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Chitosan and salicylic acid as alternatives for the control of postharvest fungal diseases in blueberries (*Vaccinium corymbosum*)

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Abstract

Blueberry (*Vaccinium corymbosum*) has characteristics that make it a highly coveted fruit by the population that seeks benefits for their health, thus giving it economic and social relevance. However, it is a very perishable fruit. In the present work, *Botrytis* sp., *Penicillium* sp., and *Alternaria* sp. were isolated from blueberry, and molecularly identified. The *in vitro* effect of chitosan (CHI) and salicylic acid (SA) on the growth of these phytopathogens was then evaluated, as well as the incidence of the disease after the application of these treatments on blueberry. CHI at 1.5% achieved an *in vitro* mycelial growth inhibition of *Botrytis* sp., *Penicillium* sp., and *Alternaria* sp. by 93, 84, and 40%, respectively. Furthermore, a complete germination inhibition of *Penicillium* sp. and *Alternaria* sp. was accomplished; *Botrytis* sp. spores were less sensitive to chitosan treatment. The germination percentage of the phytopathogens was reduced by 90% using SA at 5 mM. The *in vivo* application of CHI at 1.5% and SA at 5 mM decreased the percentage of incidence of phytopathogens in blueberries harvested after storage period at 25°C, as compared to the control. Based on these results, SA and CHI represent an alternative for the control of phytopathogens in blueberry to eliminate the use of synthetic fungicides.

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Introduction

Nowadays, research into human health is becoming more relevant, and there is a greater concern about the consumption of healthy foods with bioactive characteristics. In this sense, fruits such as blueberries (*Vaccinium corymbosum*) play a fundamental role, This fruit is highly coveted for its physicochemical properties, being low in calories, and a source of potassium, iron, and calcium (Pinedo, 2018). It has bioactive and anti-inflammatory properties since it is rich in phenolic compounds, tannins, as well as fibre that contributes to intestinal transit. Its contents of anthocyanins, vitamin C, and others give it antioxidant action, and help reduce degenerative and cardiovascular diseases, and even cancer (Chu *et al*., 2018). However, blueberry is susceptible to mechanical damage, dehydration, loss of firmness, and deterioration due to the thin exocarp, and the attack of different phytopathogens, including *Colletotrichum* spp., *Rhizopus* spp., *Alternaria* spp., *Penicillium* spp., and *Botrytis cinerea* (Mehra *et al*., 2013; Umagiliyage *et al*., 2017).

To preserve the quality of the fruit and extending its shelf life, the presence of phytopathogens must be eliminated because in the case of *Alternaria* spp. and *Penicillium* spp., they can produce mycotoxins that are harmful to both animals and humans (Anne *et al*., 2008). *Penicillium* spp. produce the mycotoxins patulin and citrinin that can induce oxidative damage in human cells (Li *et al*., 2015), while *Alternaria* spp. produce mycotoxins such as alternariol and tenuazonic acid that are involved with food poisoning (Ostry *et al*., 2009). The control of *B. cinerea* (causal agent of grey mould rot) is very relevant since it is the necrotrophic fungus with the highest incidence in blueberry that causes significant economic losses (Romanazzi *et al*., 2016).

Modified and/or controlled atmospheres and synthetic fungicides are the most common methods to control the postharvest diseases of blueberries (Ramos Bell *et al*., 2021). However, the population

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and the international market demands are focused on the policy of "zero waste". In this sense, various alternatives to fungal control have been proposed and developed, including the use of chitosan as a natural, non-toxic biopolymer with antimicrobial characteristics (Bautista-Baños *et al*., 2017). Previous work has demonstrated the functionality of chitosan as a postharvest control agent against the attack of various phytopathogens such as *Penicillium digitatum*, *Colletotrichum gloeosporioides*, *Alternaria alternata*, *Rhizopus stolonifer*, and others (Gutiérrez-Martínez *et al*., 2018a; Meng *et al*., 2020). Further, another alternative compound for the control of postharvest diseases is salicylic acid which is a natural phenolic compound present in plants (Mekawi *et al.*, 2019). The application of exogenous salicylic acid induces protein synthesis, increases the concentration of reactive oxygen species, and the production of antimicrobial phytoalexins in fruits (Hayat *et al.*, 2010).

Taking all this into account, the present work aimed to evaluate chitosan and salicylic acid as a non-polluting and eco-friendly method for the control of postharvest phytopathogens in blueberry, given the importance of this fruit.

Materials and methods

Materials

Low molecular weight commercial chitosan (45.7 kDa) with 10% acetylation (Golden-Shell Co., China) and salicylic acid (Sigma Aldrich, USA) (reagent ≥ 99.0%) were used.

Preparation of chitosan and salicylic acid

Three concentrations of chitosan (0.5, 1.0, and 1.5%) (w/v) added to 1.0% of acetic acid were prepared in sterile distilled water. The solutions were constantly stirred for 24 h, and their pH was adjusted to 5.6 with 1 N NaOH. Salicylic acid was prepared from a 50 mM stock solution in distilled water and glycerine (5%). Subsequently, dilutions were made to obtain concentrations of 2, 3, and 5 mM at pH 5.5 using a 10% (w/v) KOH solution (Ramos-Guerrero *et al.*, 2018).

Isolation of phytopathogens

Blueberry (*Vaccinium corymbosum* cv. Biloxi) with damage by phytopathogens were obtained from a commercial orchard in Nayarit, Mexico. Next, we performed cuts (1 × 1 cm) into the damaged tissue with a sterile scalpel, disinfected it in a 2% sodium hypochlorite solution, and then put it 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. Individual phytopathogens were isolated, and pure cultures were obtained (Ramos-Guerrero *et al*., 2018).

Pathogenicity assay

The pathogenicity test was carried out as described by Jiang *et al*. (2016) with some modifications. The fruits were previously disinfected in a 2% sodium hypochlorite solution and sterile distilled water. The fruits were wounded once with a 0.8 mm punch, and inoculated with 5 µL of 10^5 spores/mL solution from each fungal isolation, and then incubated at 25°C with 95% relative humidity for 7 d. The incidence percentage was determined, and the fungi were re-isolated to confirm Koch's postulates.

Morphological and molecular identification of phytopathogens

The morphology of the isolated strains was observed using an optical microscope (Motic BA 300, Canada) with the 10, 40, and 100× objectives. The shape and size of the hyphae, conidiophores, and spores were reported. Afterwards, species was determined using the taxonomic keys proposed by Barnett and Hunter (1998), and by amplifying 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 then deposited in the NCBI GenBank database.

Effect of chitosan and salicylic acid on in vitro mycelial growth inhibition

The PDA culture medium was individually homogenised with chitosan at 0.5, 1.0, and 1.5% and salicylic acid at 2, 3, and 5 mM, respectively. Afterward, it was poured into the centre of the Petri dishes (9 cm diameter). After solidified, 0.7 cm discs of 5-d old *Botrytis* sp., *Penicillium* sp., and *Alternaria* sp. were inoculated. A group of plates were

inoculated with each phytopathogen. As a control, the chlorothalonil fungicide (Bravonil® 720 SC) at 0.3% was used. The plates were incubated at 25°C for 10 - 14 d. Five plates per treatment were made, and the assay was performed in triplicate (Jiang *et al*., 2016). The mycelial diameter was quantified using the ImageJ® program, and the results were expressed as a percentage of mycelial growth inhibition (% MGI) using Eq. 1:

$$\%MGI = \frac{(Colony\ control\ diameter - Colony\ treatment\ diameter)}{(Colony\ control\ diameter - Initial\ disc\ diameter)} \times 100 \quad \text{(Eq. 1)}$$

Effect of chitosan and salicylic acid on sporulation

After 10-d incubation, 10 mL of sterile distilled water was added to the plates with treatments, and the solutions obtained were filtered through sterile gauze. The spores were counted in Neubauer chamber using serial dilutions through an optical microscope (Motic BA 300, Canada), and the spores/cm^2 produced from each isolation were determined (Ramos-Guerrero *et al*., 2018). Three replicates per treatment were performed.

Effect of chitosan and salicylic acid on germination percentage

The spore germination percentage was determined according to Jiang *et al*. (2016) with some modifications, taking 20 µL of the spore suspension obtained from each treatment and adding them to a PDA disk (20 mm in diameter). The discs were inoculated at 25°C for 8, 10, and 12 h for *Botrytis* sp., *Penicillium* sp., and *Alternaria* sp., respectively, and subsequently, 100 spores were counted using an optical microscope (Motic BA300, Canada) with the 40× objective. The spore was considered germinated when the length of the germ tube reached twice its diameter. The germination percentage was calculated with the number of germinated spores in regard to the total number of spores, using Eq. 2. Three replicates per treatment were carried out.

$$\%\ Germination = \frac{Number\ germinated\ spores}{Number\ total\ spores} \times 100 \quad \text{(Eq. 2)}$$

Effect of chitosan and salicylic acid on incidence of phytopathogens in blueberries

The incidence percentage was determined according to Jiang *et al*. (2016) with some modifications. Fruits without phytopathogen damage were washed with a 2% commercial chlorine solution, then with water for 1 min, and then dried at room temperature (25°C). Each fruit was punctured with a sterile needle, and then immersed in the best *in vitro* concentration of chitosan and salicylic acid for 2 min, and dried for 1 h. Next, they were inoculated with 5 µL of spore solution (10^5 spores/mL) of *Botrytis* sp., *Penicillium* sp., and *Alternaria* sp. Fruits were submerged in chlorothalonil as a positive control, while the negative control was fruits without treatment. Fruits were stored at room temperature (25°C) for 10 d with a relative humidity of 90 - 95%. At the end of the storage time, any fruit with visible signs of development of the inoculated phytopathogens was considered damaged. The results were expressed as a percentage of incidence using Eq. 3.

$$\%\ Incidence = \frac{Number\ infected\ fruits}{Number\ total\ fruits} \times 100 \quad \text{(Eq. 3)}$$

Statistical analysis

The results were statistically analysed 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). For the *in vitro* test, five repetitions per treatment were carried out, while the *in vivo* test was carried out with ten fruits per treatment, and the tests were carried out in duplicate.

Results and discussion

Isolation, pathogenicity, and identification

Fourteen phytopathogens were isolated from the blueberries with signs of damage, of which *Botrytis* sp. (strains: 4A, 4B, 5A, 7A, 7B), *Penicillium* sp. (strains: 2A, 12A), and *Alternaria* sp. (strains: 6B, 13A) were identified using the taxonomic keys of Barnett and Hunter (1998) (Figure 1). Based on the pathogenicity test, a higher incidence and virulence was observed for the strains 7A, 2A, and 13A. The phytopathogens were re-isolated from fruit complying with Koch's postulates.

The molecular analysis determined that the isolated strains belong to *Botrytis* sp. (accession no.: MN891765.1), *Penicillium* sp. (accession no.: MT597829.1), and *Alternaria* sp. (accession no.: MZ810519.1) with 100% identity. Previous studies

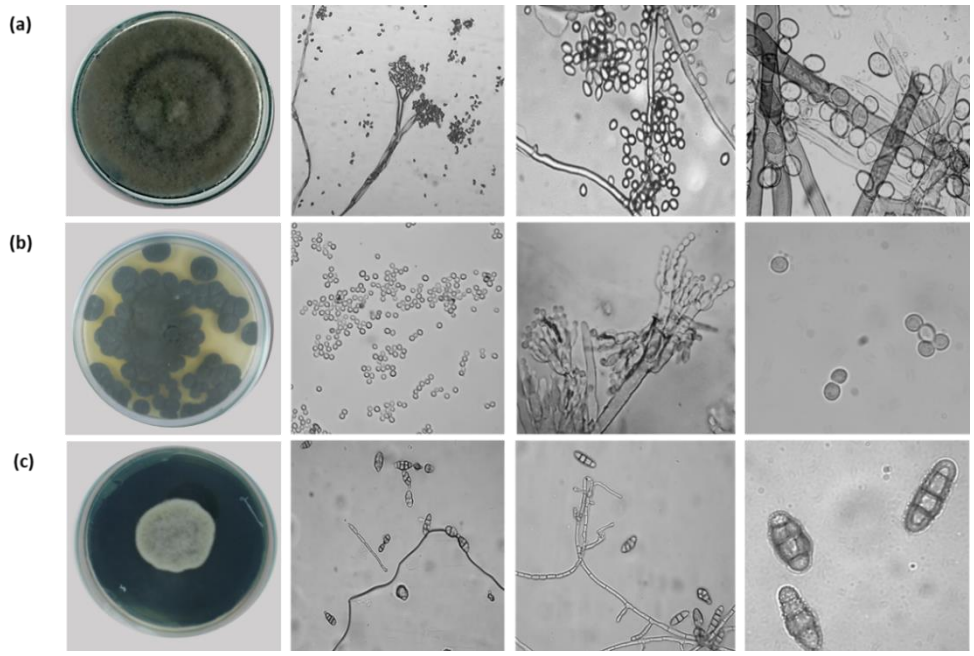

Figure 1. Colony morphology on PDA and micrographs of conidia and conidiophores (10, 40, and 100× objectives) of *Botrytis* sp. **(a)**, *Penicillium* sp. **(b)**, and *Alternaria* sp. **(c)**.

have reported that *Botrytis* sp., *Penicillium* sp., and *Alternaria* sp. are pathogenic fungi of blueberries (Greco *et al*., 2012; Liu *et al*., 2018).

Effect of chitosan and salicylic acid on inhibition of mycelial growth

Chitosan reduced the mycelial growth of *Botrytis* sp. and *Penicillium* sp. However, chitosan and salicylic acid were less efficient in the inhibition of *Alternaria* sp. (Table 1). Regarding the effect of chitosan on *Botrytis* sp., the percentage of growth inhibition in the colonies was significantly different ($p < 0.05$) for each treatment as compared to the control. Chitosan at 1.5% showed the best result with 93.38% growth inhibition. The results of the mycelial growth of *Penicillium* sp. showed no significant differences between the different concentrations of chitosan, giving the highest inhibition value of 88.92%. For *Alternaria* sp., the highest inhibition value was 40% when applying chitosan at 1.5%.

Similar results were reported by Jiang *et al*. (2016). These authors evaluated chitosan on the mycelial growth of *B. cinerea* isolated from blueberries, and found a 73% inhibition of fungal growth. On the other hand, sulfonated chitosan achieved a 90% inhibition of *Penicillium* sp. in blueberry (Liu *et al*., 2018). Likewise, González-Estrada *et al*. (2020) reported a 100% mycelial

Table 1. Effect of chitosan and salicylic acid on the percentage of inhibition of mycelial growth of *Botrytis* sp., *Penicillium* sp., and *Alternaria* sp.

Treatment	*Botrytis* sp.	*Penicillium* sp.	*Alternaria* sp.
Control	0.0 ± 0^{a}	0.0 ± 0^{a}	0.0 ± 0^{a}
Chlorothalonil 0.3%	100 ± 0^{f}	73.79 ± 0.94^{d}	21.48 ± 2.65^{d}
Chitosan 0.5%	71.71 ± 4.15^{c}	88.73 ± 1.65^{f}	7.52 ± 2.28^{c}
Chitosan 1.0%	77.57 ± 2.80^{c}	88.92 ± 1.37^{f}	7.90 ± 4.29^{c}
Chitosan 1.5%	93.38 ± 4.68^{e}	84.32 ± 2.30^{e}	40.15 ± 2.19^{e}
Salicylic acid 2 mM	4.88 ± 3.73^{b}	30.61 ± 13.49^{b}	4.29 ± 1.40^{b}
Salicylic acid 3 mM	5.29 ± 3.24^{b}	38.46 ± 6.38^{c}	5.80 ± 2.68^{b}
Salicylic acid 5 mM	48.90 ± 23.06^{d}	36.40 ± 13.93^{bc}	7.20 ± 2.83^{c}

Values are mean ± standard deviation ($n = 5$). Means followed by different lowercase superscripts in the same column are significantly different between treatments ($p < 0.05$).

growth inhibition of *P. citrinum* when using 1.0% chitosan. The effect of chitosan on *A. alternata* has been reported by Rodríguez-Romero *et al.* (2019) with similar results to those obtained in the present work, since the highest inhibition achieved was 33% with a chitosan concentration of 1.5%. Another similar study achieved a 40% inhibition of *A. alternata* growth when using the highest concentration of chitosan (El-Garhy *et al*., 2020). All these previous reports confirm the fact that chitosan has an inhibitory effect on the mycelial growth of phytopathogens due to its polycationic characteristic that facilitates it binding to the cell wall of fungi, and causes damage to the plasma membranes of mycelia and spores.

On the other hand, the effect of salicylic acid was lower as compared to chitosan for all phytopathogens. The results showed significant differences between the applied concentrations; 5 mM showed the mycelial growth inhibition values of 48.90, 36.40, and 7.20% for *Botrytis* sp., *Penicillium* sp., and *Alternaria* sp., respectively. Authors such as Cao *et al*. (2013) reported that salicylic acid did not have a direct inhibitory effect on the growth on *A. alternata*, while a more recent study demonstrated showed 100% inhibition of *A. alternata* at salicylic acid concentrations of 0.75 and 1.5 g/L (El-Garhy *et al*., 2020). On the other hand, Mekawi *et al.* (2019) treated *B. cinerea* by applying salicylic acid at 5 and 8 mM to peppers, achieving 100% of mycelial inhibition. Based on these studies, it is evident that the effect of salicylic acid as an inhibitor of phytopathogens is dependent on the concentration, as well as the fungal and plant species evaluated.

Effect of chitosan and salicylic acid on sporulation

The effect of the different treatments on the sporulation of *Botrytis* sp., *Penicillium* sp., and *Alternaria* sp. showed significant differences between chitosan and salicylic acid in comparison with the control (Figure 2). *Botrytis* sp. sporulation was dependent on the applied dose of both chitosan and salicylic acid; in the case of *Penicillium* sp., less sporulation was observed with the application of salicylic acid, while the sporulation of *Alternaria* sp. under the effect of the treatments was not reduced too much as compared to the control. In the case of the fungicide, it only inhibited the sporulation of *Botrytis* sp. These results were similar to the work performed by Peian *et al*. (2021). The authors found that the effect of chitosan at 1% on the sporulation *of B. cinerea* in grapes significantly diminished. Furthermore, they report that chitosan promoted the production of hormones related to defence, and acted on the reproductive structures of the fungus. The application of chitosan on *A. alternata* reduced but did not inhibit its sporulation, as reported by Sánchez-Domínguez *et al*. (2007), who also mentioned that the total inhibition could probably be due to the stress caused by the high doses of chitosan. Regarding the effect of salicylic acid, these results agreed with Mogollón and Castaño (2012) who confirmed that

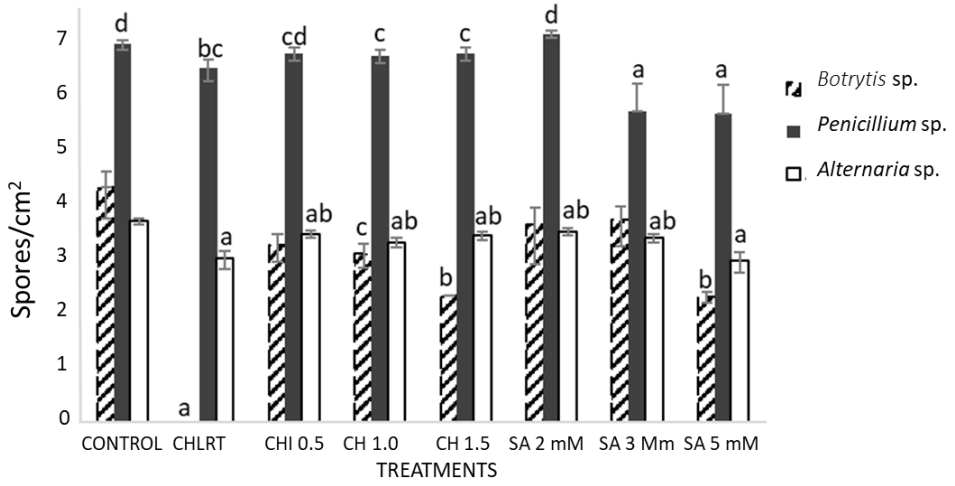

Figure 2. Effect of treatments on the sporulation *of Botrytis* sp., *Penicillium* sp., and *Alternaria* sp. CHLRT: chlorothalonil; CHI 0.5: chitosan 0.5%; CHI 1.0: chitosan 1.0%; CHI 1.5: chitosan 1.5%; and SA: salicylic acid. Bars are mean ± standard deviation ($n = 5$). Different lowercase letters within the same species indicate significant differences between treatments ($p < 0.05$).

sporulation values were reduced when evaluating the highest concentration of salicylic acid on *Mycosphaerella fijiensis* compared to the control. Similar results were also reported by Berumen-Varela *et al.* (2015) which obtained a lower sporulation/mL of *Colletotrichum* sp. for a salicylic acid concentration of 5 mM than the others evaluated.

Effect of chitosan and salicylic acid on germination percentage

The germination percentage of *Botrytis sp.*, *Penicillium* sp., and *Alternaria* sp. was affected by chitosan and salicylic acid (Figure 3). Chitosan, at the highest concentration, completely inhibited the germination of the other phytopathogens except for *Botrytis* sp., while salicylic acid at 5 mM reduced germination to 10.4, 11.3, and 1.3% for *Botrytis* sp., *Penicillium* sp. and *Alternaria* sp., respectively. The lowest germination percentage of *Botrytis* sp. was obtained with 1.5% chitosan, with a value of 41%. In other studies, a higher percent inhibition of the germination of *B. cinerea* was reported (Jiang *et al.*, 2016), and it was suggested that the difference in how chitosan acts on the growth of the mycelium and germination may be conditioned by the type of phytopathogen studied, as well as the characteristics of chitosan. Similarly, Rodríguez-Romero *et al*. (2019) inhibited 100% the germination of *A. alternata* using chitosan at 1.5%, while *P. citrinum* treated with chitosan did not germinate as described by González-Estrada *et al*. (2020). Regarding the *in vitro* application of salicylic acid, Cao *et al.* (2013) stated that the effect of this compound on the inhibition of *A. alternata* could be attributed to its biological function to trigger resistance induced by the fruit, instead of its antifungal effect on the phytopathogen.

Regarding the possible mechanism of action of chitosan, it is proposed that it has the ability to electrostatically interact with the cell wall of the fungus, thus affecting its integrity and causing morphological changes in its hyphae and reproductive structures (Gutiérrez-Martínez *et al*., 2018b).

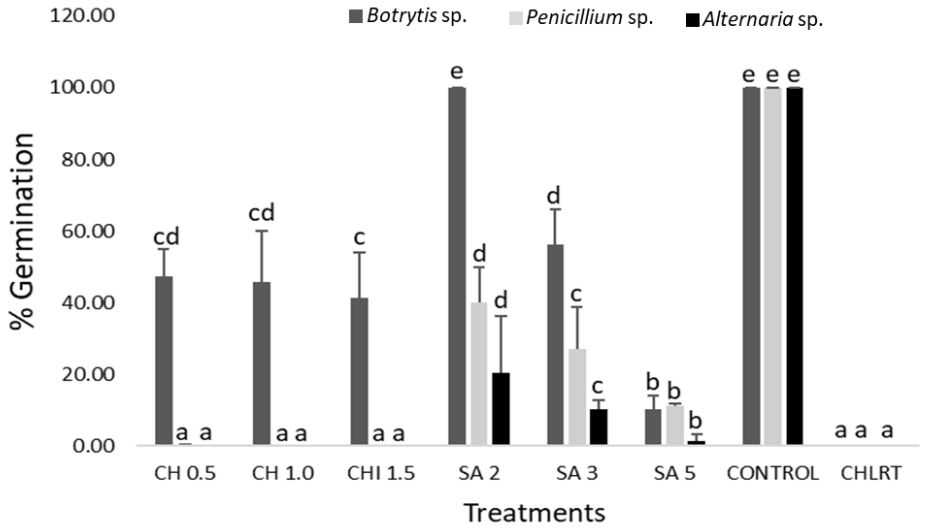

Figure 3. Effect of treatments on the germination percentage of *Botrytis* sp., *Penicillium* sp., and *Alternaria* sp. CHLRT: chlorothalonil; CHI 0.5: chitosan 0.5%; CHI 1.0: chitosan 1.0%; CHI 1.5: chitosan 1.5%; and SA: salicylic acid. Bars are mean ± standard deviation (n = 5). Different lowercase letters within the same species indicate significant differences between treatments ($p < 0.05$).

Effect of chitosan and salicylic acid on incidence of phytopathogens in blueberries

The *in vivo* evaluation of chitosan and salicylic acid on blueberry was carried out using the best treatments of each compound in the *in vitro* evaluation. The incidence of diseases caused by *Botrytis* sp., *Penicillium* sp., and *Alternaria* sp. was significantly different when applying chitosan and salicylic acid (Table 2). Based on these results, chitosan was the most effective treatment to reduce the incidence of these phytopathogens without significant statistical differences regarding the fungicide used. Damage due to the presence of phytopathogens in blueberries was evident a few days after storage, while in treated fruits, the incidence was delayed. At the end of 10-d storage, chitosan at 1.5%

showed the best results by preserving the appearance of the fruits (Figure 4). Liu *et al.* (2018) coated blueberries with chitosan (10 g/L), thus reducing their decomposition caused by *Penicillium* sp. and *B. cinerea* during storage at 28°C for 5 d. Salicylic acid has also been studied *in vivo* for the control of *A. alternata* in jujube, as an inducer of the defences of the fruit (Cao *et al*., 2013). Duan *et al.* (2019) suggest that chitosan can act as a physical barrier on the surface of fruits, thus causing a reduction in gas exchange, as well as the respiration of the fruit, which leads to prolonging fruit shelf life.

On the other hand, salicylic acid participates in the responses to different signals causing the activation of subsequent responses, such as the synthesis of antimicrobial compounds and the reinforcement mechanisms of the cell wall (Shao *et al.*, 2019). Taking into account that in the present work, the incidence was evaluated at room temperature, it was expected that the evaluation at refrigeration temperature will result in greater inhibition of the incidence of phytopathogens on postharvest blueberries.

Table 2. Percentage of incidence of *Botrytis* sp., *Penicillium* sp., and *Alternaria* sp. on blueberries at 25°C.

Treatment	*Botrytis* sp.	*Penicillium* sp.	*Alternaria* sp.
Control	100 ± 0.0^{c}	50 ± 0.94^{b}	100 ± 0.0^{b}
Chitosan 1.5%	55 ± 1.0^{a}	15 ± 1.24^{a}	80 ± 1.0^{ab}
Salicylic acid 5 mM	90 ± 0.94^{b}	35 ± 0.47^{ab}	80 ± 0.94^{ab}
Chlorothalonil 0.3%	58 ± 0.81^{a}	25 ± 0.81^{ab}	50 ± 1.63^{ab}

Values are mean ± standard deviation (n = 10). Means followed by different lowercase superscripts in the same column are significantly different between treatments ($p < 0.05$).

Figure 4. Appearance of untreated blueberries (control) and those treated with chitosan (1.5%) and salicylic acid (5 mM) at 25 ± 1°C after 10 days of storage.

Conclusion

The phytopathogens *Botrytis* sp., *Penicillium* sp., and *Alternaria* sp. were isolated and identified, both morphologically and molecularly, from damaged blueberries. The inhibition of the mycelial growth of these fungi, as well as sporulation, was dependent on the dose of chitosan used, while the percentage of germination was completely reduced. In contrast, salicylic acid had a higher effect on the spore's germination than *in vitro* growth. In the present work, the *Botrytis* sp. spores were less sensitive to the application of the treatments since a complete inhibition of these was not achieved concerning *Penicillium* sp. and *Alternaria* sp. Chitosan reduced the percentage of incidence of phytopathogens on blueberries. To determine the possible mechanisms of induction of the defences of the fruit, as well as the quality parameters, due to the effect of chitosan and salicylic acid, further evaluations should be carried out. Nevertheless, taking these results into account, both chitosan and salicylic acid could be considered as alternative systems to the fungal control of postharvest blueberries.

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