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Induction of Resistance to Papaya Black Spot Elicited by Acibenzolar-S-Methyl

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Abstract: The objective of this study was to evaluate the effect of acibenzolar-S-methyl tested at 5 concentrations (0, 1, 5, 25 and 100 μM a.i.) for its ability to protect papaya (*Carica papaya*) cv. Rainbow from black spot (*Asperisporium caricae*) following inoculation with the fungus. Effects of resistance induction treatment against black spot disease were evaluated by measuring the plant height and stem diameter. Disease symptoms were scored weekly by visually estimating disease severity of plants on the basis of a 5-class visual scale of 0 (no symptoms) to 4 (extensive lesions on leaves). Accumulation of defence-related proteins in papaya leaves were also analysed and compared. Results revealed that the level of protection against *A. caricae* was dose-dependent. Maximum reduction of the disease in leaves was obtained with 25-100 μM acibenzolar-S-methyl, with a time interval of 3 days between application of the activator and inoculation with the pathogen. The systemic resistance elicitation was characterized by an increase in 2 pathogenesis-related proteins, chitinase and β-1, 3-glucanase. These results indicate that acibenzolar-S-methyl induces partial resistance in papaya against black spot disease which may provide the grower a new option for integrated management of the disease.

Key words: *Carica papaya*, *Asperisporium caricae*, benzothiadiazole, chitinase, glucanase

INTRODUCTION

Foliar diseases caused by fungi such as black spot, caused by *Asperisporium caricae* (Speg.) Maubl., can be destructive wherever papayas are grown (Persley and Ploetz, 2003). It is one of the most serious fungal diseases of papaya, especially in Brazil, where papayas are continuously grown throughout the year in a climate conducive to outbreaks of severe epidemics (Ventura *et al*., 2003). Growers have striven hard to manage this pathogen in a number of ways. Given a choice, they usually opt for using resistant cultivars as the most efficient and effective method for disease control. Since papaya cultivars with good level of resistance to the pathogen do not exist, papaya growers have to rely on the extensive use of fungicides (Barreto *et al*., 2011). Although chemical control may provide partial protection, it is costly for small farmers, reduces the crops profitability and is harmful to the environment. Hence, there is a need to explore new strategies based on activating the plant's own immune and defense mechanism to control plant diseases.

Phenotypically, systemic resistance is manifested as protection which is long lasting and active against a broad spectrum of pathogens (Gozzo, 2003; Durrant and Dong, 2004). Different resistance-inducing compounds have been described (Kessmann *et al*., 1994; Oostendorp *et al*., 2001). Among these, benzo (1, 2, 3) thiadiazole-7-carbothionic acid-S-methyl ester (BTH) or acibenzolar-*S*-methyl (ASM) deserves particular attention for its low or no toxicity to plants, animals and the environment (Gorlach *et al*., 1996; Tomlin, 2001) and its high efficiency in protecting numerous plant species against a wide variety of pathogens (Danner *et al*., 2008; Madhusudhan *et al*., 2008; Aleandri *et al*., 2010; Huang *et al*., 2012; Carvalho *et al*., 2013; Prakongkha *et al*., 2013; Yigit, 2011). ASM increases crop resistance to diseases by activating the Systemic Acquired Resistance (SAR) signal transduction pathway. The development of SAR is associated with various cellular defence responses. These include synthesis of Pathogenesis-Related (PR) proteins, such as β-1, 3-glucanases and chitinases with antimicrobial potential (Suo and Leung, 2001; Ziadi *et al*., 2001; Edreva, 2005; Cavalcanti *et al*., 2006; Abo-Elyousr *et al*., 2010).

The aim of this study was to test ASM, applied to papaya seedling leaves, for their ability to induce resistance against *A. caricae* the causative agent of black spot disease. Accumulation of defence-related proteins in papaya leaves were also analysed and compared.

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MATERIALS AND METHODS

Plant growth: Papaya seeds were germinated in the greenhouse in flats containing Sunshine® mix No. 4 potting soil. The papaya cv. Rainbow was used. This genotype is susceptible to black spot disease. Three weeks after germination, when seedlings reached approximately 2 cm in height, they were transplanted individually into 4 in × 4 in pots containing the same potting soil. Plants were grown in the greenhouse at Komohana Research and Extension Center, Hilo and a slow-release fertilizer (Nutricote®) was applied fortnightly. The temperature was kept at of 22-28°C, the relative humidity at 68-80% and daylight of 12 h.

ASM treatment and pathogen inoculation: To determine the effect of ASM on disease reaction following inoculation with *Asperisporium caricae*, the compound was sprayed as a suspension of the formulated wettable powder (50% active ingredient) at concentrations of 0, 1, 5, 25, or 100 μM in distilled water plus 0.05% Tween 80. ASM was applied on papaya plants at 3 days before inoculation with the biotic agent.

For pathogen inoculation, mycelia carefully scraped from the lesions with *A. caricae* or infection were suspended in sterile distilled water. The suspension was filtered through cheesecloth before two repeated centrifugations at 3,000 rpm for 3 min with successive resuspensions in changes of sterile distilled water. Concentration was adjusted to 1×10^6 mL^{-1}. Inoculation was performed by thoroughly spraying the whole foliage. Inoculated plants were immediately enclosed in 17 L black plastic-lined containers under high humidity and incubated at room temperature.

Symptom evaluation: Development of black spot symptoms was weekly assessed by 3 independent observers. From 5-10 weeks after inoculation, symptoms of the disease were recorded using a 0-4 scale in which 0 = no symptoms and 4 = extensive lesions on leaves.

Effects of resistance induction treatment against this foliar disease were also evaluated by measuring the plant height and stem diameter 10 weeks after inoculation.

Protein extraction: Protein extraction followed the method of Zhu *et al.* (2003). Frozen leaves (3 g) were ground under liquid nitrogen in a 6 mL of 0.1 M phosphate buffer at pH 7, containing Phenylmethylsulfonyl Fluoride (PMSF) (1.0 mM) and 2, 2' dithiopyrindine (1.0 mM). The homogenates were filtered (Whatman No. 1) and centrifuged at 13800 g for 20 min at 4°C. The supernatants were used for enzymes assay.

Chitinase activity assay: Chitinase activity in the crude protein extracts was determined by a colorimetric assay (Zhu *et al.*, 2003). Specifically, chitin powder was washed 3 times with 0.1 M sodium acetate buffer (pH 5.2) to remove colored materials that would interfere with the enzyme assay. The reaction mixture contained 0.5 mg of washed chitin to which was added different volumes of crude enzyme extract and made up to a final volume of 0.5 mL with 0.1 M sodium acetate buffer (pH 5.2). The mixture was incubated in a shaking water bath at 37°C for 1 h then centrifuged at 12,000 g for 30 min to remove the chitin substrate. After centrifugation, an aliquot of 0.3 mL of the supernatant was placed in a 4 mL reaction tube and incubated at 37°C for 1 h with 5 μL of 25 % β-glucuronidase to hydrolyze the chitin oligomer. The amount of N-acetylglucosamine (Glc-Nac) produced in the reaction was determined by adding 0.1 mL of 0.6 M potassium tetraborate and heating the reaction mixture for 3 min in a boiling water bath. After cooling in ice, 1 mL of the color reagent diluted 1:2 with glacial acetic acid was added. The color reagent stock solution contained 10% 4-(dimethylamino)-benzaldehyde in 87.5 mL of glacial acetic acid and 12.5 mL of 11.5 M HCl. The samples were cooled and read on the spectrophotometer (Beckman DU-70) within 10 min at 585 nm. Chitinase activity was quantified from a calibration standard based on readings from 3 concentrations of N-acetylglucosamine (0.1, 0.2 and 0.4 μM). Enzyme activity is reported in katals (kat), defined as the amount of activity required to catalyze the formation of 1 M of Glc-Nac per sec.

β-1, 3-glucanase activity assay: Activity of β-1, 3-glucanases in the crude protein extracts was assayed by measuring the rate of reducing sugars production using laminarin as a substrate. Reducing sugars were assayed by the method of Nelson (Zhu *et al.*, 2003). The reaction mixture containing the crude enzyme extract and the laminarin substrate was incubated at 37°C and added to an equal volume of the Nelson alkaline copper reagent. Glucose was used as a standard. Enzyme activity is reported in katals (kat) defined as the amount of activity required to catalyze the formation of 1 M of glucose equivalents per sec.

Statistical analyses: Data on black spot severity, plant growth and PR-protein content and enzyme activities were analysed by one-way completely randomized ANOVA and means comparisons were performed by Duncan's test with $p\leq0.05$

RESULTS

Disease severity: The disease progress curves for papaya plants treated with ASM and inoculated with *A. caricae*

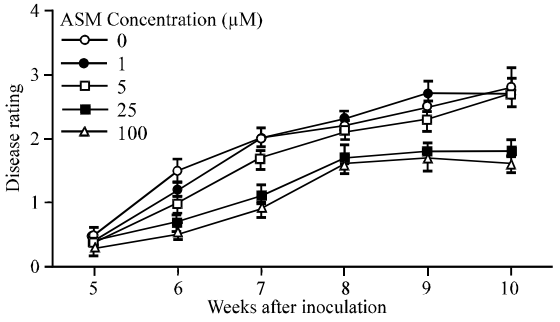

Fig. 1: Effect of foliar spray of ASM at various concentrations on black spot disease severity in papaya cv. Rainbow. Vertical bars represent±SD

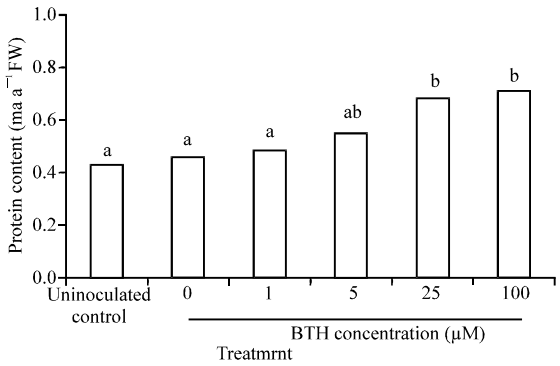

Fig. 2: Effect of foliar spray of ASM at various concentrations and *Asperisporium caricae* inoculation on defensive proteins content in papaya cv. Rainbow. Columns with identical letters are not significantly different from each other according to Duncan's multiple range test ($p \leq 0.05$)

are shown in Fig. 1. Disease development was less at concentrations providing 25-100 µM than 1-5 µM of ASM.

Pathogenesis-related proteins content: Applications of 25-100 µM ASM to 'Rainbow' papaya significantly induced PR proteins (Fig. 2) after *Asperisporium caricae* inoculation. Foliar levels of PR-proteins were about 50% higher in the plants treated with these ASM concentrations compared to the controls and 1-5 µM ASM dosages.

Chitinase and β-1, 3 glucanase activities: β-1, 3-glucanase activity in the leaves of plants treated with formulated ASM (25-100 µM) was significantly higher than in water-treated controls and lower ASM concentrations (Fig. 3). The activity of chitinase was less

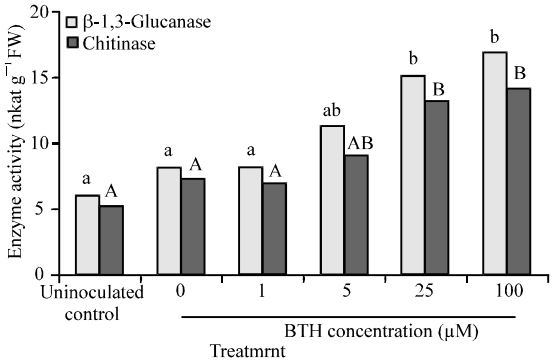

Fig. 3: Effect of foliar spray of BTH at various concentrations and *Asperisporium caricae* inoculation on enzyme activities in papaya cv. Rainbow. Columns of the same color with identical letters are not significantly different from each other according to Duncan's multiple range test ($p \leq 0.05$)

Table 1: Effect of foliar spray of ASM at various concentrations and *Asperisporium caricae* inoculation on growth of papaya cv. Rainbow

	Plant height (cm)		Stem diameter (mm)	
ASM (µM)	Uninoculated	*A. caricae*	Uninoculated	*A. caricae*
0	29.2[a]	23.4[a]	9.2[a]	7.3[a]
1	29.0[a]	24.5[a]	9.2[a]	7.4[a]
5	28.9[a]	25.5[a]	9.1[a]	7.5[a]
25	28.9[a]	28.2[b]	9.0[a]	8.5[b]
100	28.8[a]	28.9[b]	8.7[a]	8.6[b]

Columns means with the same letter are not significantly different at $p \leq 0.05$ according to Duncan's multiple range test

pronounced but also highly significant. β-1, 3-glucanase and chitinase activities induced by ASM concentrations as low as 5 µM remained very low and these treatments were not significantly different from the water-treated controls.

Plant growth: Results concerning plant growth are showed in Table 1. As expected, fungal inoculation significantly reduced plant height and stem diameter. By the end of the experiment, 10 weeks after inoculation, no significant effect was observed in plant growth of seedlings treated with up to 5 µM ASM. Plant height and stem diameter were significantly influenced by application of 25 and 100 µM ASM which were quite similar to that observed for uninoculated plants.

The treatment of ASM sprayed on papaya plants under pathogen free conditions allowed to evaluate possible phytotoxic effects. Acibenzolar-S-methyl showed no phytotoxic effects on papaya leaves during the trial.

DISCUSSION

The findings suggest that the protection of 'Rainbow' papaya seedlings from pathogen must have been due to the activation of the plant defense mechanisms, the efficacy of the induced resistance being dose-dependent. Similar enhanced disease resistance induced by ASM has been shown in a range of plant species including papaya but it has only one published study which has attempted to evaluate the elicitor effect in foliar diseases of papaya (Terra, 2009). The other reports deal with fruit rot (Dantas *et al*., 2004; Cia, 2005) and soil borne diseases (Zhu *et al*., 2003; Tavares, 2009). Our results agreed with these published studies, in that there was found a significant reduction of disease severity in ASM-pretreated papaya plants or fruits. For the development of resistance, plants need a period before being challenged with a pathogen. This interval was reported between 1 and 7 days in most cases and the pre-inoculation of plants with avirulent pathogens or abiotic elicitors was assessed for induction of resistance against several plant diseases (Heil and Bostock, 2002). In our study a 3-day-period between treatment and inoculation was satisfactory to induce resistance under our experimental conditions.

Although, uninoculated and water controls showed PR-proteins content, plants treated with ASM at 25-100 mM always exhibited more abundant protein levels. PR proteins are constitutive and inducible by different stresses, including UV light (Ziadi *et al*., 2001) which may explain why the controls showed these amount of PR proteins.

The results indicate that ASM treatment at 25-100 mM concentrations led to increases of β-1, 3-glucanase and chitinase activities in the leaves of 'Rainbow' papaya. The enhanced activities of these enzymes by ASM treatment were also found in other studies involving resistance induction in papaya. Increases in β-1, 3-glucanase activity were observed with ASM treatments which was correlated with reductions in anthracnose incidence on papaya (Dantas *et al*., 2004). In the experiment carry out by Cia (2005), the acibenzolar-S-methyl reduced in more than 50% anthracnose incidence and severity and induced the highest activity of peroxidase, chitinase and β-1, 3-glucanase and did not modify the physical-chemical characteristics of the fruits. In greenhouse experiments conducted by Tavares (2009) to evaluate the control of foot rot in papaya seedlings it was noted that plants sprayed with ASM showed increased activity of peroxidase and β-1, 3-glucanase and a highest concentration of lignin in relation to the control. However, the treatments have no effect on the activity of chitinase.

CONCLUSION

This study provides evidence that ASM induces partial resistance in papaya against black spot disease and that the induced resistance is dose-dependent.

Therefore, along with conventional fungicides, ASM may provide the farmer a new option for disease control. Further research is necessary, however, to establish a general recommendation.

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