BIOTECHNOLOGY

Exogenous application of salicylic acid to control coffee rust

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ABSTRACT. The exogenous application of salicylic acid can induce plant resistance against pathogens. However, little is known about the potential uses of this bioregulator for controlling coffee diseases. In this study, we assessed the effect of applying salicylic acid (SA – 150 mg L^{-1}) on the management of coffee rust (*Hemileia vastatrix*) in a 7-year-old coffee plantation with low crop load (651.6 kg ha^{-1} in 2017). For comparison, plants were sprayed with protectant fungicide (copper hydroxide – CH) and standard fungicides (SF) used by local farmers (boscalid, pyraclostrobin + epoxiconazole, and copper hydroxide). Non-treated plants were included as a negative control. Five monthly applications were performed from November 2016 to March 2017. Rust incidence and severity, defoliation, and growth of plagiotropic branches were evaluated monthly. The activity of catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD) and total proteins was assessed one day after the first, third, and fifth product applications. Compared to untreated plants, SA reduced the severity and incidence of rust from 36.3 to 54.7%, while CH and SF reduced disease from 31.8 to 54.6% and from 83.8 to 88%, respectively. SA reduced defoliation by 54.1%. SA increased the concentration of CAT, APX, and SOD after the first application. However, this effect was not observed after subsequent applications. Foliar application of SA reduces the severity and incidence of coffee rust and defoliation in plants with a low crop load.

Keywords: bioregulator; *Coffea arabica*; *Hemileia vastatrix*; plant resistance.

Introduction

Brazil is the world's largest coffee producer and exporter, with a cultivated area of 2.16 million hectares. Minas Gerais state produces more than 50% of Brazilian coffee. However, the occurrence of diseases limits coffee production in various Brazilian regions (Zambolim, 2016). Coffee rust, caused by *Hemileia vastatrix,* is prevalent in all coffee growing areas of the country and can cause losses of up to 50% during severe epidemics (Zambolim, 2016).

Chemical control is an essential component of foliar disease management in coffee. However, this strategy increases crop production costs and imposes risks to the environment and human health. Due to these limitations, more environmentally friendly and sustainable methods are needed to control coffee diseases. Applying resistance inducers can control diseases and reduce environmental and social impacts (Klessig, Choi, & Dempsey, 2018).

Biological and chemical inducers can stimulate plant resistance (Pieterse et al., 2014). Salicylic acid, for instance, is a phenolic compound that acts on plant signaling against biotic agents, inducing acquired systemic resistance (Kumar, 2014). Exogenous application of salicylic acid may increase the activity of enzymes that decrease reactive oxygen species, which are produced in response to stresses caused by biotic or abiotic agents (Gill & Tuteja, 2010; Asghari & Hasanloee, 2015; Csiszár et al., 2018). Ascorbate peroxidase, catalase, and peroxidase activities increase in plants treated with salicylic acid (Asghari & Hasanloee, 2015; Csiszár et al., 2018). The accumulation of proteins related to pathogenesis is also observed in plants after spraying salicylic acid (Sillero, Rojas-Molina, Avila, & Rubiales, 2012).

Plant diseases can be managed by salicylic acid, including faba bean rust (Sillero et al., 2012), cucumber damping-off (Bertoncelli et al., 2015), cucumber mosaic (Luo et al., 2011), *Ascochyta* chickpea blight (Ghazanfar, Wakil, & Sahi, 2011), and clubroot disease of cruciferous plants (Lovelock, Donald, Conlan, & Cahill, 2013). Little is known about the potential use of salicylic acid in inducing resistance to coffee diseases. One of the few studies on this topic was by Alemu, Adugna, Lemessa, and Muleta (2018), who reported that

the application of salicylic acid at 10.0 mm reduced the incidence of coffee berry disease, caused by *Colletotrichum kahawae*, in susceptible and moderately resistant coffee cultivars. Due to the potential for bioregulators in agriculture, we assessed the effect of the application of salicylic acid for the management of coffee rust under field conditions.

Material and methods

Study area

The experiment was conducted twice on a coffee (*Coffea arabica* cv. Topázio) farm from November 2016 to March 2017 in Rio Paranaíba, Minas Gerais State, Brazil (19° 11' 29.0" S, 46° 09' 41.1" W; 1,050 m altitude). During the experiment, total rainfall and mean temperature were 1,022 mm and 21.36°C.

The plants were 7-year-old, grown at 3.8 x 0.7 m spacing, and with low crop load (651.6 kg ha^{-1} in 2017). The treatments were arranged in randomized blocks with five replicates. The experimental plots were 30 m long (one row of approximately 42 plants). The four central plants within each plot were assessed. Four marked branches from the middle third of the plant were evaluated on the west- and east-facing sides.

Experimental design

Coffee plants were treated with salicylic acid, copper hydroxide (protectant fungicide used for controlling fungal and bacterial diseases in coffee), and a combination of protectant and systemic fungicides (hereafter, standard-fungicides), commonly used by local farmers to manage coffee rust (Table 1). Untreated plots were used as a negative control. Salicylic acid (PhytoTech Labs, Lenexa-KS, USA) was applied at 150 mg L^{-1}. This dosage was chosen based on previous studies (Farooq, Basra, Wahid, Ahmad, & Saleem, 2009; Najafian, Khoshkhui, Tavallali, & Saharkhiz, 2009). Five monthly applications were performed during the rainy season, starting in November 2016. Cultural practices were performed on all plots, except for spraying fungicides. The products were applied using a sprayer (model Arbus 2,000; Jacto, Pompeia, state São Paulo, Brazil) connected to a tractor, with a spray volume of 400 L ha^{-1}. To avoid plot contamination, the products were applied as follows: salicylic acid – copper hydroxide – standard fungicides.

Table 1. Treatments used for the management of coffee rust.

Treatment	Application date				
	24/11/16	19/12/16	24/01/17	22/02/17	21/03/17
Negative control	-	-	-	-	-
Copper hydroxide (CH)	2 kg ha^{-1}	2 kg ha^{-1}	2 kg ha^{-1}	2 kg ha^{-1}	2 kg ha^{-1}
Salicylic acid	150 mg L^{-1}	150 mg L^{-1}	150 mg L^{-1}	150 mg L^{-1}	150 mg L^{-1}
Standard fungicides	150 g ha^{-1} of Cantus® (boscalid) and 2 kg ha^{-1} of CH	1 L ha^{-1} of Opera® (pyraclostrobin + epoxiconazole) and 2 kg ha^{-1} of CH	1 L ha^{-1} of Opera® (pyraclostrobin + epoxiconazole) and 2 kg ha^{-1} of CH	150 g ha^{-1} of Cantus® (boscalid) and 2 kg ha^{-1} of CH	1 L ha^{-1} of Opera® (pyraclostrobin + epoxiconazole) and 2 kg ha^{-1} of CH

Negative control = Untreated plants. Copper hydroxide = Tutor®, BASF S.A, São Paulo, state São Paulo, Brazil, 691 g kg^{-1} of active ingredient. Salicylic acid = PhytoTech Labs, Lenexa-KS, USA.

Disease assessment

The natural occurrence of coffee rust (*Hemilea vastatrix*) was evaluated monthly (30 days after spraying), totaling five assessments during the experiment. On branches marked on the east- and west-facing sides, each leaf was scored for incidence and severity. Disease incidence was estimated by the proportion of diseased leaves to the total number of leaves examined. Disease severity (the proportion of diseased tissue to the total healthy leaf tissue) was quantified based on the diagrammatic scale developed by Capucho, Zambolim, Duarte, and Vaz (2011). Defoliation was estimated by the proportion of missing leaflets to the total number of leaflets on each marked branch. To estimate plant growth, we counted the number of nodes per branch.

Enzymatic analysis

Leaves were collected one day after the first, third and fifth application of the products. Samples were taken from branches not used for disease assessments and defoliation. Two healthy and completely expanded leaves were collected from the second pair of branches located in the bottom third of the plants.

The leaves were placed in 50 mL Falcon tubes, stored inside styrofoam containers packed with ice and transported to the laboratory for processing. The total soluble proteins and the activity of the enzymes superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) were assessed on the day of collection. To extract soluble proteins, approximately 100 mg of plant tissue were ground in liquid nitrogen and again in 1.5 mL of Tris-HCl buffer. The ground material was centrifuged for 10 min. at 6,000 g. An aliquot of 100 µL of the supernatant was mixed with 3 mL of Bradford's reagent (LGC Biotechnologia, Cotia, state São Paulo, Brazil) and incubated at room temperature for 5 min. Absorbance was measured at 595 nm and soluble protein concentration was calculated from the standard curve constructed with bovine serum albumin (BSA; Bradford, 1976).

To determine enzymatic activity, a crude extract was prepared from 200 mg of fresh leaves. The material was macerated in liquid nitrogen, followed by the addition of 1,500 µL of the extraction medium (750 µL of 200 mM potassium phosphate buffer pH 7.8, 15 µL of 10 mm ethylenediamine acid – EDTA, 150 µL of 200 mm ascorbic acid, 585 µL Milli-Q water). The sample was poured into a 2 mL Eppendorf tube and centrifuged at 12,000 g for 20 min. at 4°C. The supernatant was collected and stored on ice until reading (Sperotto, 2014).

The SOD activity was assayed by the methods of Beauchamp and Fridovich (1971) and Giannopolitis and Ries (1977), which are based on the amount of enzyme able to inhibit 50% of nitro blue tetrazolium (NBT) at 560 nm, expressed in U min.$^{-1}$ mg^{-1} protein.

To determine CAT, a 12.5 µL aliquot of the crude enzymatic extract was used according to Havir and McHale (1987) and Anderson, Prasad, and Stewart (1995). CAT activity was determined as a decrease in absorbance at 240 nm for 1 min. (with 10 s intervals) due to the degradation of H_2O_2. Results were expressed as µmol min.$^{-1}$ mg protein^{-1}.

APX activity was evaluated from a 12.5 µL aliquot of the enzymatic extract according to Nakano and Asada (1981), and the enzymatic activity was calculated using the molar extinction coefficient of 2.8 mm^{-1} cm^{-1}. Results were expressed as µmol min.$^{-1}$ mg protein^{-1}. For all enzymes and total soluble proteins, absorbance assessments were performed using a spectrophotometer (UV-Vis Evolution 300, Thermo Scientific).

Statistical analysis

Incidence and severity data were converted into area under the disease progress curve (Campbell & Madden, 1990). Due to the homogeneity of variance between experiments 1 and 2 (replicates), the data were pooled and submitted to analysis of variance (ANOVA, $p < 0.05$), followed by the Tukey test ($p < 0.05$).

Results and discussion

The application of salicylic acid, copper hydroxide and the standard fungicides reduced the severity and incidence of rust in coffee plants with low crop load (Figure 1). The intensity of rust in plants treated with salicylic acid was similar to those treated with copper hydroxide, a protectant fungicide, and the standard fungicides, a combination of protectant and systemic fungicides commonly used by local farmers. The exception was for leaves located on the west-facing side, which had a lower incidence and severity of rust when treated with the standard fungicides than with salicylic acid (Figure 1). Compared to untreated plants, salicylic acid reduced the incidence and severity of rust from 36.3 to 54.7%. The application of copper hydroxide and standard fungicides reduced the disease from 31.8 to 54.6% and from 83.8 to 88%, respectively.

Salicylic acid, copper hydroxide and standard fungicides reduced defoliation from 54.1 to 92.1% (Figure 2A). Defoliation of plants treated with salicylic acid was 20.74 versus 45.2% in non-treated plants. Only 3.5% of leaves fell off in plots treated with standard fungicides.

The number of nodes per plagiotropic branch was higher in plants sprayed with standard fungicides. The lowest number was observed in the negative control (Figure 2B). Plants treated with salicylic acid had a similar number of nodes to the untreated plants (Figure 2B).

Foliar application of salicylic acid altered the concentration of total soluble proteins (TSP), catalase (CAT), ascorbate peroxidase (APX), and superoxide dismutase (SOD) in coffee plants (Table 2). After the first application, plants treated with salicylic acid had TSP levels lower than those observed in the other treatments. The concentration of TSP however increased after the last application of salicylic acid, reaching levels similar to those observed in plants treated with copper hydroxide and standard fungicides. The levels of APX, CAT and SOD in plants treated with salicylic acid were at least twice as high as the other treatments after the first application (Table 2). After the second application, the levels of CAT were the same in all

treatments. Plants treated with salicylic acid had higher concentrations of SOD and APX than the control (Table 2). After the third application, the levels of TSP and antioxidant enzymes were similar among all treatments (TSP = 23.9 ± 3.17; CAT = 1.79 ± 1.37; APX = 30.19 ± 4.14; SOD = 2.47 ± 0.16).
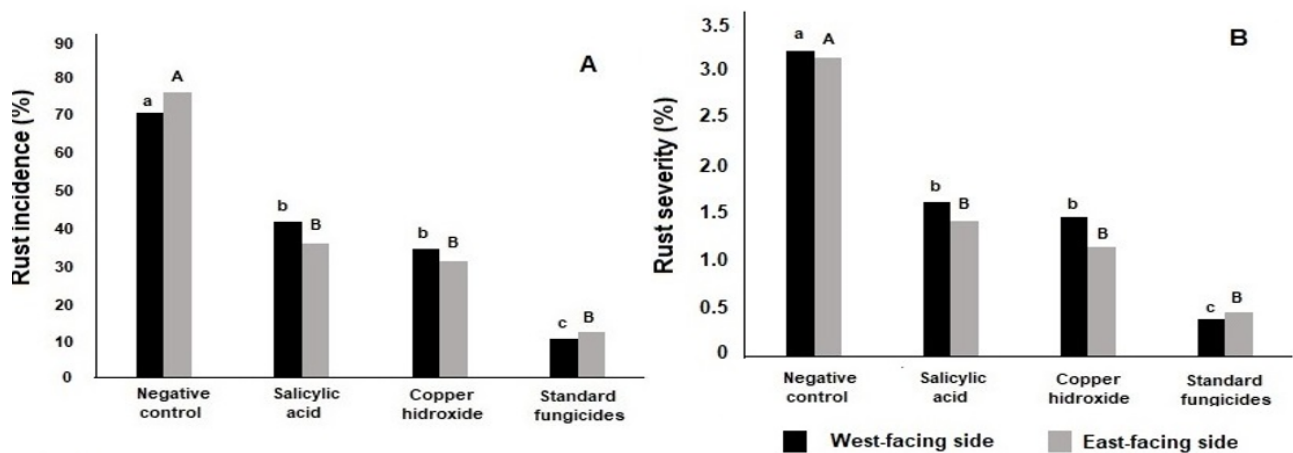

Figure 1. Mean area under the disease progress curve for incidence (A) and severity (B) of coffee rust, caused by *Hemileia vastatrix*, on west-facing and east-facing sides of plants treated with five applications of salicylic acid (150 mg L^{-1}), copper hydroxide (2 kg ha^{-1}), and standard fungicides used by local farmers (boscalid, copper hydroxide, and pyraclostrobin + epoxiconazole). Means followed by the same capital letter (east-facing side) and the same lowercase letter (west-facing side) are not significantly different by the Tukey test (p < 0.001).
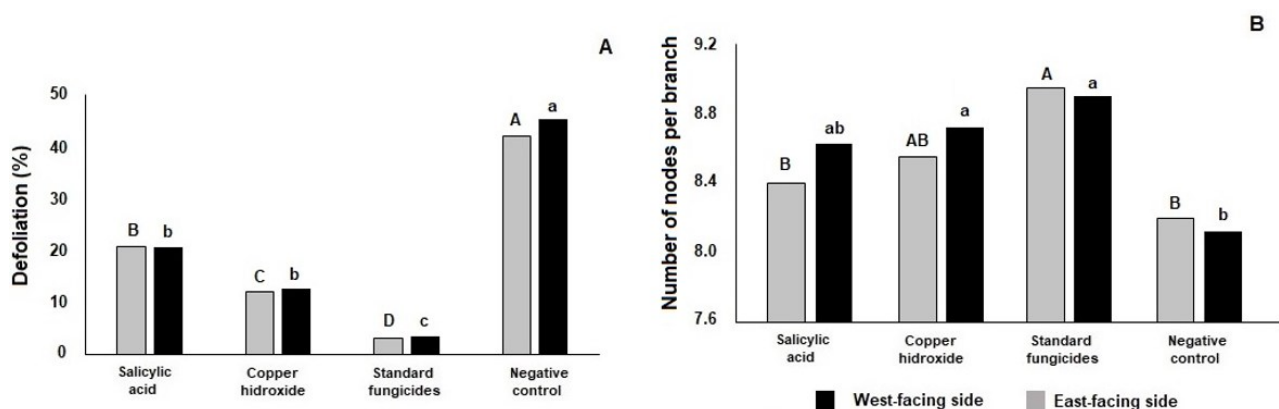

Figure 2. Defoliation (A) and number of nodes per plagiotropic branch (B) on west-facing and east-facing sides of plants treated with five applications of salicylic acid (150 mg L^{-1}), copper hydroxide (2 kg ha^{-1}), and standard fungicides used by local farmers (boscalid, copper hydroxide, and pyraclostrobin + epoxiconazole). Means followed by the same capital letter (east-facing side) and the same lowercase letter (west-facing side) are not significantly different by the Tukey test (p < 0.001).

Table 2. Concentration of total soluble proteins (TSP) (mg of protein g of fresh mass^{-1}), superoxide dismutase (SOD) (U min.$^{-1}$ mg^{-1} of protein), ascorbate peroxidase (APX) (μmol min.$^{-1}$ mg of protein^{-1}) and catalase (CAT) (μmol min.$^{-1}$ mg of protein^{-1}) in coffee plants treated with salicylic acid (150 mg L^{-1}), copper hydroxide (2 kg ha^{-1}), and standard fungicides used by local farmers (boscalid, copper hydroxide, and pyraclostrobin + epoxiconazole).

Treatment	TSP	SOD	APX	CAT	TSP	SOD	APX	CAT
		25/11/16				25/01/17		
Salicylic acid	10.49 b	6.99 a	117.11 a	7.81 a	8.08 b	7.14 a	59.89 a	2.71 ns
Copper hydroxide	29.69 a	2.76 b	51.96 b	3.45 b	11.41 ab	5.32 ab	43.85 b	4.50
Standard fungicides	25.57 a	2.35 b	40.86 b	2.78 b	11.99 ab	4.31 b	44.48 ab	3.03
Negative control	30.01 a	1.86 b	33.26 b	0.95 b	15.55 a	3.81 b	37.84 b	3.73

Means followed by the same letter within a column are not significantly different by the Tukey test (p < 0.05). Ns = not significant by the F test (p < 0.05).

Under low crop load conditions, foliar application of salicylic acid reduces the intensity of coffee rust to levels similar to those observed with the application of a combination of protectant and systemic fungicides, including copper hydroxide, boscalid, epoxiconazole + pyraclostrobin. Defoliation is the most common damage caused by coffee rust (Zambolim, 2016), and the application of salicylic acid reduces defoliation by controlling the disease. The incidence of coffee rust was higher than 70% in untreated plants, which confirms that the conditions were conducive to the disease, even under low crop load. The application of salicylic acid

may have activated the plant defense mechanisms against *H. vastatrix*, reducing the establishment of the pathogen in coffee tissues (Kumar, 2014). Salicylic acid is an important signaling molecule involved in acquired systemic resistance (Klessig et al., 2018). To enhance the effect of salicylic acid as a resistance inducer, spraying must be performed before pathogen arrival, as recommended for protectant fungicides.

Protein consumption was higher after the first application of salicylic acid, culminating with increased production of the antioxidant enzymes superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT), which is consistent with the findings of Rasmussen, Hammerschmidt, and Zook (1991). Thus, these enzymes act against reactive oxygen species in plants started soon after the application of salicylic acid (Herrera-Vásquez, Salinas, & Holuigue, 2015). Excessive production of oxygen-reactive species, such as superoxide anion (O_2) and hydrogen peroxide (H_2O_2), may be one of the plant's first responses to pathogen attack and, consequently, triggers cell death. SOD, CAT and APX are enzymes involved in the detoxification of reactive oxygen species (Sharma, Jha, Dubey, & Pessarakli, 2012) and the application of salicylic acid increases the activity of these enzymes in plants, including apple (Zhang et al., 2016) and wheat (Sorahinobar et al., 2015). As the intensity of coffee rust increased over the course of the experiment, the signaling process induced by the exogenous application of salicylic acid was less evident. Plants under attack by pathogens react by activating defense mechanisms, including by increasing the concentration of reactive oxygen species (Herrera-Vásquez et al., 2015).

Coffee development was not impaired by the application of salicylic acid, as determined by the number of nodes per plagiotropic branch. The activation of resistance mechanisms by exogenous application of bioregulators may require energy for defense and limit plant growth (Vicente & Plasencia, 2011). The highest growth in plants treated with the standard fungicides is possibly due to the more efficient control of coffee rust. Epoxiconazole and pyraclostrobin also increase photosynthesis and enhance the antioxidant system of coffee plants (Honorato Júnior, Zambolim, Aucique-Pérez, Resende, & Rodrigues, 2015). Further studies may reveal whether the application of salicylic acid controls coffee rust in plants with high crop load and whether salicylic acid-induced signaling interferes with the yield of coffee beans.

Conclusion

Foliar application of salicylic acid (SA – 150 mg L^{-1}) reduces the severity and incidence of coffee rust from 36.3 to 54.7% and defoliation by 54.1% in plants with low crop load. SA increases the activity of the antioxidant enzymes ascorbate peroxidase, superoxide dismutase, and catalase after the first application with no significant effect after subsequent applications. The application of SA may activate the plant defense mechanisms against *H. vastatrix*, reducing the establishment of the pathogen in coffee tissues. To enhance the effect of SA as a resistance inducer, spraying must be performed before pathogen arrival, as recommended for protectant fungicides.

Acknowledgements

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