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Antifungal activity of salicylic acid against *Penicillium expansum* and its possible mechanisms of action

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ABSTRACT

Apple is a fruit widely produced and consumed around the world. Blue mold (*Penicillium expansum*) is one of the main postharvest diseases in apples, leading to a wide use of fungicides and the search for alternative products. The aim of this study was to assess the effect of salicylic acid (SA) against *P. expansum*, elucidating its mechanisms of action. The antimicrobial effect was determined by exposing conidia to a 2.5 mM SA solution for 0 to 120 min, followed by incubation. The effect of pH on the efficacy of SA against *P. expansum* was assessed both *in vitro* and *in situ*. The action mechanisms were investigated through fluorescence assays, measurement of protein leakage, lipid damage, and transmission electronic microscopy. SA was capable of inhibiting 90% of the fungal germination after 30 min, causing damage to the conidial plasma membrane and leading to protein leakage up to 3.2 μg of soluble protein per g of mycelium. The pH of the SA solution affected the antimicrobial activity of this secondary metabolite, which inhibited the germination of *P. expansum* and the blue mold incidence in apples in solutions with pH ≤ 3 by 100%, gradually losing its activity at higher pH.

1. Introduction

Apples (*Malus domestica* Borkh.) are one of the most cultivated and consumed fruits worldwide, reaching a global production above 70 million tons in 2012 (Faostat, 2014). There are several diseases that can affect apples, including blue mold caused by *Penicillium expansum*, a very aggressive cosmopolitan fungus, tolerant to many adverse environmental conditions. It can spread quickly and produce the mycotoxin patulin, which can be deleterious to human health (da Rocha et al., 2014).

From harvesting to storage, apples should be exposed to several procedures that, if well performed, can significantly reduce the incidence of blue mold. This pathogen requires small injuries in the epidermis or natural openings to infect apples (Filonow, 2005; Mondino et al., 2009).

In general, disinfection procedures are mostly overlooked, in favor of the use of fungicides as the main way to control postharvest diseases (Calvo et al., 2007; Karabulut et al., 2002). Synthetic fungicides based on imidazole and dicarboximide groups are commercially available and recommended by the Ministry of Agriculture, Livestock and Food Supply in Brazil for the control of the blue mold.

However, the development of isolates resistant to synthetic chemicals, associated with growing public concerns related to health and environmental pollution, have increased the efforts to research alternative methods of fruit protection against pathogens (Mills and Golding, 2015). Among these, the use of phenolic compounds is of interest, not only due to the important roles of these compounds in plant defenses against pathogens, but also because they have proven to be beneficial to human health exhibiting antioxidant and anti-carcinogenic effects (Sanzani et al., 2010).

Salicylic acid (SA), a phenolic acid with an aromatic ring linked to a hydroxyl group or functional derivatives, is an endogenous plant hormone involved in plant growth development and cell signaling (Asghari and Aghdam, 2010; Tian et al., 2007). Many studies have evaluated its antifungal activity against several postharvest pathogens, including *P. expansum* (Yu and Zheng, 2006), *Botrytis cinerea* (Wang et al., 2011), and *Rhizopus stolonifer* (Panahirad et al., 2012). However, the mechanisms of action of SA against those pathogens have not been fully elucidated to date.

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Thus, this study aimed to assess the effect of SA on the germination and growth of *P. expansum* and elucidate its mechanisms of action. Such approach is assumed to be crucial for the development of efficient alternative strategies to replace the use of fungicides to control blue mold in apple fruits.

2. Materials and methods

2.1. Fungal isolate

P. expansum was isolated from an infected apple fruit showing typical symptoms of blue mold, identified and provided by Dr. Rosa Maria Sanhueza, and stored in the mycology collection of the Phytopathology Laboratory (Federal University of Santa Catarina, Florianópolis, Brazil) with the code MANE 138. The isolate was grown and maintained in potato dextrose agar (PDA) culture medium, at 25 °C for two weeks prior to use. The conidial suspension was prepared in apple juice (4%, v/v) and calibrated to the final concentration with the aid of a Neubauer chamber (hemacytometer).

2.2. Chemicals

The caffeic, chlorogenic, cinnamic, salicylic, and syringic phenolic acids were acquired from Sigma-Aldrich Co. (St. Louis, MO, USA). They were diluted in sterile distilled water with the aid of a magnetic bar and a stirrer until solutions were homogeneous (Shalmashi and Eliassi, 2008). The concentrations of the phenolic acids varied according to the experiments.

Calcofluor White Stain and propidium iodide were also acquired from Sigma-Aldrich Co. (St. Louis, MO, USA) and used at Sections 2.7.1 and 2.7.2 to evaluate the cell wall damage and the plasma membrane integrity of the conidia, respectively.

Finally, bovine serum albumin (BSA) was acquired from Sigma-Aldrich Co. (St. Louis, MO, USA) to quantify total protein and used as standard in Section 2.7.3.

2.3. Screening of phenolic acids

The antimicrobial potential of the different phenolic acids was assessed in concave slides. For this, 25 μL of a phenolic acid solution (cinnamic, caffeic, syringic, chlorogenic or salicylic) at 2.5 mM and 25 μL of *P. expansum* suspension (10^5 conidia/mL) were added to the slide cavity. Sterile distilled water and sodium hypochlorite (0.5%, v/v) were used as positive and negative controls, respectively. The concave slides were placed inside Petri dishes and incubated for 20 h, at 25 °C ± 1 °C, under high relative humidity. Each treatment was replicated four times and each replicate was represented by a cavity in the concave slide. The germination of 100 conidia was evaluated for each replicate with the aid of an optical microscope (FWL1500 T, Feldmann Wild Leitz). The experiment was conducted twice.

2.4. Minimum contact time between salicylic acid and P. expansum

The minimum contact time between SA and *P. expansum* was determined according to Liu et al. (2007), with modifications. Ten milliliters of a *P. expansum* suspension (2×10^8 conidia/mL) prepared in apple juice were added to 10 mL of sterile distilled water (negative control), 0.5% sodium hypochlorite (positive control) or 2.5 mM SA, under constant stirring, at room temperature. Aliquots (500 μL) were collected after 0, 5, 15, 30, 60, and 120 minute incubation and being centrifuged (8000 *g*, 5 min, 4 °C). The conidia were collected, washed twice with a 50 mM phosphate buffer (pH 7.0) and re-suspended in apple juice (4%, v/v) to a final concentration of 10^5 conidia/mL. Finally, the germination test in concave slides described above was performed with the conidial suspension. The experiment was conduced three times.

2.5. Effect of acidification or alkalization of the SA solution on P. expansum germination

Twenty milliliters of the conidial suspension (10^5 conidia/mL) prepared in apple juice were added to 20 mL of a 2.5 mM SA solution. Then the pH values of the final mixture were adjusted to 2.0, 2.5, 2.8, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 or 6.0 by the addition of 0.05 N HCl or 2 N NaOH. As control, apple juice (4%, v/v) was used without SA addition at the same pH values described above. The resulting conidial suspensions were submitted to the germination test described above. The experiment was repeated three times.

2.6. Effect of acidification or alkalization of the SA solution on the incidence of P. expansum rot in apples

Standardized apple fruits cv. Fuji, acquired from COOPERSERRA (São Joaquim/SC, southern Brazil), were disinfected with a chlorine solution (0.5%, v/v), injured twice in the equatorial zone with a standard needle (1 mm × 5 mm) and immersed into a *P. expansum* conidial suspension (10^4 conidia/mL) prepared in sterile distilled water or in salicylic acid solution (both pH ranging from 2 to 6, adjusted with 0.05 N HCl or 2 N NaOH). These suspensions were stirred for 30 min followed by the immersion of apples for 2 min. The fruits were then transferred to plastic containers and kept at 25 °C under high relative humidity, in the dark, throughout the experimental period. Three replicates per treatment were made, where a plastic container containing five fruits represented one replication.

The rate of growth of the lesions was determined by measuring the diameter of the lesion (cm) of each injury made in every single fruit, with a standard ruler, every 3 days (first evaluation was performed after 3 days of incubation). Based on the average value of the lesion diameters over time, the lesion growth rate was estimated in each tray as follows: $LGR = (\Sigma (\theta_t - \theta_{t-1}) / t)$; where "θ" represents the average diameter of the lesion at time "t". The results were expressed in cm/day.

Moreover, the incidence was calculated at the end of the experiment by the division of the number of injuries made in the apples presenting blue mold symptoms by the total number of injuries made in these fruits. The average results were expressed in %.

2.7. Action mechanisms of SA against P. expansum

2.7.1. Damage to the cell wall of P. expansum conidia

The evaluation of the SA effects on the pathogen cell wall was performed according to Cerioni et al. (2010), with modifications. Firstly, the conidia of *P. expansum* were suspended in 2.5 mM SA or sterile distilled water (control) to a final concentration of 10^8 conidia/mL. The suspensions were maintained at room temperature under constant stirring. Samples (500 μL) were collected at intervals of 0, 5, 15, 30, 60, and 120 min, centrifuged (8000 *g*, 5 min, 4 °C), double washed with a 50 mM sodium phosphate buffer (pH 7.0) and sterile distilled water, centrifuged (8000 *g*, 5 min, 4 °C), and re-suspended in apple juice (4%, v/v) to a final concentration of 10^5 conidia/mL. Aliquots of 50 μL were then transferred to concave slides and incubated as described above. The samples were then transferred to microscope slides, 10 μL of Calcofluor White Stain reagent (Sigma-Aldrich, USA) at 50 μg/mL and 10 μL of 10% (w/v) potassium hydroxide added, and incubated in the dark for 15 min. The conidial morphology and germ tubes were examined with the aid of a fluorescence microscope (Eclipse 50i, Nikon) at the wavelengths of 395 nm (excitation) and 440 nm (emission). The images were recorded with a Nikon digital camera (Coolpix P500) and the experiment conducted twice.

2.7.2. Damage to the plasma membrane of P. expansum conidia

Damage to the plasma membrane of *P. expansum* conidia exposed to SA was determined as described by Liu et al. (2007), with modifications. Conidial suspensions were prepared in 2.5 mM SA or sterile distilled