SILVER NANOPARTICLES AND RESISTANCE INDUCERS IN THE CONTROL OF BANANA ANTHRACNOSIS

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ABSTRACT: The search for techniques to control anthracnose (*Colletotrichum musae*) of lower toxicity to human health and environmental impact is essential for the development of a sustainable system in the banana production chain. Among these strategies, with great potential for use in agriculture and still lacking efficiency studies, silver nanoparticles (AgNPs) and resistance inducers stand out. Thus, the aim of this work was to evaluate the *in vitro* antifungal activity of AgNPs and resistance inducers in the control of post-harvest banana anthracnose. In *in vitro* experiment, the fungus was cultured in PDA culture medium added of different AgNP concentrations (0, 25, 50, 100 and 200 ppm) and resistance inducers (*Bacillus subtilis*, acibenzolar-S-methyl and *Saccharomyces cerevisiae*). The variable analyzed was mycelial growth inhibition (%), while in the post-harvest experiment, banana fruits were immersed for three minutes using the same treatments as the *in vitro* study. In this case, incidence (% of fruits with lesions) and severity (% of epidermis covered by lesions) were evaluated. Colloidal silver concentration at 100 ppm, as well as the resistance of *Bacillus subtilis* and acibenzolar-S-methyl inducers were the most efficient treatments in the control of post-harvest banana anthracnose caused by *Colletotrichum musae*.

KEYWORDS: *Colletotrichum musae*, colloidal silver, post-harvest, elicitors.

NANOPARTÍCULAS DE PRATA E INDUTORES DE RESISTÊNCIA NO CONTROLE DA ANTRACNOSE NA BANANA

RESUMO: A busca por técnicas de controle da antracnose (*Colletotrichum musae*) de menor toxicidade a saúde humana e impacto ambiental é essencial para o desenvolvimento de sistema sustentável na produção da banana. Entre essas estratégias, com grande potencial para uso na agricultura e que ainda carecem de estudos de eficiência temos a utilização de nanopartículas de prata (AgNPs) e indutores de resistência. Assim, o objetivo do trabalho foi avaliar a atividade antifúngica de AgNPs e de indutores de resistência *in vitro* e no controle da antracnose da banana em pós-colheita. No experimento *in vitro*, cultivou-se o fungo em meio de cultura BDA acrescido das diferentes concentrações de AgNPs (0, 25, 50, 100 e 200 ppm) e os indutores de resistência (*Bacillus subtilis*, acibenzolar-S-metil e *Saccharomyces cerevisiae*). A variável analisada foi inibição do crescimento micelial (%). Enquanto, que no experimento em pós-colheita foi feito imersão dos frutos de banana por três minutos, utilizando os mesmos tratamentos do estudo *in vitro*. Nesse caso, foi avaliado a incidência (% de frutos com lesão) e severidade (% da epiderme coberta por lesões). A concentração de 100 ppm de prata coloidal, bem como os indutores de resistência *Bacillus subtilis* e acibenzolar-S-metil foram os tratamentos mais eficientes no controle da antracnose em frutos de banana causada por *Colletotrichum musae* em pós-colheita.

PALAVRAS CHAVE: *Colletotrichum musae*, prata coloidal, pós-colheita, elicitores.

INTRODUCTION

The use of pesticides for pathogen control is an attractive strategy for farmers due to their simplicity, low use of manpower and satisfactory short-term results; however, the positive aspects are suppressed over time and a succession of disadvantages emerge, for example, accumulation of chemical residues in soil and water, occurrence of resistant microorganisms and environmental imbalance due to the lack of selectivity of products used (Ghini and Bettiol, 2002).

Among crops of greater social, economic and cultural importance in Brazil, with high use of chemical pesticides, banana stands out (*Musa* spp.), which is the fruit with the highest *per capita* consumption (20 kg / inhabitant / year) in Brazil (IBGE, 2017). According to Faostat (2017), the national banana production is 6.6 million tons, which ranks Brazil in the 4th position in the world production, in addition to the notorious quality and sweetness of the Brazilian fruit.

Despite this scenario of relevance and considering the area of 465 thousand hectares, it could be verified that the national productivity is low (14.3 t / ha) compared to the productive potential of the crop, which shows 60 t / ha in countries such as Indonesia and Nicaragua (FAOSTAT, 2017). This is in part due to the attack of phytopathogens such as *Colletotrichum musae*, causal agent of anthracnose disease, responsible for fruit deterioration during transport, storage and marketing. Infection begins in the field and remains quiescent until the beginning of maturation, forming lesions with large depressed and necrotic areas, causing losses of up to 40% (Silva et al., 2016).

To maintain the productive potential, producers intensify the use of high-toxicity pesticides, which increases production costs and causes greater ecosystem imbalances, favoring the resurgence of phytosanitary problems, emergence of new pests previously considered secondary, or also resistance to products used (Prabhu and Poulose, 2012). Thus, the search for alternative products with high efficiency and low or zero toxicity for phytosanitary control is extremely necessary in an attempt of minimizing losses and contamination resulting from the traditional system. In this context, the possibility of using AgNPs and resistance inducers is highlighted.

Colloidal silver consists of clusters of suspended silver atoms, which may vary in shape and size, with sizes ranging from 1 to 100 nm, being considered nanoparticles (Kholoud, Abou and Ala`a, 2010). AgNPs have been used to improve seed germination, plant growth, acting as antimicrobial agents in the control of phytosanitary diseases (Lamsal et al., 2011a; Gupta, Agarwal and Pradhan, 2018). Studies conducted by Min et al. (2009) demonstrated significant inhibition of *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *S. minor* germination after the use of AgNPs, suggesting the possibility of using this compound as an alternative to conventional chemical control.

In addition to colloidal silver, another alternative is the use of resistance inducers, which act by triggering plant defense mechanisms, which reduces infection and the development of pathogens (Agrios, 2004). They have systemic characteristics, with low risk to human health and environment (Conrath, Pieterse and Mauch-Mani, 2002). The effect of resistance inducers such as acibenzolar-S-methyl (ASM) was evidenced by several authors in different pathosystems such as: *Trichothecium roseum* in melon (Ge et al., 2015) and *Fusarium oxysporum* in lettuce (Gilardi et al., 2016). *Bacillus subtilis* (BS) showed soft rot control (Rhizopus stolonifer) in Baifeng peaches (Wang et al., 2013). Chen et al. (2017) used the same inducer and also found control of *Fusarium oxysporum* pathogen in *Artemisia selengens*. In addition, an inducing effect of *Saccharomyces cerevisiae* yeast on citrus (Toffano, Fialho and Pascholati, 2017) and apple was observed (Stella et al., 2013).

Thus, the aim of this work was to evaluate the *in vitro* antifungal activity of AgNPs and resistance inducers and in post-harvest banana anthracnose control.

MATERIAL AND METHODS

The work was conducted at the Laboratory of Plant Health and Biology, Federal Institute of Santa Catarina, Campus of Santa Rosa do Sul from February to November 2018.

Two *in vitro* experiments were carried out, the first testing different AgNP doses and the second evaluating resistance inducers, in a completely

randomized design, with five treatments in the first experiment and four treatments in the second, and seven replicates with a Petri dish with potato-dextrose-agar (PDA) culture medium as sample unit. In the experiment with AgNP, treatments used were: AgNP doses of 0, 25, 50, 100 and 200 ppm. To evaluate the control of resistance inducers, BS (10 mL of commercial product (p.c.) L^{-1}), ASM (60 mg p.c.L^{-1}) and *Saccharomyces cerevisiae* (SC) (1 mL p.c.L^{-1}) were used. Control treatment consisted of not adding anything to the medium. For the *in vitro* study, Petri dishes were inoculated with a mycelium disc from a pure *C. musae* colony at 5 days 25 ± 2ºC, 12-h photoperiod, previously isolated from fruits with symptoms of the disease. Mycelium discs used had 5mm in diameter and height, being extracted with the aid of a stainless-steel furrower.

To obtain the colloidal silver, a suspension of commercial gelatin was prepared by dissolving 5g in 800 mL of water at 40 ° C. About 50 mL of 1 mol L^{-1} silver nitrate solution was added and after stirring, 250 ml of 25% glucose solution were added under stirring. The solution was poured into 1 liter amber flask and kept at 75 ° C for 44 hours. After this step, 6 mL of 10% sodium carbonate solution were added and heated (75 ° C) for about 4 hours. The procedure was repeated until reaching, at a 1: 400 dilution, 1.35 absorbance units at wavelength of 410 nm.

Petri dishes were then maintained at 25 ± 2 ° C with 12-h photoperiod in BOD (Biochemical Oxygen Demand) incubators. Mycelial growth was evaluated by measuring the diameter of colonies (average of two diametrically opposite measurements) with the aid of a caliper 120 hours after subculture, and the mycelial growth inhibition percentage was quantified in relation to control treatment.

To verify disease control, two *in vivo* experiments were conducted in a completely randomized design with six replicates using the same treatments as the *in vitro* study. Fruits were harvested from a commercial orchard of Santa Rosa do Sul / SC in pre-climatic maturation stage, skin color 1, totally green, on a scale ranging from 1 to 7 (CEAGESP, 2015). Fruits were removed from the middle region of the bunch, separated into bouquets of three fruits with average diameter of 34 mm, composing the sample unit.

In a container, six liters of water were added and appropriate treatments were individually performed. The bouquets of each treatment were emerged in the solution for a period of three minutes. Then, bouquets were placed on a workbench and stored for 23 days at 20°C, simulating the shelf and consumption period, when anthracnose incidence and severity was evaluated.

Incidence was evaluated in percentage of fruits that presented symptoms of the disease in relation to total fruits. For severity, the mean area of lesions resulting from the mean length of the largest and smallest diameter of lesions was considered. Data obtained in *in vitro* and *in vivo* experiments were submitted to analysis of variance (p≤0.05). If statistical significance was found, regression analysis was performed when the different concentrations of silver nanoparticles were evaluated and comparison of means by the Tukey test (p≤0.05) for the evaluation of resistance inducers.

RESULTS AND DISCUSSION

The effect of colloidal silver (Figure 1) and resistance inducers (Table 1) concentrations on the mycelial growth of *Colletotrichum musae* was observed in the *in vitro* study. From the polynomial equation, it was observed that inhibition increased to the maximum point (113.2 ppm colloidal silver) when the variable reached 73% inhibition (Figure 1). Lamsal et al. (2011a) evaluated the effect of different silver nanoparticle concentrations on bell pepper anthracnose and also found, at dose of 100 ppm, maximum fungal growth inhibition compared to control. Kim et al. (2018) also evaluated fungal growth inhibition of colloidal silver on eighteen pathogens, showing that this concentration had the greatest effect on the evaluated pathogens.

Microscopy studies have shown that silver nanoparticles act to control pathogens as a result of damage caused by the separation of cell wall layers and collapse of hyphae (Min et al., 2009), in addition to the damage caused to the composition of sugars, proteins, n-acetyl glucosamine and lipids constituting pathogens (Ouda, 2014).

Figure 1. *Colletotrichum musae* mycelial growth inhibition (%) resulting from the use of different colloidal silver concentrations, evaluated 120 hours after subculturing in Petri dishes. Means obtained from seven replicates in relation to control treatment (0 ppm).
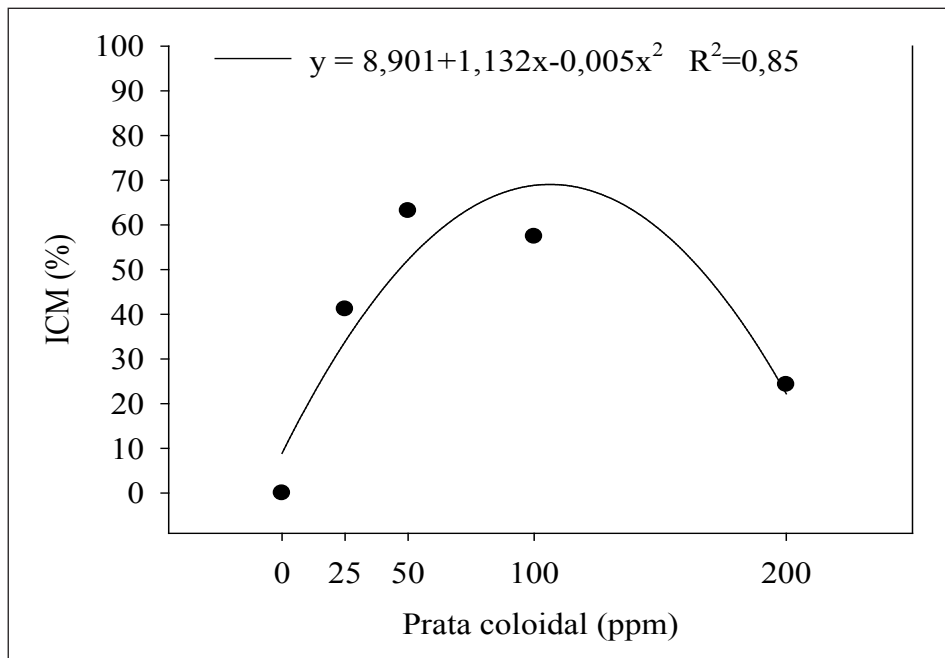
Table 1. Effect of different resistance inducers on *Colletotrichum musae* mycelial growth inhibition (%), evaluated 120 hours after subculturing in Petri dishes. Means obtained from seven replicates in relation to control treatment (0 ppm).

Treatments	Dose (p.c.L⁻¹)[A]	ICM (%)[B]
Control	-	0 c
Bacillus subtilis	10 ml	76.79 a
Acibenzolar-S-Methyl	60 mg	53.58 b
Saccharomyces cerevisiae	1 ml	1.93 c
CV (%)	7.11	

[A] Commercial Product.

[B] Averages followed by different lowercase letters in the column differ statistically using the Tukey test (p≤0.05).

Figure 1 shows a decline in mycelial growth inhibition at colloidal silver concentration of 200 ppm, demonstrating that the effect on the pathogen is dependent on the dose used. Using high concentrations, nanoparticle aggregation may have occurred, which increased its size, modifying its morphology and ion release capacity, consequently decreasing toxicity on the pathogen.

Regarding the effect of resistance inducers on the *in vitro* study, it was found that BS showed reduction in mycelial growth of the pathogen compared to control treatment (Table 1). These results are related to the bacterial lipopeptide production, which act on the cell membranes of fungal reproductive structures, inducing abnormal hyphae elongation, as well as edema and

conidial ruptures (Srikhong et al., 2018). Pyoung Il et al. (2010) identified iturin A, fengicin and surfactin A lipopeptides produced by BS involved in antifungal activity on *Colletotrichum gloeosporioides*.

Saccharomyces cerevisiae-based inducer did not differ from control in the mycelial growth evaluation (Table 1). Studies have shown the efficiency of the use of this inducer in the control of *Colletotrichum* pathogens (Lopes et al., 2015), contrary to the results obtained, but it is noteworthy that the antibiosis response may be dependent on yeast concentration, time interval between yeast applications and contact with the pathogen, in addition to the pathogen species itself. Gurgel et al. (2017) in a study with resistance inducers and control of *Etlingera elatior* anthracnose,

evaluated the activity of β-1,3-glucanase, important defense proteins in the resistance induction process (Van Loon and Van Strien, 1999). These authors did not identify significant difference between the use of *S. cerevisiae* based product and the control treatment; therefore, there was no action of these enzymes on (1,3) -β-glucan and chitin components of the fungal cell wall (Stangarlin et al., 2011), which could directly act in the pathogen suppression.

Regarding the *in vivo* experiment, increasing the dose reduced the incidence and severity of the disease at minimum colloidal silver concentrations of 141.9 and 110.8 ppm, respectively (Figures 2 and 3). Lamsal et al. (2011b) corroborate these results, as they present an *in vivo* study where maximum fungal hyphae growth inhibition and conidial germination occurred at similar silver nanoparticle dose (100ppm).

Figure 2. Anthracnose (*Colletotrichum musae*) incidence (%) in bananas immersed in different colloidal silver concentrations evaluated at 23 days of storage at 20ºC.
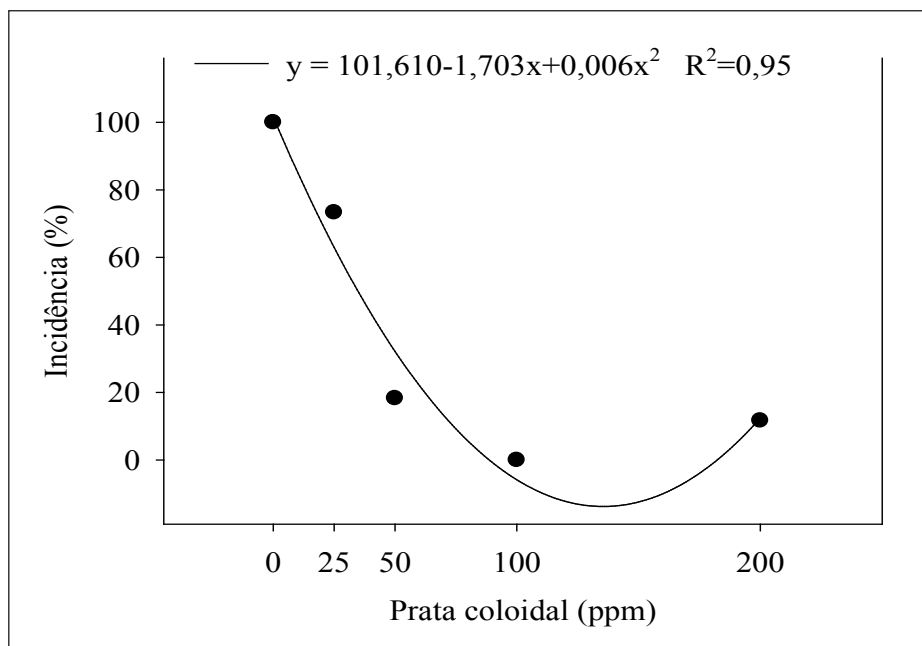
Figure 3. Anthracnose (*Colletotrichum musae*) severity (%) in bananas immersed in different colloidal silver concentrations evaluated at 23 days of storage at 20ºC.
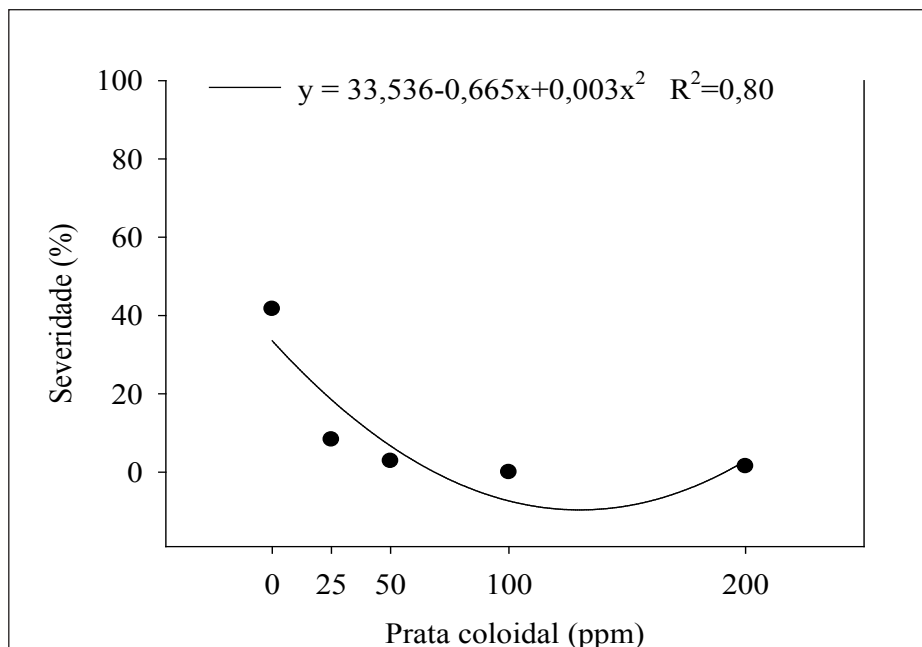
In evaluating the incidence and severity of resistance inducers, similar to *in vitro* results, BS and ASM's role in anthracnose control is highlighted (Table 2). It is assumed that in addition to the deleterious effect on fungus structure, another factor related to the response of these compounds to the pathogen is the induction of gene expression in host defense. Study by Gond et al. (2014) demonstrated that BS-treated corn seedling roots demonstrated induction of pathogenesis-related genes, including PR-1 and PR-4, related to plant defense against fungal pathogens (acquired systemic resistance - ASR). Similarly, ASR-related gene induction in ASM-treated apples was also found, decreasing the severity of fire blight disease (*Erwinia amylovora*) (Maxson-Stein et al., 2002).

Table 2. Anthracnose (*Colletotrichum musae*) incidence (%) and severity (%) in bananas immersed in different resistance inducers evaluated at 23 days of storage at 20°C.

Treatments	Dose (p.c.L⁻¹)[A]	Incidence (%)[B]	Severity (%)[B]
Control	-	100 a	41.67 a
Bacillus subtilis	10 ml	0 c	0 b
Acibenzolar-S-Methyl	60 mg	0 c	0 b
Saccharomyces cerevisiae	1 ml	8.3 b	4.3 b
CV (%)		4.76	23.07

[A] Commercial Product.

[B] Averages followed by different lowercase letters in the column differ statistically using the Tukey test (p≤0.05).

In addition to the increased expression of genes involved with ASR, it is hypothesized that ASM activates the phenylpropanoid pathway to increase the activity of related enzymes, strengthening the cell wall of fruits, which hinders pathogenic invasion. A study conducted with melon immersed in 0.1 g / L ASM solution for 10 minutes showed that this inducer increased the activity of enzymes phenylalanine ammonia lyase, tyrosine ammonia lyase, cinnamate 4-hydroxylase, 4-coumarate coenzyme A ligase, peroxidase and laccase (Liu et al., 2014). In addition, the authors showed increase in caffeic acid and ferulic acid content, precursors of lignin biosynthesis with the use of ASM.

Based on results obtained in this work, it could be concluded that colloidal silver, especially at 100 ppm concentration, as well as BS and ASM resistance inducers were efficient in the control of banana anthracnose caused by post-harvest *Colletotrichum musae*. Such a strategy within an integrated management program may reduce the use of conventional fungicides.

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