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In vitro development of *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. isolated from coffee in the presence of chitosan and salicylic acid

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Abstract: Several factors have contributed to reduced coffee production in Brazil, such as the anthracnose disease (Colletotrichum gloeosporioides). This study aimed to evaluate the effect of chitosan and salicylic acid (SA) on the development of C. gloeosporioides. SA was used at 0, 1.25, 2.5 and 5 mM and chitosan at 0, 1, 2 and 4 mg mL^{-1}. The controls were 10% ethanol (0 mM SA) and 0.05 N HCl (0 mg mL^{-1} chitosan). Conidia germination was verified using 100 µL from each treatment and 100 µL conidia suspension which were placed in each well of the ELISA plate. After incubated for 24 h the germinated conidia were enumerated. The mycelial growth was evaluated by the transfer of a mycelial disc (9 mm) to the centre of Petri plates containing potato dextrose agar and various concentrations of SA and chitosan. The diameter of the colony was measured daily. Chitosan did not inhibit the germination of conidia (p = 0.397), while SA inhibited up to 58% (5 mM SA) of the germination. There was a significant reduction in the mycelial growth at all SA concentrations, but chitosan was only effective at 4 mg mL^{-1}.

Key words: Anthracnose, Antimicrobial, Fungi.

Desenvolvimento in vitro do *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. isolado do café, na presença de quitosana e ácido salicílico

Resumo: Vários fatores contribuem para a redução da produção do café no Brasil, como a antracnose (*Colletotrichum gloeosporioides*). Este estudo avaliou o efeito da quitosana e ácido salicílico (AS) sobre o desenvolvimento do *C. gloeosporioides.* O AS foi usado a 0; 1,25; 2,5 e 5 mM e a quitosana a 0, 1, 2 e 4 mg mL^{-1}. Os controles foram etanol 10% (AS 0 mM) e HCl 0,05 N (Quitosana 0 mg mL^{-1}). A germinação de conídios foi verificada usando 100 µL de cada tratamento e 100 µL da suspensão de conídios os quais foram depositados em poços de placa de ELISA. Depois de incubados por 24h, os conídios germinados foram contados. O crescimento micelial foi avaliado pela transferência de um disco de micélio (9 mm) para o centro de placas de Petri contendo meio batata, dextrose e ágar e concentrações de AS e quitosana. O diâmetro da colônia foi mensurada diariamente. A quitosana não inibiu a germinação de conídios (p = 0,397), enquanto o AS inibiu até 58% (AS 5 mM) a germinação. Houve uma redução significativa do crescimento micelial em todas as concentrações de AS, mas a quitosana foi efetiva somente a 4 $mg.mL^{-1}$.

Palavras chave: Antracnose, Antimicrobiano, Fungo.

Introduction

Brazil is one of the biggest coffee (*Coffea arabica* L.) producing countries and exporters, worldwide. It is the second largest consumer of coffee reaching a production in 2017 of 44.77 million 60 kg bags of processed coffee Ministério de Agricultura, Pecuária e Abastecimento (Brasil, 2017). Nowadays, the general objectives are geared toward productivity features, resistance to pests, diseases and fruit quality, to suit the various consumer markets and, consequently, increase profitability (Meletti & Bruckner, 2001).

In Brazil and other coffee producing countries, the coffee crop is affected by various plant health problems. Of particular mention, are those caused by the fungus C*olletotrichum gloeosporioides* (Penz.) Penz. & Sacc., which is present at all stages of coffee development (Santos, 2012). *C. gloeosporioides* is the causal agent of anthracnose disease (Chen, 2002), which is characterised by the presence of spots or rot on the fruit surface as well as quiescent infections that occur in the field (Benato, 1999). The fruits are often discarded when presenting these symptoms, compromising its marketing and commercial value (Teixeira, 1995). Furthermore, irregular, greyish, necrotic patches occur in the leaves. The fungus attacks the young leaves at the tips of the branches, causing the leaves and branches to die, symptoms known as tip blight (Marcolan et al., 2009).

Plants have naturally developed inducible defence mechanism against pests and pathogens. However, in most crop plants, natural resistance against pathogens is rare. Induced resistance, which exploits the natural defence machinery of plants, is gaining increasing attention as a nonconventional, economical and ecologically friendly approach for plant protection (Stadnik & Rivera, 2001). Elicitors are compounds that activate chemical defence in plants. They include both substances of pathogen origin (exogenous elicitor) and compounds released from plants by the action of the pathogen (endogenous elicitor) (Bertoncelli et al., 2015, Pascholati & Dalio, 2018). As stated by Sticher, Mauch Mani and Métraux (1997), elicitors are classified as physical or chemical, biotic or abiotic, and complex or defined, depending on their origin and molecular structure.

Chitosan and salicylic acid (SA) are both well-known natural inducers of disease resistance (Deffune, 2001). Chitosan is a high molecular weight cationic polysaccharide β-1,4-glucosamine produced by the deacetylation of chitin. It displays direct antimicrobial activity against a wide variety of plant pathogens (Badawy, Rabea, Taktak, 2014, Badawy Rabea, 2013, Azevedo et al., 2007, Bhaskara Reddy et al., 2000, Devlieghere Vermeulen, Debevere, 2004, El Ghaouth et al., 1992 & El Ghaouth et al., 1997). Several factors affect its antimicrobial potential, such as the degree of deacetylation, molecular weight, pH of the medium and temperature. Although the mechanisms responsible for its antimicrobial action are not yet fully elucidated (Devlieghere, Vermeulen, & Debevere, 2004), research suggests that the pH-sensitive polycationic amino groups of chitosan interact with the negative charges present on the cell membrane of the microorganism, causing changes in permeability of the plasma membrane and loss of intracellular components (Avadi et al., 2004, Yadav, Bhise, 2004, Stamford, Stamford, & Franco, 2008).

SA is a phenolic compound and plays an important role in signalling of plant defence mechanisms against pathogens (Mauch-Mani & Métraux, 1998). According to Campos (2009), SA has the capacity to activate the expression of resistance genes against pathogens including the synthesis and accumulation of pathogenesis-related proteins, which degrade structural polysaccharides of the fungi cell wall and cause disruption of its architecture, hindering the development of the organisms and preventing their growth (Zareie, Melanson, & Murphy, 2002). Like chitosan, SA also has a dual effect: it activates several plant defence mechanisms during host-pathogen interactions and acts as an antimicrobial abiotic agent (Cia, Pascholati, & Benato, 2007). SA has been shown to cause leakage of the pathogen's proteins to the medium, measurable lipid damage, and intracellular disorganisation (Rocha, 2014).

Considering these aspects, this study aimed to evaluate the effect of chitosan and SA on the *in vitro* development of *C. gloeosporioides*, responsible for affecting coffee production.

Materials and methods

C. gloeosporioides fungus was obtained from leaves showing symptoms of anthracnose, which were sterilised in 70% ethanol for 30 s, followed with 1% sodium hypochlorite for 1 min and then washed in sterile distilled water (Vieira et al., 2012). The tissues were dried on sterile filter paper and then transferred to Petri plates

containing agar-water culture and incubated in a growth chamber (biological oxygen demand-BOD) at 25 °C for 7 days adapted from Silva et al. (2006). The pure culture of the fungus was obtained from the new plates containing potato dextrose agar (PDA) for further experiments (Bozza, 2009).

Suspension plates containing sporulated fungi (12 days) were used to obtain conidia. The conidia were removed with the aid of a cover slip and sterile distilled water (10 mL), according to Maia (2013). The resulting suspension was filtered through cheesecloth to remove PDA debris. A haemocytometer was used to determine the conidia concentration, which was adjusted to 10^4 conidia mL^{-1}.

SA (Sigma Aldrich Chemicals, SP, Brazil) was dissolved in 10% ethanol at 0, 1.25, 2.5 and 5 mM. Low molecular weight Chitosan with 75–85% deacetylation degree (Sigma Aldrich Chemicals, SP, Brazil) suspensions (0, 1, 2 and 4 mg mL^{-1}) were prepared according to Bhaskara Reddy et al. (2000), using 0.05 N HCl as a solvent and adjusting the final pH to 5.6. The fungicide with active principle carbendazim (0,5L para 100 L de solução), used as a negative control for its efficiency against *Colletotrichum gloeosporioides* (Nakpalo et al., 2017), was prepared according to the manufacturer's specifications and used as a positive control. Solutions of HCl and ethanol alone were used as negative controls for chitosan and SA, respectively.

In order to evaluate the effect of the compounds on the germination of conidia, 100 µL aliquots of fungal conidial suspension (10^4 conidia mL^{-1}) were deposited in ELISA plate wells (Regente, 1997). Then, 100 µL of the various chitosan and SA concentrations were added. After incubation at 25 °C for 24 h under a photoperiod of 12 h in the light and 12 h in the dark, the percentage of spore germination was evaluated. A spore was considered germinated when the length of the germination tube was equal to or greater than the smallest diameter of the conidia. The conidia were enumerated in the quadrants of the haemocytometer (Optik Labor) until 100 conidia in each replicate (Overall, 400 conidia per treatment), with the aid of an optical microscope (Olympus CX31). The percentage of germination inhibition (PGI) was determined by the equation, according to Celoto et al., (2008):

$$PGI = \frac{\text{control germination} - \text{treatment germination x 100}}{\text{control germination}}$$

In the assessment of mycelial growth, chitosan was prepared as described above, autoclaved at 120 °C and then added to the autoclaved PDA medium. The desired SA concentrations (0, 1.25, 2.5 and 5 mM) were prepared in PDA medium and then autoclaved. These concentrations were used according studies evaluating antimicrobial activity of SA (Rocha, 2015 & Wang et al., 2011). The treatments were poured into Petri dishes. After solidification of the medium, a 4 mm diameter mycelial disc was removed from the edge of the colonies grown on PDA medium and placed in the centre of each Petri dish. The plates were sealed with plastic film and placed in a BOD incubator at 25 °C. The mycelial growth was assessed daily, by measuring the colony diameter in two perpendicular axes until the mycelia of at least one of the treatments reached the edge of the plate. The resulting data were used to calculate the mycelial growth speed index (MGSI), according to the following index (Oliveira, 1991): $MGSI = \Sigma(D-Da)\ N/N$, where D = current colony average diameter, Da = colony average diameter from the previous day and N = number of days after subculturing.

The experiments were performed in a completely randomised design with four replicates per treatment. Each repetition consisted of a well from the ELISA plate or a Petri dish for the spore germination and mycelial growth experiment, respectively.

The data were analysed for normality (Shapiro-Wilk) and those that did not show a normal distribution were log10-transformed. All data were then evaluated by one-way analysis of variance (ANOVA) and Tukey's multiple comparison test.

Results and discussion

According to the *C. gloeosporioides* conidial germination results in the presence of SA (0, 1.25, 2.5 and 5 mM) (Table 1), a significant difference between treatments ($p = 0.001$) was observed.

Particularly, the 5 mM SA treatment differed from the controls (water and 0 mM SA, respectively) but had similar values to the fungicide, promoting 58% conidial germination inhibition (Figure 1). The concentrations of 1.25 and 2.5 mm SA are not significantly different with controls (Figure 1 and Table 1). Bonaldo & Pascholati (2007) observed that conidial

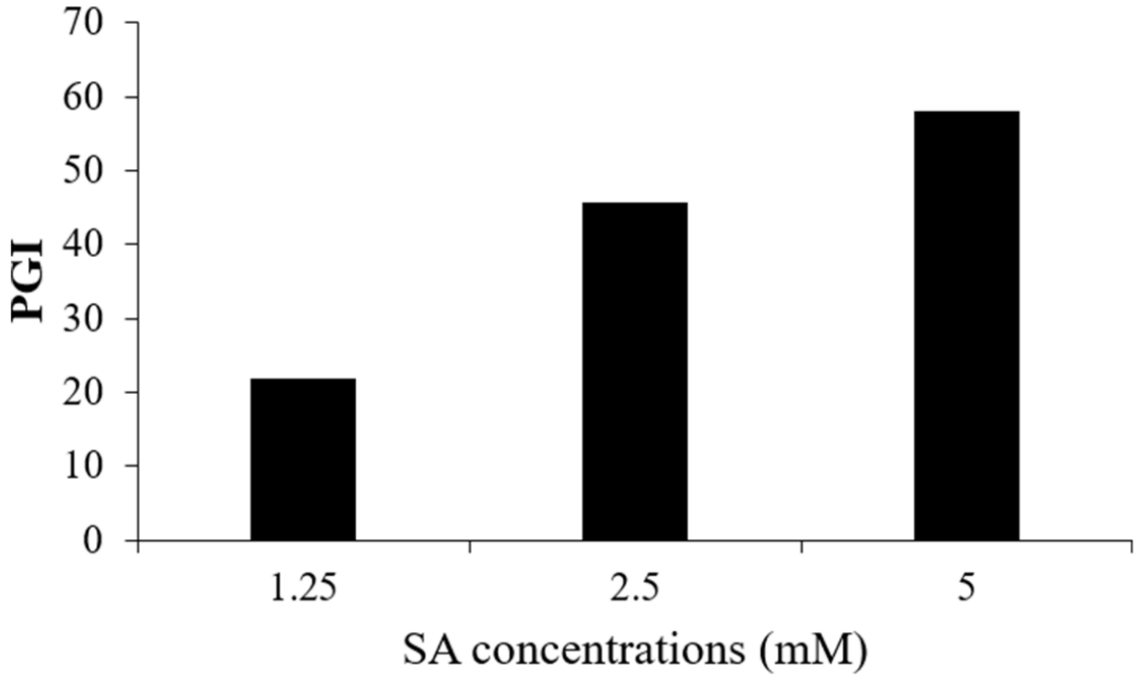
germination in the control (water) was than 50%, as well as the present work.

Table 1 - Conidial germination percentage of *Colletotrichum. gloeosporioides* in different salicylic acid (SA) concentrations and fungicide Carbendazim. Water and 0 mM SA represent the controls.

Treatments	Average ± standard error
Water	42.8±7.1a*
0 mM SA	40.0±6.3a
1.25 mM SA	31.3±5.7a
2.5 mM SA	21.8±2.8ab
5 mM SA	16,8±2,6b
Carbendazim	0,5±0,5b

* Mean ± standard error followed by the same letter do not differ by Tukey test at 5% significance.
Coefficient of variation = 3,6

Figure 1 - Percentage of germination inhibition (PGI) conidial of *Colletotrichum gloeosporioides* in different salicylic acid (SA) concentrations, compared to the control (AS 0 mM).



Source: Research Data.

In contrast to SA, under the experimental conditions used, chitosan did not inhibit conidial germination at any of the concentrations used (p = 0.397) (Table 2).

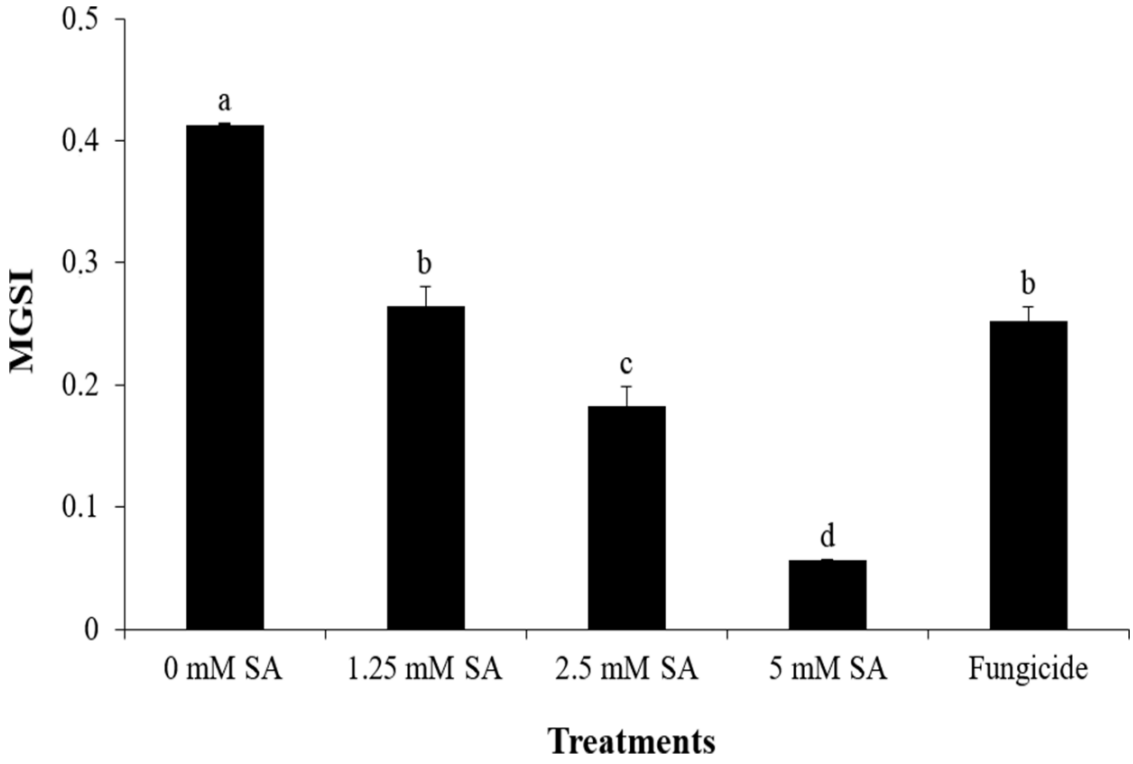
Regarding the mycelial growth, all SA concentrations reduced the MGSI in a dose-dependent manner, compared to 0 mM SA (p < 0.0001), with the greatest inhibition at 5 mM SA (Figure 2). At 1.25 mM, SA showed a similar effect to the fungicide, whereas, at 2.5 and 5 mM, SA showed a greater reduction than the fungicide. Compared to 0 mM SA, the 1.25, 2.5, and 5 mM SA reduced MGSI by 36, 56 and 86%, respectively.

Table 2 - Conidia germination percentage of *Colletotrichum. gloeosporioides* in different chitosan and fungicide Carbendazim. Water and 0 mg.mL^{-1} chitosan represent the controls.

Treatments	Average ± standard error
Water	46,8±16,5
0 mg.mL^{-1} Chitosan	70,0±5,7
1 mg.mL^{-1} Chitosan	56,8±9,0
2 mg.mL^{-1} Chitosan	32,3±5,9
4 mg.mL^{-1} Chitosan	42,3±7,0
Carbendazim	37,5±23,9
P value	0,397

Coefficient of variation = 14,6

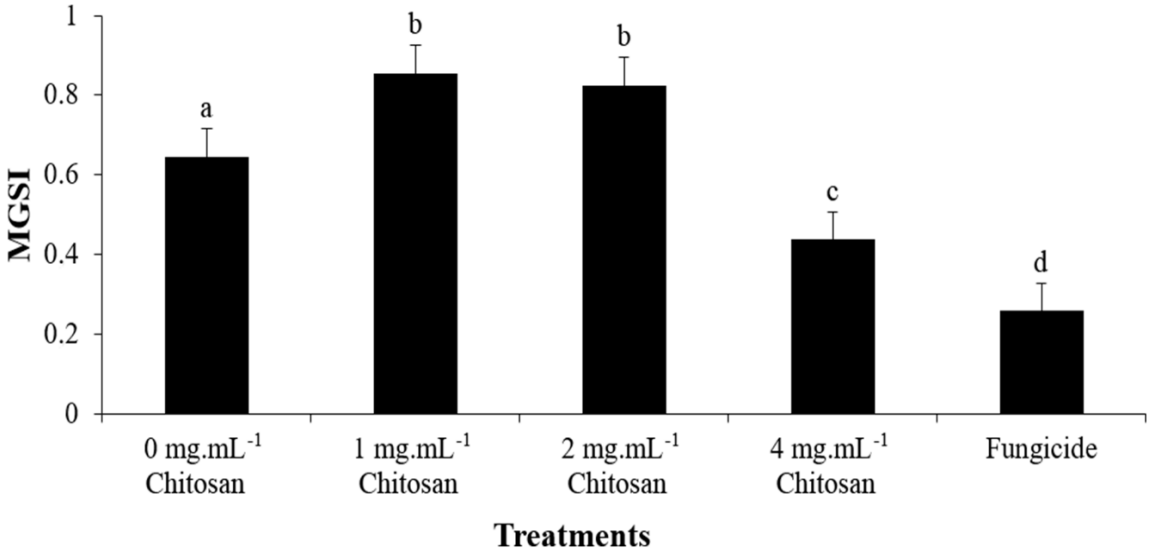
Figure 2 - Mycelial growth speed index (**MGSI**) of *Colletotrichum gloeosporioides* in different salicylic acid (SA) concentrations and fungicide carbendazim. Bars ± standard error followed by the same letter do not differ by Tukey's test at 5% significance.



Source: Research Data.

There was also a significant difference in the MGSI between the various chitosan concentrations and the controls ($p < 0.0001$). Specifically, 4 mg mL^{-1} chitosan proved effective at reducing the mycelial growth, while 1 and 2 mg mL^{-1} concentrations were not effective in controlling mycelial growth when compared to control (Figure 3).

Figure 3 - Mycelial growth speed index (**MGSI**) of *Colletotrichum gloeosporioides* in different chitosan concentrations and fungicide carbendazim. Bars ± standard error followed by the same letter do not differ by Tukey's test at 5% significance.



Source: Research Data.

SA acts as an endogenous signal in plant defence against pathogens (Mauch-Mani & Métraux, 1998). It plays a major role in both local resistance, systemic induced resistance to initial phytopathogen attack and hypersensitive cell death of infected cells (Zhao, Davis, & Verpoorte, 2005). The entire response is seemingly mediated by a complex signal transduction pathway regulated by several stress signals. The action of SA in plant defence, in response to various types of stress, has been proven by Taiz and Zeiger (2004).

Besides having an effect on the defence of the plant, studies have shown that SA can directly affect pathogens. In particular, SA can cause degradation of the plasma membrane of some pathogenic spores, such as *Penicillium expansum* (Link) in tomato (Wang et al., 2011). It was supposed that salicylate ions penetrate the fungal cell wall and their interaction with hydrogen ions present in the plasma membrane can cause disruption of the lipid bilayer or damage to the plasma membrane proteins associated with fungal spores (Rocha, 2014).

Evaluating the antifungal effect of SA on *P. expansum* in apple, Rocha (2014) showed that 2.5 mM SA inhibited 100% conidia germination, causing changes in the plasma membrane with no effect on the pathogen cell wall. In this study, it was observed that 5 mM SA was sufficient to inhibit 58% of *C. gloeosporioides* germination. A similar result was observed by Iqbal (2010), who demonstrated that SA acted as a fungicide against *Penicillium* sp. in citrus, noting a 60% decrease of conidia germination at 2 mM and 100% at 6 mM. Inhibition of conidial germination prevents the formation of the promycelium and establishment of the pathosystem, limiting the capacity of pathogen and disease evolution.

Beyond the effect of SA on the conidia germination of *C. gloeosporioides*, it was found that the compound also acts on mycelial growth at the lowest concentration tested (1.25 mM). At the maximum SA concentration used (5 mM), there was an 86% inhibition of the MGSI, compared to the control. In contrast, 0 mM SA (10% ethanol), had no effect on mycelial growth, showing that ethanol does not influence the SA antimicrobial activity.

Other studies performed *in vitro* also showed that the SA is able to act directly on the mycelial growth of *C. gloeosporioides*. Cia (2005) studied the effect of SA on *C. gloeosporioides* from papaya and observed that 10 mM of the compound directly decreased mycelial growth, reducing the fungus colony diameter and completely inhibiting its development, but there was no effect on spore germination. The SA fungitoxic role on *C. gloeosporioides* in bananas was demonstrated by Viana, Oliveira, Gomes

Pessoa, and Martins (2012), who tested 0.3 g SA, incorporated into 100 mL of PDA medium plus tetracycline (50 µg mL^{-1}) and noticed 100% inhibition of the fungus mycelial growth.

Chitosan has also demonstrated a direct antifungal effect. El Ghaouth et al. (1992) observed a reduction in conidia germination of *Botrytis cinerea* (Pers.) and *Rhizopus stolonifer* (Ehrenb.) Vuill. in strawberry when incubated with chitosan. Also, Di Piero and Garda (2008) found reduced germination of *Colletotrichum lindemuthianum* (Sacc. & Magnus) Briosi and Cavara on common bean with chitosan (20 µg mL^{-1}). Although the current study did not display reduction in conidia germination, it was observed that chitosan at 4 mg mL^{-1} reduced the mycelial growth of *C. gloeosporioides* on coffee leaves. This condition should not favor the development of fungus in the plant tissue and consequently in the disease progress of plant, by directly impeding its development.

Corroborating these results, Camilli et al. (2007) evaluated the inhibitory effect of chitosan against *B. cinerea* (Pers.) *in vitro*, finding that mycelial growth was completely inhibited at 1.5 and 2.0% (v/v) chitosan. However, no reduction in *B. cinerea* germination was noted after 24 h incubation.

Similarly, Roller and Covill (1999), analysed morphologic changes in *Zygosaccharomyces bailii (Lindner) Guillierm,* which causes deterioration of apple juice, in the presence of 5.0−0.5 g L^{-1} chitosan and revealed that there was no reduction in conidia germination when used at 0.5 g L^{-1}, as morphological changes observed initially returned to their initial state. This showed that at low concentrations, the effects of chitosan were reversible.

Maia et. al. (2010), studying the *in vitro* effect of chitosan at 0, 20, 40, 80 and 160 mg L^{-1} on the development of *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni and *Elsinoe ampelina Shear*, using Bordeaux mixture as default, observed that after 24h incubation, the Bordeaux mixture presented a greater reduction in germination when compared with chitosan. As a natural biodegradable polymer, chitosan probably has a less residual effect (Forbes-Smith, 1999).

Studies suggest that the action of chitosan on the spore germination of some pathogens occurs by changes in the cell wall and damage to the plasma membrane and vacuole. Liu et al. (2007) conducted a study to evaluate the effect of chitosan on the germination of *P. expansum* and *B. cinerea*. They observed that in the presence of chitosan, the germination was reduced, with a greater effect on *P. expansum*. In contrast, the mycelial growth of *B. cinerea* was the most affected by increasing the chitosan content in the medium. Thus, the plant pathogens sensitivity to chitosan varies according to the species and stage of development and the effect of product used (Fellipini, 2011).

Fellipini and Di Piero (2009) demonstrated that at 4 µg mL^{-1}, chitosan reduced 41% of *Colletotrichum acutatum* (J.H. Simmonds) mycelial growth on apple, while 100 µg ml^{-1} inhibited the conidia germination up to 66%. However, the most technical viability for the control of the disease was chitosan at 10 g L^{-1} (pH 4), which reduced the severity by 26%. El Ghaouth et al. (1994) illustrated the antifungal activity of chitosan against *Pythium aphanidermatum* (Edson) Fitzp, in cucumber. Di Piero and Garda (2008) found a greater inhibition of *C. lindemuthianum* mycelial growth with increased chitosan concentration evidencing for a dose-dependent effect of elicitor, that is, higher concentrations of chitosan has higher antifungal effect.

The bioactivity of chitosan is associated with its molecular weight, chain length, its degree of acetylation, and the distribution and conformation of the acetyl groups in its molecular chain (Pacheco et al., 2008). *In vitro* studies demonstrated that lower molecular weight chitosans are more effective in controlling the growth and germination of *Leptographium procerum* (W.B. Kendr.) M.J. Wingf. and *Sphaeropsis sapinea (Fr.) Dyko & B. Sutton* conidia than high molecular weight chitosans, due to the ease of penetration of the molecule through the fungi cell wall, interfering with their physiological activities (Chittenden & Singh, 2009).

In this study, low molecular weight chitosan was used, which may have contributed to the reduction in the mycelial growth of *C. gloeosporioides*. The cationic characteristics of chitosan exert a unique role in its action against pathogens. The positive charges can interact with negatively charged residues in the pathogenic cells, interfering with the permeability of the invading agent (Coqueiro & Di Piero, 2011).

It is likely that chitosan causes morphological and structural changes, disrupting the molecules of the pathogen cells Hadwiger et al., 1986 cited by Prapagdee et al. (2007) and inhibiting the growth of fungi (Cia et al., 2007). By forming a biofilm barrier, chitosan also promotes a

reduction in nutrient availability, consequently, the pathogens is unable to access the supplies essential to their mycelial growth, preventing the establishment of the pathosystem (Cia, 2005).

Conclusions

Salicylic acid caused a significant reduction in mycelial growth and conidia germination of *C. gloeosporioides*, in vitro bioassays.

Chitosan was able to reduce *C. gloeosporioides* mycelial growth, however, it had no effect on conidial germination.

Studies involving the antimicrobial activity of SA and chitosan must be performed to further elucidate their mechanisms of action against *C. gloeosporioides*.

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