, Universidad Autónoma de Nayarit. Ciudad de la Cultura "Amado Nervo", Tepic, Nayarit, México.*

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Abstract

The blueberry crop is mainly attacked by the phytopathogen *Botrytis cinerea*, being very aggressive for this plant, showing resistance to synthetic fungicides. Non-chemical alternatives such as chitosan and salicylic acid have been proposed for its control. Therefore, the objective of this study was to evaluate the combination of these compounds for the control of *Botrytis cinerea*. The *in vitro* evaluation showed percentages of fungal growth inhibition of 60% and 100% inhibition of sporulation and germination of the phytopathogen. The micrographs showed damage to fungal structures of *B. cinerea* under the application of the treatments due to the additive effect demonstrated by combining chitosan and salicylic acid. Likewise, there was a great reactivity when combining these compounds, favoring the attack on the phytopathogen and increasing the *in vivo* effect by reducing the decomposition rate of blueberries by 60% compared to the control.
Keywords: *B. cinerea*, blueberry, chitosan, salicylic acid.

Resumen

El cultivo de arándano es atacado principalmente por el fitopatógeno *Botrytis cinerea*, siendo muy agresivo para esta planta y ha demostrado resistencia a fungicidas sintéticos. Para su control se han propuesto alternativas no químicas como el quitosano y ácido salicílico. Por ello el objetivo de este estudio fue evaluar la combinación de estos compuestos para el control de *Botrytis cinerea*. La evaluación *in vitro* mostró porcentajes de inhibición de crecimiento fúngico de 60 % y un 100% de inhibición de esporulación y germinación del fitopatógeno. Las micrografías mostraron daños en estructuras fúngicas de *B. cinerea* bajo la aplicación de los tratamientos, debido al efecto aditivo demostrado al combinar quitosano y ácido salicílico. Así mismo, hubo una gran reactividad al combinar estos compuestos favoreciendo el ataque al fitopatógeno y aumentando el efecto *in vivo* al disminuir la tasa de descomposición de los arándanos a un 60% con respecto al control.
Palabras clave: *B. cinerea*, arándano, quitosano, ácido salicílico.

* *Corresponding author. E-mail*: pgutierrez@ittepic.edu.mx
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1 Introduction

The production and marketing of blueberries are increasing, with a worldwide production in 2020 of 1 million 261 tons. Mexico is the 6th largest producer with 50 293 tons (FAO, 2020). This crop has gained a lot of attention due to its physicochemical characteristics that make it a fruit of high biological value, it is a rich source of powerful antioxidants such as flavonoids, anthocyanins, chlorogenic acid, and procyanidins (Liu *et al*. 2019).

However, the quality and its physicochemical characteristics are compromised when it is attacked by different phytopathogens. It has been reported that the main fungal species that cause the rotting of these fruits during the post-harvest stage is *Botrytis cinerea* (Ramos Bell *et al*. 2021). This phytopathogen is very aggressive because it can attack from the flowering stage of the fruit, remaining in a latent or quiescent state, expressing its symptoms during the post-harvest stage of the fruit. Synthetic fungicides such as Switch® 62.5 WG (Ciprodinil + Fludioxonil), Carbendazim® 50, Pyrus® 400 SC, and Quadris® are usually used for its control (Sautua *et al*., 2019; Milinči *et al*. 2020). These fungicides have been reported to have harmful effects on health, causing diseases such as diabetes, neurological deficits, and respiratory diseases (Narenderan *et al*., 2020).

For this reason, efficient alternatives to control phytopathogens, safe for the consumer and the natural environment are been sought. Chitosan has been studied as a biodegradable and non-toxic compound for the control of different phytopathogens including *Botrytis cinerea* with high percentages of inhibition of these fungi (Bautista-Baños *et al*., 2017).

Chitosan has been evaluated to control a variety of pathogens in different fruits such as soursop, jackfruit, and avocado (Ramos-Guerrero *et al*. 2020; Coronado-Partida *et al*. 2021). This compound has been able to induce the activity of enzymes such as phenylalanine ammonium lyase in avocado and preserve its physicochemical characteristics (Herrera-González *et al*. 2022).

Likewise, it has been described that chitosan can induce the biosynthesis of antioxidant phenolic compounds of interest for the prevention of degenerative diseases (Berumen-Guerrero *et al*. 2020).

On the other hand, the use of salicylic acid as a naturally occurring phenolic compound, present in many plants has been studied with good results for fungal control on fruits and vegetables (Mekawi, Khafagi, and Abdel-Rahman, 2019). Grapefruits with a lower percentage of disease symptoms caused by *B. cinerea* were observed, showing an increase in enzymatic activity and phenolic compounds (García-Pastor *et al*. 2020). Similarly, some studies reveal the possible effect that the combination of chitosan and salicylic acid could cause to achieve the inhibition of some fungal diseases (Shi *et al*., 2018; Nasannova *et al*., 2020).

In order to obtain a greater antifungal effect on blueberry fruits, the main objective of this research was to evaluate the effect of the combination of chitosan and salicylic acid as an alternative treatment to the chemical control of *Botrytis cinerea*.

2 Materials and methods

2.1 Materials and fruits

Commercial chitosan (47.5 kDa, 90% deacetylation, Golden-Shell Co., China) and salicylic acid (Sigma Aldrich, USA) were used. Blueberry fruits were obtained from an orchard in the state of Nayarit, Mexico at physiological maturity and selected for the study without any apparent physical damage or decomposition by pathogens.

2.2 Pathogen. Isolation and identification

Botrytis cinerea was isolated from blueberry fruits with damage by phytopathogens. Next, we performed cuts 1 x 1 cm of damaged tissue with a sterile scalpel, disinfected in a 2% sodium hypochlorite solution, and then in sterile distilled water for 2 min. Finally, the tissue (free of humidity) was placed in Petri dishes with potato dextrose agar (PDA) (Difco, France) and incubated at 25 °C for a period between 24 to 72 h. Single phytopathogens were isolated and pure cultures were obtained (Ramos-Guerrero *et al*., 2018). The species of each isolation was identified by amplification of the internal transcribed region ITS-5.8S of rDNA using ITS 1 (5'-TCCGTAGGTGAACCCTGCGG-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') primers. The PCR products were cleaned using ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) and sequenced directly on a Genetic Analyzer 3130 Sequencer (Applied Biosystems Thermo Fisher Scientific) at the facilities of the Colegio de Postgraduados, Mexico.

The sequences were deposited in the NCBI GenBank database. For its reactivation, it was sown in Potato Dextrose Agar (PDA) and incubated at 25 °C for 7 days.

2.3 Preparation of chitosan and salicylic acid

Chitosan at 0.5, 1.0, and 1.5% (w/v) was prepared in sterile distilled water with the addition of acetic acid (1%). The solutions were constantly stirred for 24 h and their pH was adjusted to 5.6 with 1N NaOH. The concentrations of 0.03, 0.04, and 0.07% (w/v) of salicylic acid, dilutions were made starting from a stock solution of 50 mM in distilled water and glycerin (5%), adjusted to pH 5.5 using a 10% (w/v) KOH solution (Ramos-Guerrero *et al*., 2018).

2.4 Evaluation of mycelial growth

The chitosan concentrations (0.5, 1.0, and 1.5% (w/v)) were previously mixed by mechanical agitation with salicylic acid and then poured into the Potato Dextrose Agar (PDA) in 9 cm diameter Petri dishes. Discs (0.7 cm diameter) of *Botrytis cinerea* mycelium with 5 days of growth were placed and incubated at 25 ºC for 8 days (Jiang *et al*., 2016). As a positive control, plates were incubated with PDA plus chlorothalonil fungicide (Bravonil® 720 SC) at 0.3% (w/v). After 8 days of storage, mycelial diameter measurements were made using the ImageJ® software. Five repetitions per treatment were performed and the experiment was performed in duplicate. The mycelial growth inhibition percentage (%MGI) was calculated according to Equation 1.

$$\%MGI = \frac{(\text{Control colony diameter} - \text{Treatment colony diameter})}{(\text{Control colony diameter})} \times 100 \quad (1)$$

2.5 Sporulation

Spore solutions were prepared from Petri dishes with the chitosan and salicylic acid treatments from 8 days of growth. In this context, 10 mL of sterile distilled water was added and then the mycelium was spread and filtered through sterile gauzes. Spore counting was performed with the Neubauer camera using an optical microscope (Motic BA 300, Canada) with the 10X and 40X objective lenses (Saito, Obenland, and Xiao, 2020).

2.6 Germination percentage

We evaluated the percentage of germination according to the methodology described by Jiang *et al*. (2016) with some modifications. In this regard, 20 μL of the spore suspension obtained from the chitosan and salicylic acid treatments were taken and added to the A disk (20 mm in diameter). Subsequently, the discs were inoculated at 25 ºC for 7 h, spore germination was counted considering 100 spores per treatment using an optical microscope (Motic BA300, Canada). The spore was considered germinated when the length of the germ tube reached twice its diameter. Three repetitions per treatment were carried out and the germination percentage was calculated according to Equation 2.

$$\%Germination = \frac{(\text{Number of germinated spores })}{(\text{Total number of spores})} \times 100 \quad (2)$$

2.7 Scanning electron microscopy

To observe the possible alterations at the morphological level caused by chitosan and salicylic acid to the phytopathogen, the Scanning Electron Microscope was used following the methodology described by Ramos-Guerrero *et al*. (2018) with modifications. Samples of 8 mm diameter were taken from the different treatments and placed in a vial in contact with a 3% glutaraldehyde solution for 72 h at 4 ºC. After, washings were carried out with ethanol in concentrations from 30-100% v/v for 50 min and for the 100% concentration three times for 20 min. Subsequently, the samples were dried at 30 ºC for 12 h and placed in sample holders with a double-sided carbon tape and coated with gold. For this analysis of the samples, a scanning electron microscope (MINI-203 SEM SNE-3200M, South Korea) was used with an acceleration voltage of 20 kV, a voltage of -5 kV, and a distance of -20 mm.

2.8 *Chitosan-salicylic acid combination effect*

To determine what type of effect the combination of chitosan and salicylic acid would have on the growth of *B. cinerea*, the Abbott index (Equation 3) was estimated according to the methodology described by (Peralta-Ruiz *et al*. 2020), where: %MIobs corresponds to the percentage of mycelial inhibition observed for the combination of chitosan and salicylic acid. The %MIexp is calculated using equation 4, where %MICHobs and %MISAobs correspond to the percentage of mycelial inhibition of chitosan and salicylic acid individually determined using equation 1. According to the result of the Abbott index, a synergistic effect was assigned if AI ≥ 1.5, an additive effect if AI ≥ 0.5-1-5 and an antagonistic effect if AI ≤ 0.5.

$$\text{Abbott Index (AI)} = \frac{(\%MIobs)}{(\%MIexp)} \tag{3}$$

$$\%MIexp = (\%MICHobs - \%MISAobs) - (\%MICHobs * \%MISAobs)/100 \tag{4}$$

2.9 *Chemical interaction between chitosan and salicylic acid*

To visualize the potential interactions between chitosan and salicylic acid, the molecules were obtained from the PubChem platform, being selected chitosan (CID 71853) (NCBI, 2022a) and salicylic acid (CID 338) (NCBI, 2022b). Afterward, a minimization of the respective energies was carried out, using the Hartree-fock algorithm of the Discovery Biovia software (Studio Visualizer v17.2.0.16349, San Diego). The visualization of the hydrogen acceptor and donor zones was carried out following the protocols established for activation by the software, generating also the interactions using the minimization protocol for the molecules individually.

2.10 *Blueberry decomposition rate*

Fruits without any apparent damage were washed and disinfected with 2% sodium hypochlorite and distilled water for 2 min, according to the methodology described by Jiang *et al*. (2016). The fruits were immersed in four different treatments (distilled water as control, chitosan 1.5%, salicylic acid 0.07%, and the combination of both compounds). The fruits were immersed for 2 min in the different treatments and allowed to dry at room temperature for one hour. Then the fruits were wounded once with a 0.8 mm punch and inoculated with 5 μL of *B. cinerea* spore solution adjusted to 10^5 spores/mL. Blueberries were stored for 10 days with relative humidity conditions of 90-95% and a temperature of 25°C. To estimate the percentage of incidence of the disease 30 fruits were used per treatment and photographic evidence of the evolution of the infection in the fruits was taken daily until the end of 10 days, any fruit with a visible sign of the fungus was considered damaged. The decomposition rate was calculated according to equation 5.

$$\%Decay\ rate = \frac{(No.\ infected\ fruits)}{(No.\ totals\ fruits)} \times 100 \tag{5}$$

2.11 *Statistical analysis*

A complete factorial design was carried out, considering the different treatments as independent variables. The results were statistically analyzed by analysis of variance (ANOVA) and the LSD Fisher test ($P < 0.05$) was used to determine the comparisons of means using the statistical program Statistica v12.0 (StatSoft Inc., 2013).

3 Results and discussion

3.1 *Evaluation of mycelial growth*

The combination of chitosan and salicylic acid on the inhibition of *B. cinerea* growth inhibition was higher than the percentage of inhibition obtained when evaluating the compounds individually (Table 1). On the other hand, the fungicide chlorothalonil did not allow mycelial growth of *B. cinerea*. This fungicide acts by binding to the sulfhydryl groups of the amino acids of the fungus, inactivating the enzymes involved in the production of ATP, thus causing cell death of the phytopathogen (Mogollón Ortiz *et al*., 2012). Despite its effectiveness, the damage represented by the use of this type of fungal control, either by ingestion or exposure to it, is reported. In addition, these not only affect target organisms, but also damage the atmosphere, soil, water, and living beings (de Souza *et al*., 2020).

The best combination was chitosan at 1.5% with salicylic acid at 0.07% with 97% inhibition of the phytopathogen. This agrees with the results obtained in previous works that show that antimicrobial activity increases as the concentration of evaluated compounds increases (Peralta-Ruiz *et al*. 2020).

Table 1. Effect of chitosan and salicylic acid at different concentrations on the inhibition of mycelial growth and sporulation of *Botrytis cinerea*.

Treatments	Concentration (%)	Growth inhibition (%)
Chitosan (CH)	0.5	71.71 ± 0.9275 c
	1	77.57 ± 0.6275 c
	1.5	93.38 ± 1.0483 d
Salicylic acid (SA)	0.03	4.88 ± 0.8360 a
	0.04	5.29 ± 0.7253 a
	0.07	48.90 ± 5.1565 b
CH - SA	0.5 - 0.07	73.50 ± 1.0024 c
	1.0 - 0.07	87.54 ± 0.6106 d
	1.5 - 0.07	97.80 ± 0.6382 e
Chlorothalonil	0.3	100 ± 0 e
Control	-	0.0 ± 0 a

*Values are expressed as mean ± standard error (n = 5). Different letters in each column indicate significant differences between treatments. Fisher's LSD test ($p \leq 0.05$).

The increase in the antifungal activity of the combination of chitosan and salicylic acid can be attributed to a collective effect of the mechanisms of action of both compounds when combined. In this sense, it is proposed that the antimicrobial property of chitosan is attributable to its acetylated units (N-acetylglucosamine and CH_3CONH_2), which form hydrogens and hydrophobic interactions. On the other hand, when chitosan dissolves at acid pH, the amino groups (NH_2) of glucosamine are protonated and the cationic polyelectrolyte develops electrostatic interactions with the anionic groups, in this case, the negatively charged phospholipids of the fungal cell membrane (Ramírez-Benítez *et al*. 2019). These characteristics of chitosan allow it to interact with organic and inorganic molecules, with the proteins present in the fungal cell wall it forms weak electrostatic interactions, while white non-protonated amino groups, it has a high affinity with most of the metals from the cytoplasm, forming ionic and chelating interactions (Herrera-Gonzalez *et al*., 2021). On the other hand, salicylic acid is capable of causing damage to the lipid bilayer of the phytopathogen, leading to a leak of intracellular material (da Rocha Neto *et al*. 2016). Further, it was observed that when mixing salicylic acid and chitosan for postharvest control of green mold in grapefruit, there was an increase in endogenous salicylic acid levels, which is related to the induction of the fruit's defense system (Shi *et al*., 2018).

3.2 Sporulation evaluation

The development of sporulation was significantly different depending on the treatments evaluated, being lower for the highest concentration of chitosan and salicylic acid, while when combining them, *B. cinerea* was not able to sporulate (Table 2). This result is similar to those found by Peian *et al*. (2021) in which the effect of the application of chitosan at 1% on the sporulation of *Botrytis cinerea* in grapes was significantly diminished. These authors report that chitosan promotes the production of enzymes such as phenylalanine ammonium lyase, peroxidase, and polyphenoloxidase related to defense and acts on the reproductive structures of the fungus. Similar results were also reported by Berumen-Varela *et al*. (2015) who obtained a lower sporulation/mL of *Colletotrichum* sp. for a concentration of salicylic acid of 5mM with respect to the lower concentration of this. The combination of chitosan and salicylic acid enhanced its effect on the reproductive structures of the fungus, completely preventing its development. Similar behavior was observed by Rayón-Díaz *et al*. (2021) who combined chitosan concentrations greater than 0.5% with sodium silicate, and completely inhibited the sporulation of *Colletotrichum gloeosporioides*. This effect may indicate that chitosan not only directly affects the fungal cell wall but can also induce changes at the molecular level by interfering with the synthesis of proteins and DNA of the phytopathogen (Song *et al*., 2016).

Table 2. Effect of chitosan and salicylic acid at different concentrations on the sporulation of *Botrytis cinerea*.

Treatments (%)	**Sporulation (spores/mL)**
Chitosan 0.5	$4.20 \times 10^6 \pm 1.83$ c
Chitosan 1.0	$3.10 \times 10^6 \pm 1.22$ c
Chitosan 1.5	$7.5 \times 10^5 \pm 0.00$ b
Salicylic acid 0.03	$9.60 \times 10^6 \pm 6.18$ d
Salicylic acid 0.04	$8.30 \times 10^6 \pm 5.69$ d
Salicylic acid 0.07	$7.00 \times 10^6 \pm 1.29$ b
CH 0.5 - SA 0.07	0 ± 0 a
CH 1.0 - SA 0.07	0 ± 0 a
CH 1.5 - SA 0.07	0 ± 0 a
Control	$3.00 \times 10^7 \pm 2.00$ e
Chlorothalonil 0.3	0 ± 0 a

*Values are expressed as mean ± standard deviation (n = 5). Different letters in the table indicate significant differences between treatments. Fisher's LSD test ($p \leq 0.05$).

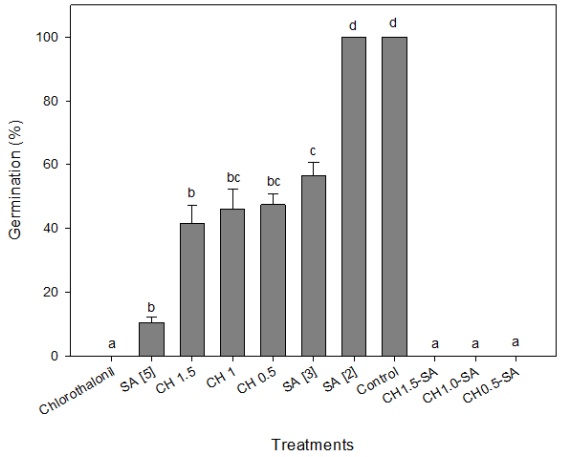

Figure 1. Effect of treatments on the germination of *B. cinerea*. Chitosan (CH) (0.5, 1.0 and 1.5%), salicylic acid (SA) (0.03, 0.04 and 0.05%), Combination salicylic acid 0.05% - chitosan (SA-CH). Different letters in the bars indicate significant differences between treatments. Fisher's LSD test ($p \leq 0.05$).

3.3 Germination percentage evaluation

By combining chitosan with salicylic acid, it is possible to completely inhibit the germination of the phytopathogen (Figure 1), demonstrating the effect that these compounds can exert together on *B. cinerea*. Regarding the application of the compounds separately, salicylic acid at 0.07% almost totally inhibited spore germination, followed by chitosan at 1.5%, achieving only 41% germination. Chitosan, as mentioned above, can interact with different metabolic or structural cellular components that interfere with the normal physiological processes of microorganisms (Mejía *et al*., 2020). While salicylic acid can also affect the normal development of the phytopathogen, causing damage to its cellular respiration (da Rocha Neto *et al*., 2015). Once again, this analysis demonstrates the advantage offered by combining two compounds whose effect would result in the sum of the action of both to completely stop the development and growth of the fungus.

3.4 Scanning electron microscopy

The images obtained from the scanning electron microscopy reveal structures affected by the chitosan and salicylic acid treatments evaluated compared to the control (Figure 2). *Botrytis cinerea* without treatment (Figure 2a) presents a structure with smooth septate hyphae and without the presence of damage. In contrast, the application of chitosan at 1.5% caused wrinkled and broken hyphae, and a transparent-looking layer can be seen around the spores (Figure 2b). This effect on spores was reported by Palma-Guerrero *et al*. (2008) who suggest that this material that surrounds the spore could be chitosan that binds to the glycoproteins present in the spore of the fungus to be able to penetrate inside the fungal cell. Wrinkled hyphae with abnormal ramifications were observed when chitosan was applied to *Aspergillus ochraceus* (Meng *et al*. 2020) and this effect was attributed to the interaction that exists between the positively charged groups of chitosan with the compounds present in the cell wall of the phytopathogen with a negative charge.

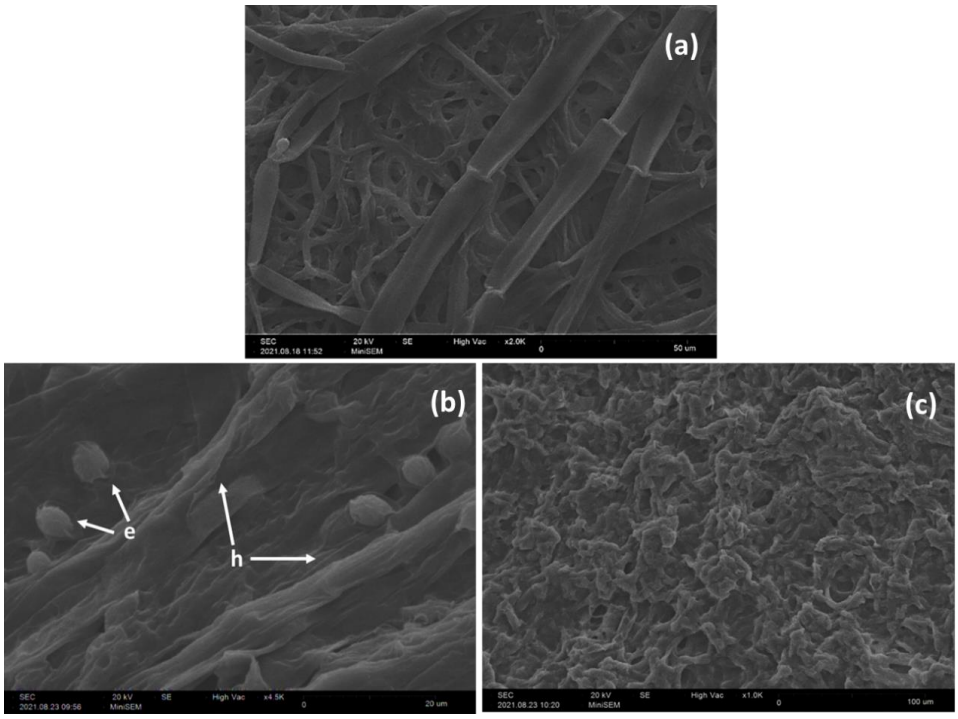

Figure 2. Micrographs of *Botrytis cinerea* under the action of chitosan and salicylic acid. (a) Hyphae (h) of *Botrytis cinerea* without treatment. (b) Hyphae and spores (e) of *B. cinerea* with chitosan. (c) Hyphae of *B. cinerea* against salicylic acid.

Table 3. Effect of the combination of chitosan (CH) and salicylic acid (SA) according to the Abbott index.

Treatments (%)	**Mycelial inhibition obs (%)**	**Mycelial inhibition exp (%)**	**Abbott index**	**Effect**
CH 0.5 - SA 0.07	73.5	85.54	0.86	ADDITIVE
CH 1.0 - SA 0.07	87.54	88.53	0.99	ADDITIVE
CH 1.5 - SA 0.07	97.8	96.62	1.01	ADDITIVE

*Different letters in the table indicate significant differences between treatments. Fisher's LSD test ($p \leq 0.05$).

Other authors have also described this damage to fungal hyphae and spores by chitosan (Ramos-Guerrero *et al*. 2018; Rayón-Díaz *et al*, 2021). Treatment with 0.07% salicylic acid caused compaction of *B. cinerea* hyphae (Figure 2c). A similar effect was observed by Kong *et al*. (2021) who observed wrinkled and bulging *Fusarium solani* hyphae when treated with salicylic acid, which is associated with the hydrophobicity of this compound that allows interaction and directly affects the integrity of the phytopathogen cell wall. This supports the previously reported antifungal action of salicylic acid by causing protein and lipid leakage from the cell membrane of *Penicillium expansum* (da Rocha Neto *et al*. 2016).

3.5 Effect of the combination of chitosan-salicylic acid

For all the combinations of chitosan and salicylic acid, an additive effect was obtained according to the Abbott index (Table 3), for which it is hypothesized that there is a sum of mechanisms of action by chitosan and salicylic acid. Peralta-Ruiz *et al*. (2020) also obtain an additive effect by combining chitosan with an essential oil and mention that the antimicrobial effect of the essential oil is enhanced by the antifungal activity of chitosan. An increase in antibacterial activity when combining salicylic acid and chitosan was also reported by Yang, Fang, and Ji (2016), this effect is attributed to the possible hydrogen

bond interaction that occurs between the hydroxyl or amide group of chitosan with the carboxyl group of salicylic acid. To inhibit the growth of *Colletotrichum gloeosporioides*, chitosan was combined with sodium silicate, obtaining an additive effect determined by the Abbott index (Rayón-Díaz, *et al*., 2021). Other salts, including potassium sorbate, also showed an additive effect when adding chitosan to control *Geotrichum candidum* in tomato (Rodriguez Guzman *et al*., 2022).

3.6 Chemical interaction between chitosan and salicylic acid

To explain the effect exerted by the combination of chitosan and salicylic acid on the control of *Botrytis cinerea*, the possible chemical interaction that could exist was determined. Chitosan (Figure 3A) has a molecular weight of 45,700 g/mol, with 47 hydrogen acceptors and 29 donors, therefore, it has a polar surface area of 808 A2 (Figure 3B). In the case of salicylic acid (Figure 3C), the molecule has a molecular weight of 138.12 g/mol with 3 hydrogen acceptors and 2 hydrogen donors (Figure 3D), salicylic acid has a polar surface of 57.5 A2, which is lower (7%) than chitosan. According to the simulation of the possible interaction between chitosan and salicylic acid, it is observed that there are no potential interactions between it and chitosan. Although it is appreciated that there is an area where there is no hydrophilic potential (Figure 4A), this area is equivalent to a surface area of 155.34 A2, which is lower compared to the total area of chitosan (1308 A2). Nevertheless, the OH attached to the phenol that makes up salicylic acid appears as a potential hydrogen acceptor source. Since the applied molar concentration of salicylic acid was 5 mM, and the molar concentration of chitosan was 0.328 mM, the volume of chitosan is higher compared to salicylic acid (2364.94 Armstrong for chitosan and 113.81 Armstrong for salicylic acid), this could explain why there is an additive effect and not a synergistic effect. Chitosan and salicylic acid generate a zone of great reactivity (Figure 4B) caused by the OH groups present and no steric hindrance to the activity potentially attributable to the amino group.

A similar analysis reported by Nasonova *et al*. (2020) mentioned a possible union between chitosan and salicylic acid molecules through the formation of hydrogen bonds and the presence of a hydrophobic skeleton in the structure of the salicylic acid molecule.

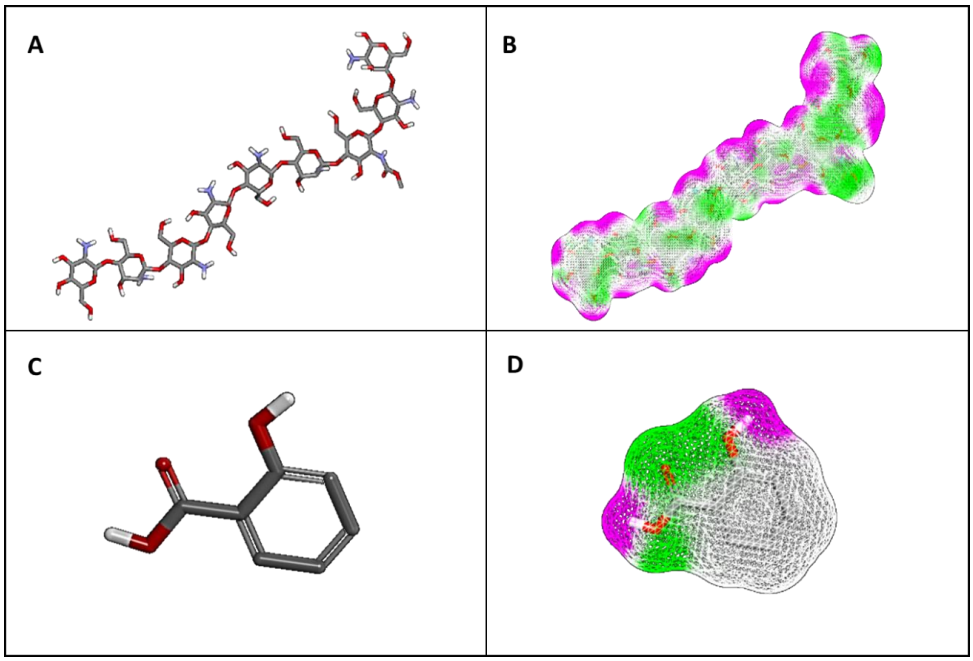

Figure 3. Chitosan structure (A). Acceptor zones (magenta) and hydrogen donor zones (green) in the chitosan structure (B). Salicylic acid structure (C). Hydrogen acceptor (magenta) and hydrogen donor (green) zones in the salicylic acid structure (D).

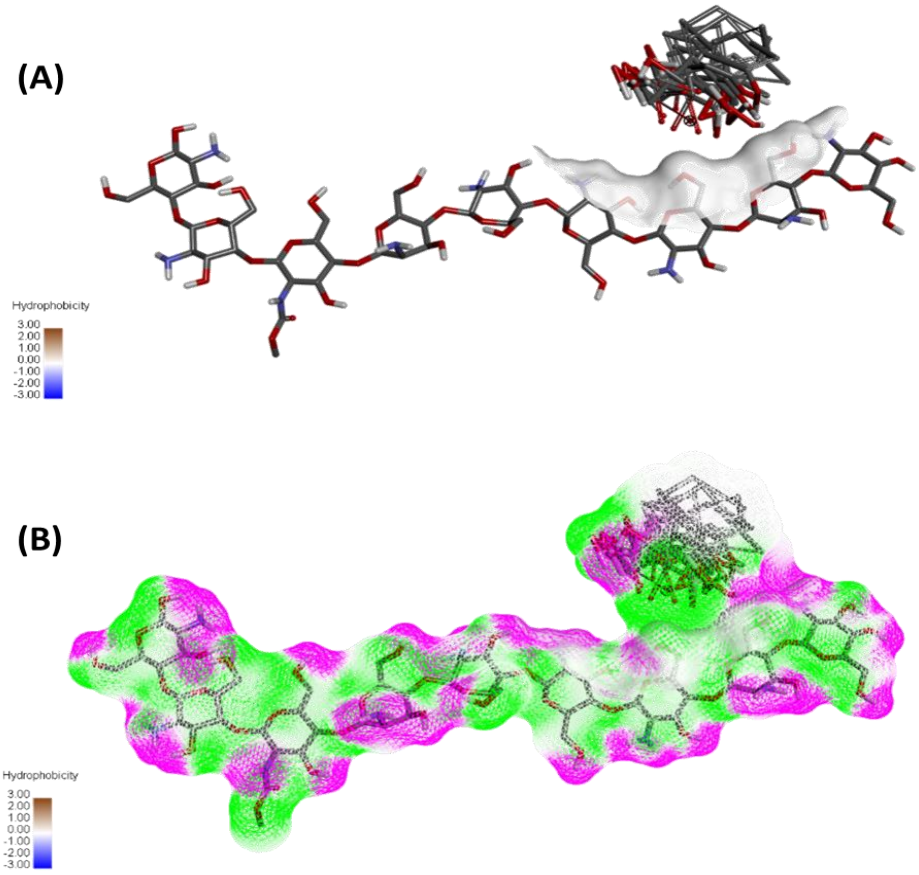

Figure 4. Zones of high hydrophobicity (white) in the surface area of interaction between chitosan and salicylic acid molecules (A). Acceptor zones (magenta) and hydrogen donors (green) in the structure of salicylic acid and chitosan (B).

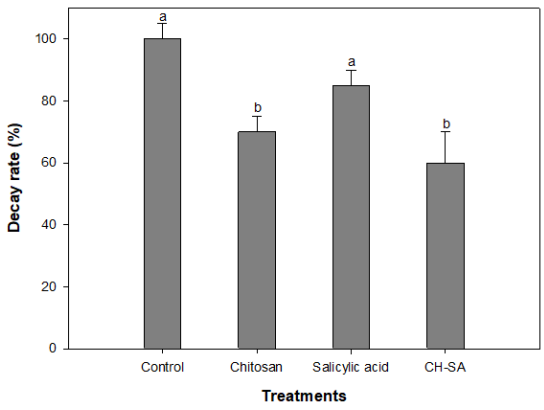

Figure 5. The decomposition rate of blueberries under application of chitosan (CH), salicylic acid (SA), and their combination. Different letters in the bars indicate significant differences between treatments. Fisher's LSD test ($p \leq 0.05$).

3.7 Blueberry decomposition rate

The rate of decomposition of blueberry fruits caused by *Botrytis cinerea* was significantly different when applying chitosan and salicylic acid (Figure 5), the incidence rate was lower when combining these compounds with a value of 60% compared to 100% incidence of the disease for the control fruits. These results confirm what has been proposed regarding the action of chitosan as an agent capable of reducing the action of some phytopathogens (Duan *et al.* 2019), through the collection of mechanisms such as direct action on fungal structures (Gutierrez-Martinez *et al.* 2018) or the induction of fruit defense responses (Bautista-Baños *et al.* 2017). While salicylic acid participates in responses to different signals, causing the activation of subsequent responses, such as the synthesis of antimicrobial compounds and

cell wall reinforcement mechanisms (Shao *et al*. 2019). Although salicylic acid decreased the rate of decomposition of blueberries relative to the control, this effect was not as successful. In this context, a similar study discusses that salicylic acid had no *in vivo* effect on the control of *P. expansum* (da Rocha Neto *et al*., 2016) and suggests that salicylic acid, having low viscosity, is not capable of forming a physical barrier on the surface of the fruit that can protect from the attack of phytopathogens. A report on the combined effect of chitosan and salicylic acid for the control of *Penicillium digitatum* states that the incidence of the disease is reduced by applying these combined treatments with respect to their application separately (Shi *et al*., 2018). On the other hand, as a cell wall reinforcement treatment, through the lignin deposition and consequent disease control, the combined effect of chitosan nanoparticles and salicylic acid on corn plants was demonstrated (Kumaraswamy *et al*., 2019).

Conclusions

The combination of chitosan and salicylic acid inhibited the *in vitro* mycelial growth of *Botrytis cinerea* by 97%, while the sporulation and germination of the phytopathogen were completely inhibited. Chitosan caused wrinkling of hyphae and loss of spore turgidity, as well as salicylic acid, which also caused damage to hyphae and spores of *B. cinerea*, observed under scanning electron microscopy. On the other hand, an additive effect was obtained when combining the chitosan and salicylic acid treatments, reflected by an increase in the inhibition effect of these on the phytopathogen; and it was detected that when combined they generate a zone of great reactivity caused by the OH groups present in their structures. The *in vivo* evaluation showed that the combination of chitosan and salicylic acid can reduce the rate of decomposition of blueberries by 40% compared to untreated fruits. The results of the application of chitosan and salicylic acid show that these compounds could act as an eco-friendly alternative and directed towards compliance with the zero-residue policy.

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