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Research article

Salicylic acid and acibenzolar-S-methyl induce disease resistance to banana leaf spot caused by *Curvularia eragrostidis*

Noplapat Bussabong, Veeranee Tongsri*, Pattavipha Songkumarn

Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Bangkok 10900, Thailand

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Abstract

Leaf spot is a harmful disease that reduces the quantity and quality of banana yields in Thailand. This research aimed to investigate the molecular identity of a pathogen causing banana leaf spot, the effect of salicylic acid (SA; 0, 0.5, 1 and 1.5 mM) and acibenzolar-S-methyl (ASM; 0, 1, 2, 3, 4 and 5 mM) on pathogen growth *in vitro*, the reduction of disease severity and the induction of disease resistance in host plants. The virulent fungal pathogen was identified as *Curvularia eragrostidis*. Treatments of SA at 1.5 mM and ASM at all tested concentrations significantly inhibited the mycelial growth of the pathogen. Additionally, all concentrations of both activators significantly reduced disease severity by up to 77.8% for SA and 100% for ASM. Banana plants treated with 0.5 mM SA produced an early response of activating total phenolic compounds (TPCs) within 24 hr after inoculation (hai) and extremely stimulated the activity of peroxidase (POX) at 96 hai. In the 1 mM ASM treatment, the highest accumulations of endogenous SA and TPCs occurred at 24 and 96 hai, respectively. Plants treated with ASM also had the highest phenylalanine ammonia-lyase (PAL) and β-1,3-glucanase (GLU) activity levels at 96 hai. The activity of POX in the ASM treatment slightly increased at 24 hai. These findings revealed that the reduction of leaf spot disease in bananas occurred through the involvement of both activators in the direct suppression of the pathogen and the induction of disease resistance against *C. eragrostidis*.

Introduction

Banana (*Musa acuminata* L.) is one of the most economically important fruit crops in tropical and subtropical regions. In Thailand, the banana cultivar "Hom Thong" (AAA Group) is the most planted cultivar in diverse areas for export as fresh produce. It is also the most expensive cultivar among other famous cultivars, including "Khai" and "Namwa". Ordinarily, leaf spot disease of bananas grown throughout the world is primarily caused by a diversity of fungal species in the genus *Mycosphaerella* spp. and they produce negative effects on the quantity and quality of banana yield (Chang et al., 2016).

* Corresponding author.
E-mail address: fagrvnt@ku.ac.th (V. Tongsri)

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In Thailand, leaf spot disease caused by *Curvularia* sp. has been reported as the most harmful foliar disease that is abundantly distributed in "Hom Thong" bananas in Saraburi province (Bussabong et al., 2018). Generally, *Curvularia* sp. is not considered as a major pathogen of bananas; it has only been found as a minor pathogen on banana plant parts (Kaewjan et al., 2012). However, *Curvularia* species have often been reported as causal agents of diseases in other plant families (Marin-Felix et al., 2017).

A variety of approaches to control leaf spot diseases of bananas have been previously reported. The use of antagonistic bacteria has been found to substantially suppress banana leaf spot disease (Gutierrez-Monsalve et al., 2015). Resistant varieties of bananas have also been introduced in cultivated areas to manage black Sigatoka disease (Irish et al., 2013). Additionally, synthetic fungicides have been widely used to manage banana leaf spot in various growing areas. Although the effectiveness of the synthetic chemicals used for disease control has been noted in several studies, these chemicals had negative impacts on human health and the environment. Furthermore, chemicals can promote fungicide resistance by pathogens, and because of these limitations on fungicidal use, alternative control strategies are needed for plant diseases. One of the chemical activators, salicylic acid (SA), has been shown to increase the resistance of plants against several diseases (Dessalegn et al., 2013; Zhu et al., 2016). The resistance mechanisms induced by exogenous SA are involved in the synthesis of pathogenesis-related proteins, defense-related enzymes and secondary metabolites against plant pathogens (Luo et al., 2011; Zhang et al., 2016; Zhu et al., 2016). Another chemical activator, acibenzolar-S-methyl (ASM), which is an SA analog, has also been used to induce disease resistance in several plants infected by pathogens (Smith-Becker et al., 2003; Korpraditskul et al., 2006). The mechanisms elicited by ASM against plant diseases were similar to those triggered by exogenous SA (Felipini et al., 2015).

The species of fungi causing leaf spot in banana in Thailand have been poorly studied, and limited research exists linked to the induction of foliar disease resistance in bananas using SA or ASM. The current research aimed to (i) verify the identity of pathogen species associated with leaf spot disease in bananas, (ii) demonstrate the effect of SA and ASM on the *in vitro* growth of the pathogen, (iii) investigate the application of SA and ASM on the reduction of "Hom Thong" banana leaf spot, and (iv) evaluate the contents of endogenous SA and total phenolic compounds (TPCs) and the activity of defense-related enzymes, such as phenylalanine ammonia-lyase (PAL), β-1,3-glucanase (GLU) and peroxidase (POX), which accumulate in banana plants in response to SA and ASM in a dose- and time-dependent manner. This study should provide information that can be used to inform alternative control methods for leaf spot disease in banana plants.

Materials and Methods

Pathogen isolation and pathogenicity test

"Hom Thong" banana leaves infected with leaf spot disease were collected from banana orchards in Saraburi province, central Thailand. The fungus causing the disease was isolated from the diseased leaves using a tissue transplanting technique. The lesion edges were cut into sections 3 mm^2 in area. Then, pieces of the diseased tissues were surface sterilized using 1% sodium hypochlorite solution for 5 min, rinsed twice with sterile distilled water and dried in an aseptic cabinet. Subsequently, they were placed on a potato dextrose (PDA) Petri dish and incubated at room temperature (28–30°C) for seven days. The fungus grown from the diseased tissues was collected and kept in PDA slants for further pathogenicity testing using the detached leaf technique. Then, a piece of "Hom Thong" banana leaf (20 cm × 15 cm in size) was inoculated with the fungus agar disks (5 mm in diameter) and incubated in a moist transparent plastic box at room temperature for 7 d. Then, the lesion size produced after inoculation was measured and the pathogen was reisolated. The experiment was repeated three times.

Molecular identification of pathogen

DNA extraction

The most virulent isolate of the pathogens was cultured in malt broth (malt extract 20 g/L) for 72 hr. Then, 50 mg of mycelia were macerated with liquid nitrogen using a mortar and pestle, transferred to a 1.5 mL centrifuge tube to which was added 300 µL of each extraction buffer (100 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0 and 1 M KCl) and chloroform. The mixture was centrifuged at 12,000×*g* for 10 min. Then, the supernatant was transferred to a new microtube, where 20 µL of 3 M sodium acetate (pH 5.2) and 200 µL of isopropyl alcohol were added for nucleic acid precipitation. The reaction tube was centrifuged at 12,000×*g* for 10 min and the supernatant was discarded. The pellet was washed with 70% ethyl alcohol and centrifuged at 12,000×*g* for 10 min. After washing, the DNA was suspended with 30 µL of nuclease-free water and stored at -20°C.

Polymerase chain reaction amplification

Polymerase chain reaction (PCR) was conducted to amplify the DNA using the primer pairs for ITS1 (5′GCCGTAGGTGAACCTGCGG3′) and ITS4 (5′TCCTCCGCTTATTGATATGC3′) (White et al., 1990). The DNA amplification was performed in 25 µL of reaction mixture containing 0.25 mM of deoxynucleotide triphosphate, 0.5 U Taq polymerase (Thermo Scientific; USA), 0.1 µM of each primer and 50 ng of DNA template. The PCR amplifications were conducted in a C1000 thermal cycler system (Bio-RAD, USA) using the following program: 95°C for 5 min, followed by 30 cycles of 95°C for 45 s, 60°C for 45 s, 72°C for 45 s and a final cycle of 72°C for 5 min. Amplification products were examined using electrophoresis in 1.0% agarose gels and visualized under an ultraviolet transilluminator. The PCR product was purified and sequenced by a commercial sequencing service provider (Pacific Science Co., Ltd.; Thailand).

Sequence alignment and phylogenetic analysis

DNA sequences of the tested isolate were refined using the BioEdit sequence alignment editor (Hall, 1999). The consensus sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) to identify and analyze homologous sequences with the pathogen species deposited in the GenBank database (Altschul et al., 1990). The nucleotide sequences were aligned using the MEGA version 7.0 software (Kumar et al., 2016) and the phylogenetic analysis was bootstrapped based on 1,000 replicates.

Effect of salicylic acid and acibenzolar-S-methyl on pathogen growth using the poisoned food technique

The virulent pathogen was cultured in Petri dishes of PDA containing different concentrations of SA (0, 0.5, 1 and 1.5 mM) or ASM (0, 1, 2, 3, 4 and 5 mM). A mycelial plug (5 mm diameter) of the pathogen was placed on the center of a Petri dish with a 9 cm diameter. Then, it was incubated at 25°C under a 12 hr photoperiod. The colony diameter of the pathogen was measured at 7 d after incubation. The experiment was conducted with five replicates of each concentration of the chemicals. The percentage of mycelial growth inhibition was calculated based on the formula ((M1 - M2) / M1) × 100, where M1 is the pathogen colony diameter in the control Petri dish and M2 is the pathogen colony diameter in the SA or ASM Petri dish.

Individual effect of exogenous salicylic acid and acibenzolar-S-methyl application on the severity of banana leaf spot disease

Seven-month-old banana plants cv. "Hom Thong" (Musa AAA group) were grown in clay pots (38 cm diameter). The banana plants were individually sprayed with different concentrations (200 mL per plant) of either SA (0, 0.5, 1 and 1.5 mM) or ASM (0, 1, 2, 3, 4 and 5 mM) before inoculation with the pathogen for 24 hr. The experiment was conducted with three replicates per concentration, and each replicate consisted of three banana leaves. The control treatment was sprayed with distilled water. A needle was used to inflict 10 wounds to each of the old leaves and the wounds were inoculated with pathogen agar disks (5 mm diameter). Then, the inoculated plants were incubated in moist plastic bags and kept in a greenhouse environment for 48 hours after inoculation (hai). After this, the plastic bags were removed. Disease severity was determined by measuring the lesion size 7 d after inoculation. Each spot symptom was measured in two directions of perpendicular length. The average lesion size of all treated leaves was classified into five levels of severity of leaf spot in the scoring scale as follows: 0 = no disease appearance, 1 = tiny chlorotic spots of less than 1 mm in size, 2 = necrotic spots of about 1–3 mm in size, 3 = necrotic spots of about 4–6 mm in size and 4 = necrotic spots of greater than 6 mm in size. The percentage reduction of diseases severity was calculated based on the formula ((D1 - D2) / D1) × 100, where D1 is the disease severity score of leaves treated with distilled water and D2 is the disease severity score in leaves treated with SA or ASM.

Induced resistance to the leaf spot disease on banana plants

Plant material preparation

Three-month-old "Hom Thong" banana seedlings from tissue culture propagation were used in this trial. The seedlings were grown in black plastic pots (13 cm diameter) with black rice-husk substrate planting material and 1 g of a slow-release fertilizer (25-25-6 of N-P-K) per pot. The pots were placed under greenhouse conditions (28 ± 2°C). They were supplied with the same levels of water and nutrient. After 5 mth, healthy and uniform banana plants (30–40 cm high) were selected for further experiments.

Pathogen inoculum preparation

A single conidium of the pathogen was cultured on a PDA Petri dish and incubated at 25°C for 7 d. The conidial suspension was prepared by flooding the culture with 20 mL of sterile distilled water and filtering it through double-layer cloth. The inoculum was adjusted to 1×10^5 conidia/mL using a hemocytometer.

Application of plant resistant inducers

Banana plants were individually sprayed with 100 mL of either 0.5 mM SA or 1 mM ASM (Bion® 50 WG; Syngenta; Switzerland) and kept for 24 hr before inoculation with the pathogen. Plants sprayed with distilled water followed by conidial suspension were used as the control treatment. Then, the treated plants were transferred to moist plastic bags with 85% relative humidity. All treated leaves from one plant were collected at 0, 24, 48, 72, 96 and 120 hai, and they were cut into small pieces (1 mm^2 in area). The pieces of banana leaves were mixed well, wrapped in aluminum foil, immediately frozen in liquid nitrogen for 20 s and then stored at -80°C for further analysis.

Determination of the concentration of endogenous salicylic acid and total phenolic contents

A 1 g frozen leaf sample was macerated with liquid nitrogen using a mortar and pestle to obtain a fine powder that was homogenized in 20 mL of 90% methanol. The homogenate was placed in 2 mL microtubes and centrifuged at 13,000×*g* for 15 min at 4°C. The supernatant was used to determine levels of endogenous SA and TPCs.

The concentration of endogenous SA was determined following modified protocols from Hemsanit and Prathuangwong (2009). The reaction tube included 500 µL of sample extract and 2,500 µL of 0.02 M ammonium iron (III) sulfate and was incubated for 5 min at 30°C. Subsequently, absorbance was assessed at 530 nm using a spectrophotometer (Shimadzu, UV-160A; Japan) and it was interpolated from a standard curve of SA. The concentration of endogenous SA was expressed in milligrams of SA equivalents per fresh weight. The experiment was repeated twice with three replicates per treatment.

Levels of TPCs were determined using the Folin-Ciocalteu method following Lichanporn et al. (2009). Samples of 50 µL of extract were added to 3,950 µL of distilled water and mixed thoroughly with 250 µL of Folin-Ciocalteu reagent. Then, 750 µL of 20% (w/v) sodium carbonate was added to the mixture and it was incubated for 60 min at room temperature in the dark. The absorbance was measured at 765 nm. The TPC content was calculated using a standard curve for gallic acid. The results were expressed as milligrams of gallic acid equivalent per mL. The experiment was repeated twice with three replicates per treatment.

Determination of the activity of phenylalanine ammonia-lyase, β-1,3-glucanase and peroxidase

An assay (Assis et al., 2001) was used to assess the PAL activity. A 1 g sample of frozen leaf was macerated with liquid nitrogen using a mortar and pestle to obtain a fine powder that was homogenized in 20 mL of 0.05 M phosphate buffer (pH 8.8) and 7.8 µL of β-mercaptoethanol. The homogenate was placed in 2 mL microtubes and centrifuged at 21,000×*g* for 20 min at 4°C. The supernatant was used to conduct the PAL activity assay. Samples of 200 µL of enzyme extract were mixed with 2,800 µL of 0.05 M borate buffer (pH 8.8) containing 20 mM of L-phenylalanine and they were incubated for 60 min at 37°C. The reaction was stopped with 1 mL of 1 M HCl. The PAL activity was measured at absorbance of 290 nm. The activity units were expressed per milligram of protein. The experiment was repeated twice with three replicates per treatment.

GLU activity assay was conducted following Velasquez and Hammerschmidt (2004). A 1 g sample of frozen leaf was macerated with liquid nitrogen using a mortar and pestle to obtain a fine powder that was homogenized in 15 mL of 0.05 M sodium acetate buffer (pH 5.0) containing 150 µL of 100 mM phenylmethylsulfonyl fluoride and 0.45 g of polyvinylpyrrolidone. The homogenate was placed in 2 mL microtubes and centrifuged at 21,000×*g* for 20 min at 4°C. The supernatant was further used for GLU activity assay. The reaction was set up with 500 µL of enzyme extract and 130 µL of laminarin in 0.05 M sodium acetate buffer (pH 5.0). Then, the mixture was incubated for 30 min at 35°C and heated for 2–3 min. After heating, 290 µL of 3,5-dinitrosalicylic acid and 2,000 µL of 0.05 M sodium acetate buffer (pH 5.0) were added to the reaction tubes and they were heated for 5 min. When the reaction tubes had cooled, 1,000 µL of distilled water was added. The GLU activity was measured at absorbance of 540 nm. The activity units were expressed per milligram protein. The experiment was repeated twice with three replicates per treatment.

POX activity was determined through the oxidation of guaiacol following Lichanporn et al. (2009). A 1 g sample of frozen leaf was macerated with liquid nitrogen using a mortar

and pestle to obtain a fine powder that was homogenized in 20 mL of 0.05 M phosphate buffer (pH 7.0) and 0.2 g of polyvinylpyrrolidone. The homogenate was placed in 2 mL microtubes and centrifuged at 19,000×*g* for 20 min at 4°C. The supernatant was used to conduct the POX activity assay. A measure of 2,780 µL of 0.05 M phosphate buffer (pH 7.0), 100 µL of 20 mM H_2O_2, 100 µL of 20 mM guaiacol and 20 µL of protein extract was mixed in a test tube. The oxidation of the substrate was measured spectrophotometrically at 470 nm at 0 and 150 s. One unit of enzyme activity was defined as the amount of enzyme causing a 0.01 change in absorbance per minute The experiment was repeated twice with three replicates per treatment.

The total protein of each enzyme extract was measured following Bradford (1976), using a standard curve of bovine serum albumin. All enzymatic analyses were conducted in three replicates per treatment.

Statistical analysis

The analyses were conducted using the SPSS software version 25 (IBM Corp.; USA). Data were presented as mean ± SD and analysis of variance was used to test for significant effects of treatments, followed by Duncan's multiple range test for comparisons of means. Differences were considered significant at $p < 0.05$.

Results

Pathogen isolation and pathogenicity test

Eight fungal isolates were obtained from the diseased leaves. Based on the colony characteristics and conidial shapes, the fungi were characterized as *Curvularia* spp. (seven isolates) and *Nigrospora* sp. (one isolate). All tested isolates produced different levels of disease severity on the pieces of banana leaves. The SIH1 isolate displayed the highest virulence with the largest mean (± SD) lesion size (1.93 ± 1.52 cm × 0.55 ± 0.36 cm), as shown in Table 1. This isolate caused dark brown necrotic lesions with narrow elliptical shapes surrounded by large yellow halos on the banana leaf pieces. To fulfill Koch's postulates, reisolated cultures from the artificially inoculated leaf pieces were identified using conidial characteristics as *Curvularia* sp.

Molecular identification of Curvularia sp. isolate SIH1

A PCR product of about 549 bp was obtained using the ITS1/ITS4 primers. The BLAST search of the ITS region (ITS1-5.8S rDNA-ITS2) indicated that the SIH1 isolate was 99% consistently matched with *C. eragrostidis*. Phylogenetic tree analysis of ITS1, 5.8S, ITS2 and 28S gene sequences revealed that the SIH1 isolate clustered in the same clade as *C. eragrostidis* with accession numbers MK886805.1, MT740182.1, KU232932.1, MW082794.1, KX447646.1, MW082787.1 and KU856617.1 (Fig. 1). This sequence was deposited in the GenBank database under accession number MK182359.

Effect of salicylic acid and acibenzolar-S-methyl on pathogen growth determined using the poisoned food technique

Concentrations of SA at 0.5 and 1 mM had no negative effect on the mycelial growth of *C. eragrostidis* SIH1 compared with the control, but its higher concentration (1.5 mM) significantly inhibited pathogen growth by 12.7%. However, all concentrations of ASM (1–5 mM) markedly suppressed the pathogen growth by 22.0% to 41.5%. At 1 mM of both activators, ASM exhibited a stronger inhibitory effect on the mycelial growth than SA (Table 2).

Individual effect of exogenous salicylic acid and acibenzolar-S-methyl application on banana leaf spot disease severity

All concentrations of exogenous SA (0.5–1.5 mM) substantially reduced disease severity by 77.8%. Similarly, all concentrations of ASM (1–5 mM) extremely suppressed disease severity by 63.9–100% (Table 3 and Fig. 2).

Table 1 Pathogenicity test on pieces of "Hom Thong" banana leaf based on inoculation with diverse isolates of fungi obtained from leaf spot disease of banana and incubated in moist plastic boxes at room temperature (28–30°C) for 7 d

Fungal isolate	Lesion size (cm)
Curvularia sp. isolate SIH1	1.93±1.52 × 0.55±0.36
Curvularia sp. isolate SIH2	1.50±1.11 × 0.69±0.53
Curvularia sp. isolate SSnH1	0.44±0.43 × 0.17±0.22
Curvularia sp. isolate SSnH2	0.75±0.27 × 0.16±0.07
Curvularia sp. isolate SRW1	0.56±0.26 × 0.22±0.11
Curvularia sp. isolate SRW2	0.58±0.34 × 0.26±0.20
Curvularia sp. isolate SRW3	0.37±0.36 × 0.14±0.17
Nigrospora sp. isolate SRW4	0.73±1.03 × 0.23±0.31
Potato dextrose agar disk (control)	0.00

Values shown as mean ± SD of five replicates

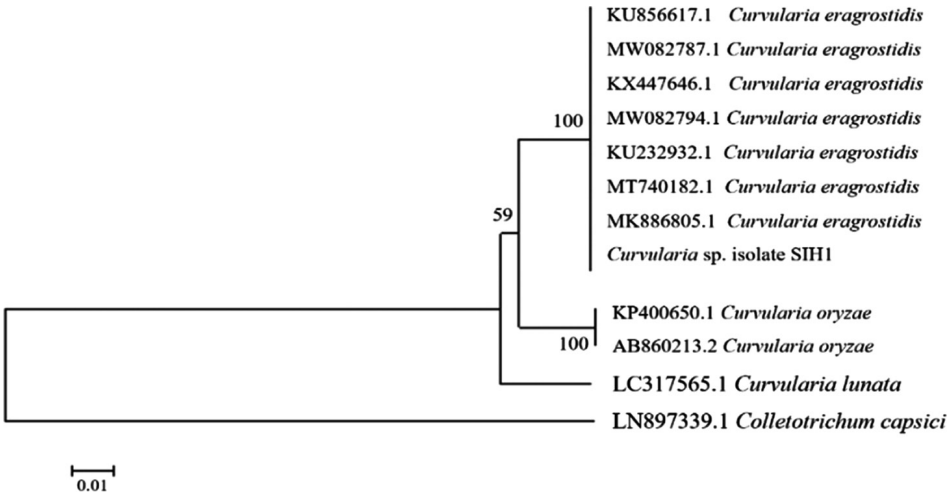

Fig. 1 Phylogenetic tree generated from ITS1, 5.8S, ITS2 and 28S gene sequences of *Curvularia eragrostidis*, where tree was generated with Mega 7.0 software using 1,000 bootstrap replicates and *Colletotrichum capsici* was used as an out group. Bootstrap values are shown at each node.

Table 2 Effect of different concentrations of salicylic acid (SA) and acibenzolar-S-methyl (ASM) on colony diameter and percentage of mycelial growth inhibition of *Curvularia eragrostidis* SIH1 *in vitro*, incubated for 7 d at room temperature

Treatment	Colony diameter (cm)	Mycelial growth inhibition (%)
distilled water (Control)	9.00±0.00^{f}	-
0.5 mM SA	8.90±0.06^{f}	1.1
1.0 mM SA	8.83±0.17^{f}	1.9
1.5 mM SA	7.86±0.06^{e}	12.7
1.0 mM ASM	7.02±0.16^{d}	22.0
2.0 mM ASM	6.25±0.09^{c}	30.6
3.0 mM ASM	5.80±0.11^{b}	35.6
4.0 mM ASM	5.40±0.10^{a}	40.1
5.0 mM ASM	5.27±0.05^{a}	41.5
F test	*	
Coefficient of variation (%)	21.62	

Values (mean ± SD of five replicates) in a column superscripted by different lowercase letters are significantly ($p < 0.05$) different.

Table 3 Effect of different concentrations of salicylic acid (SA) and acibenzolar-S-methyl (ASM) on disease severity score of banana leaf spot and percentage of disease severity reduction under greenhouse conditions after inoculation with *Curvularia eragrostidis* SIH1 at 7 d

Treatment	Disease severity score	Reduction of disease severity (%)
Distilled water (control)	3.6±0.5^{c}	-
0.5 mM SA	0.8±1.2^{ab}	77.8
1.0 mM SA	0.8±1.1^{ab}	77.8
1.5 mM SA	0.8±0.9^{ab}	77.8
1.0 mM ASM	0.0^{a}	100.0
2.0 mM ASM	1.3±1.2^{b}	63.9
3.0 mM ASM	0.2±0.4^{a}	94.4
4.0 mM ASM	0.5±0.7^{ab}	86.1
5.0 mM ASM	0.2±0.4^{a}	94.4

Values (mean ± SD of three replicates) in a column superscripted by different lowercase letters are significantly ($p < 0.05$) different.

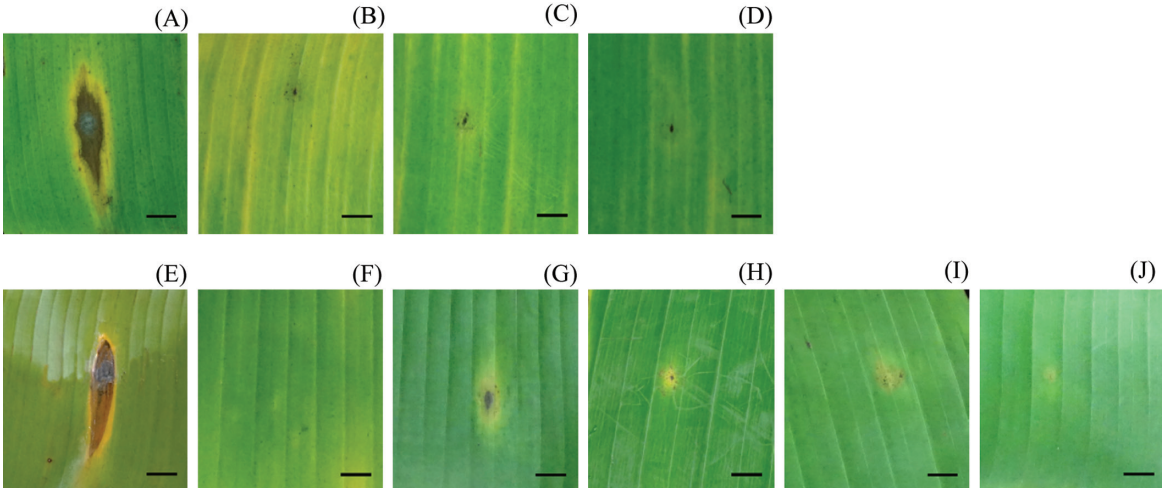

Fig. 2 Effect of different concentrations of salicylic acid at 0, 0.5, 1 and 1.5 mM (A–D, respectively) and acibenzolar-S-methyl at 0, 1, 2, 3, 4 and 5 mM (E–J, respectively) on the severity of banana leaf spot under greenhouse conditions after inoculation with agar disks of *Curvularia eragrostidis* SIH1 at 7 d and scale bar = 10 mm

Determination of concentrations of endogenous salicylic acid and total phenolic contents

The amounts of endogenous SA and TPCs in banana leaves after treatment with either 0.5 mM exogenous SA or 1 mM ASM before inoculation with *C. eragrostidis* SIH1 were significantly different in each experimental period. The application of exogenous SA produced a late response by activating the amount of endogenous SA at 120 hai. However, the ASM treatment produced the highest level of endogenous SA at 24 hai, but it was substantially decreased at 48 hai. Additionally, ASM slightly activated the amount of endogenous SA through 96 hai with a higher amount than those of other treatments (Fig. 3A).

The TPC levels were initially stimulated at 24 hai by exogenous SA, and subsequently, there was a pattern of alternating decreasing and increasing in phenolic compound accumulation for the rest of the time points. In the ASM-treated plants, the TPC levels steadily increased to 96 hai, and thereafter, the TPC contents declined (Fig. 3B).

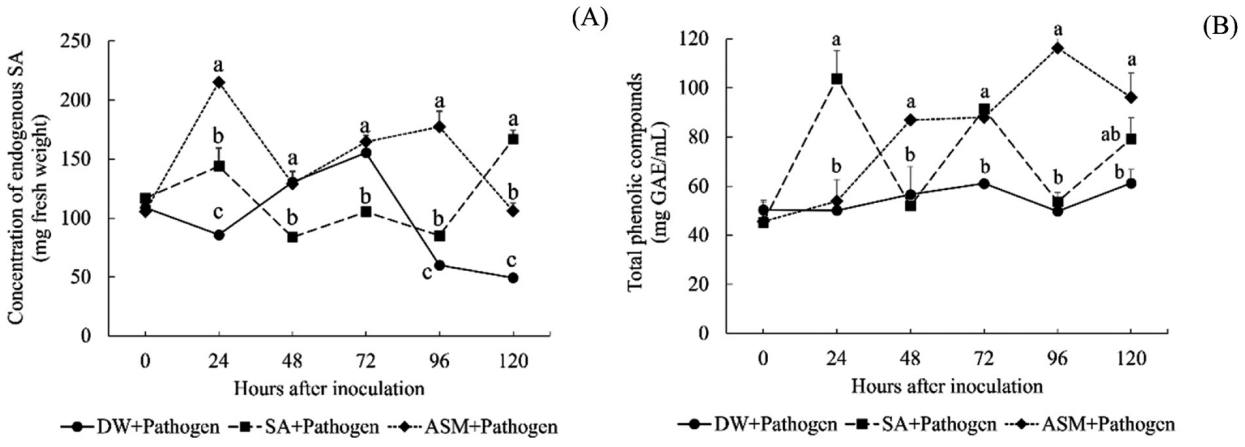

Fig. 3 Concentration of endogenous SA (A) and total phenolic compounds (B) on leaf tissues of "Hom Thong" banana plants after treatment with exogenous 0.5 mM salicylic acid (SA) or 1 mM acibenzolar-S-methyl (ASM) for 24 hr before inoculation with *Curvularia eragrostidis* SIH1 compared with inoculated plants after the treatment of distilled water at different periods, where experiment was repeated twice with three replicates per treatment, Error bars represent ± SD. Different lowercase letters at each time point indicate significant ($p < 0.05$) different between treatments

Determination of the activity of phenylalanine ammonia-lyase, β-1,3-glucanase and peroxidase

In both the SA and ASM treatments, PAL activity was significantly increased at 96 hai. The highest PAL activity was observed in ASM-treated plants and then it decreased substantially at 120 hai (Fig. 4A). The GLU activity slightly increased at 24 hai through the application of exogenous SA. In the ASM treatment, initially, lower activity of GLU occurred at 48 hai and then, activity drastically increased at 96 hai (Fig. 4B). The POX activity accumulated the most in the exogenous SA-treated plants at 96 hai, while its activity was first induced by ASM treatment at 24 hai with slight accumulation. The control treatment, pathogen inoculated plants, also displayed a high activity of POX at 96 hai, but the activity was lower than that of the exogenous SA treatment. Subsequently, the POX activity of exogenous SA and control treatments declined substantially at the end of trial period (Fig. 4C).

Discussion

Several of the isolates of *Curvularia* spp. that were obtained from the leaf spot disease of banana were pathogenic on the pieces of healthy "Hom Thong" banana leaves. This was consistent with the findings of another study that showed that some species of the genus *Curvularia*, such as *C. lunata*, caused disease in organic banana (Kamel et al., 2016). In the current study, *C. eragrostidis*, the most virulent isolate SIH1, could also produce symptoms of necrotic oval spots surrounded by yellow halos on banana leaves. This concurred with other studies that found that *C. eragrostidis* was pathogenic on many economically important crops, including orchids, rice and pineapples (Středa et al., 2013; Kusai et al., 2015).

In the current study, the application of both inducers (SA and ASM) effectively reduced the disease severity of leaf spot in banana infected by *C. eragrostidis* SIH1. This result was in agreement with many reports that showed that when SA or ASM or both were exogenously applied

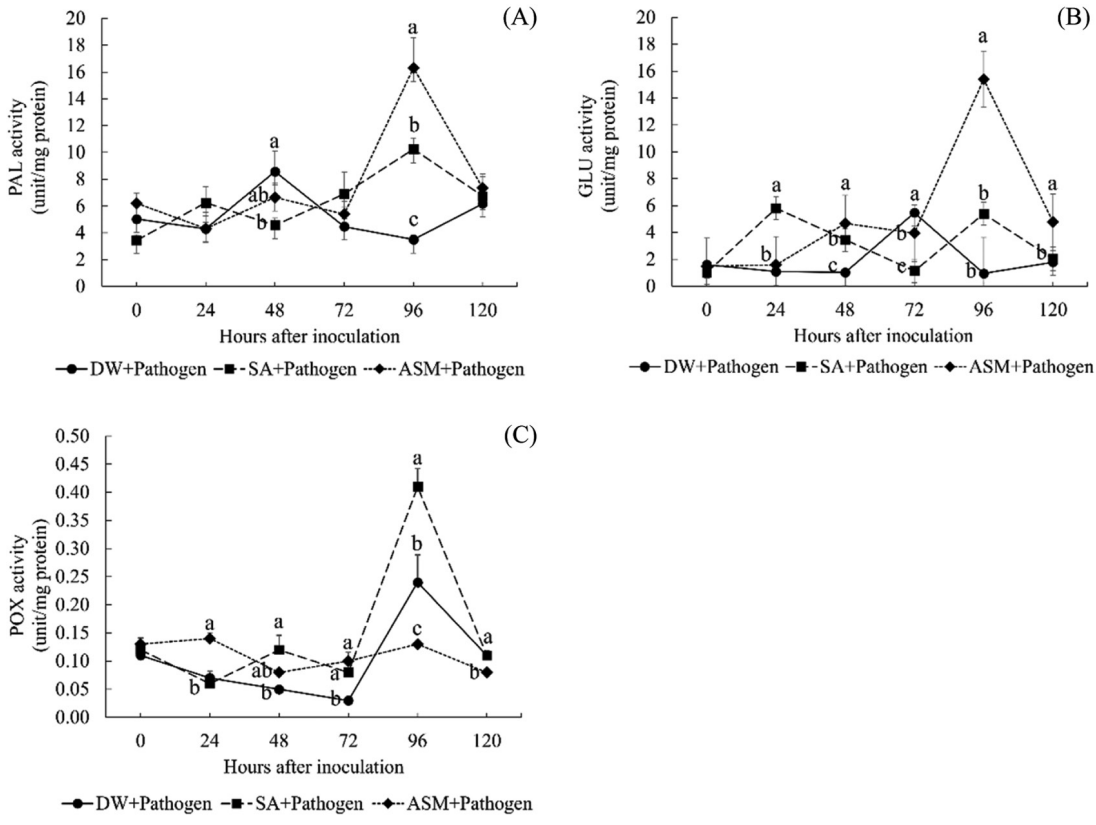

Fig. 4 Activity levels of phenylalanine ammonia-lyase (A), β-1,3-glucanase (B) and peroxidase (C) on the leaf tissues of "Hom Thong" banana plants after treatment with exogenous 0.5 mM SA or 1 mM ASM for 24 hr before inoculation with *Curvularia eragrostidis* SIH1 compared with inoculated plants after the treatment of distilled water at different periods, where experiment was repeated twice with three replicates per treatment. Error bars represent ± SD. Different lowercase letters at each time point indicate significant ($p < 0.05$) different between treatments

to plants, they acted as foliar disease suppressors of early blight in tomato and of anthracnose in the common bean (Costa et al., 2018; Aslam et al., 2019). The application of SA or ASM or both also reduced the incidence of soilborne diseases, such as wilt and clubroot, which are caused by *Fusarium* sp. and *Plasmodiophora* sp. (McGrann et al., 2016; Chavez-Arias et al., 2020).

Based on the current results, it is suggested that the mechanisms causing the reduction in disease severity by the two chemicals might be associated with either a direct toxic effect on fungal morphological development or chemically-induced resistance in the host. The former speculation (due to the fact that there was a varying degree of the inhibitory action on pathogen mycelial growth after treatments of SA and ASM) was observed *in vitro*. Treatments of SA at 1.5 mM and ASM at all concentrations (1–5 mM) significantly inhibited fungal growth. However, contradictory reports exist on the effects of SA on pathogen growth. The mycelial growth of *Glomerella cingulata* was not found to be affected by SA treatment (Zhang et al., 2016), whereas *F. solani* was found to be extremely sensitive to higher concentrations of SA at 80 mM (Ramteke, 2019). Similarly, researchers found that ASM treatments also directly inhibited the mycelial growth of *Rhizoctonia solani* (Faessel et al., 2008). However, no negative effects of ASM on the mycelial growth of *Cercospora* and *C. lindemuthianum* (Felipini et al., 2015; Costa et al., 2018) have been reported.

Apart from the inhibitory mechanism, the conjecture of the SA and ASM mechanism in inducing resistance in banana plants was based on the following evidence. The low concentration of SA treatments (0.5 mM and 1.0 mM) with no mycelial inhibitory effect could minimize disease severity to the same extent as the higher concentration (1.5 mM), which showed inhibitory action. Notably, strong evidence was observed in the ASM treatment at the lower-level concentration of 1.0 mM, as the lowest degree of inhibitory effect on mycelial growth was noticed with the high percentage of reduction in disease severity (100%). The current results were in accordance with other reports showing that the application of exogenous SA and ASM provided substantial disease resistance against several pathogens (Zhang et al., 2016; de Oliveira et al., 2018).

The mechanisms of plant defense induced by chemical activators have been elucidated in several plant species. Defense mechanisms usually induce plant defense genes, leading to increased defensive enzyme activities (Adamuchio-Oliveira et al., 2020) and the accumulation of pathogenesis-related proteins and secondary metabolites (Taif et al., 2020). Endogenous SA was monitored in the plants treated with chemicals to determine whether SA and ASM induced plant defense responses that reduced the severity of banana leaf spot symptom. Both SA and ASM treatments caused higher accumulation of endogenous SA within 24 hai than the control treatments of water. The rapid accumulation of endogenous SA could suppress disease severity, as shown in the aforementioned result. Indeed, throughout the periods observed, the maximum level of endogenous SA was achieved earlier (24 hai) in the ASM treatment than other treatments and it continued to increase after decreasing at 48 hai for a long period (from 48 hai to 96 hai). Therefore, rapid, higher and prolonged accumulation of endogenous SA in the ASM treatment could support the observation of the substantial reduction (100%) in the severity of banana leaf spot disease. Endogenous SA also accumulated substantially during the early stage of exogenous SA application to rubber leaves against *Phytophthora palmivora* (Deenamo et al., 2018). SA is synthesized in plants via either isochorismate synthase or the phenylalanine ammonia-lyase pathway where they are associated with the production of many antimicrobial substances (Lefevere et al., 2020).

The phenolic compounds in plant tissues are generally toxic to pathogens and they are capable of inducing disease resistance to pathogens (Lattanzio et al., 2006). The current study found that SA stimulation increased the TPC level within 24 hai. The high accumulation of TPCs was detected throughout the observation period after 48 hai in the ASM-treated plants, but it was only partly detected at some time points in the SA-treated plants. These results were in agreement with Prakash et al. (2020), who demonstrated a strong association between the reduction in the severity of *Alternaria* blight disease and a high level of accumulation of phenolic compounds in tomato leaf tissues after an exogenous application of SA. Furthermore, Felipini et al. (2015) found that ASM application could build up TPCs in beet tissues treated against *Cercospora* leaf spot.

The analysis of defense-related enzymes showed that the highest increases in the activities of PAL, GLU and POX were observed in the banana leaves treated with both activators. At 96 hai, the maximum activity levels of PAL and GLU were in the ASM treatment, while the highest activity of POX was in the exogenous SA application. PAL is an essential enzyme in the phenylpropanoid pathway, and it is related to the generation of lignin and phenolic compounds in plant tissues (Li et al., 2014; Yadav et al., 2020).

Enhanced enzyme activity levels of PAL and other defense-related enzymes have been noted in other previous studies. For example, Deenamo et al. (2018) demonstrated that exogenous SA elevated the contents of PAL, POX, catalase,

H_2O_2 and phytoalexin in rubber trees, leading to a reduction in disease severity caused by *P. palmivora*. Furthermore, exogenous SA promoted PAL and POX activity in tomato plants against a leaf blight pathogen (Prakash et al., 2020).

Both GLU and POX are members of pathogenesis-related protein families. The application of exogenous SA in *Panax notoginseng* was shown to induce an expression of *PnGlu1* (β-1,3-glucanase) in root cells and gene overexpression was found to confer resistance against *Fusarium* wilt (Taif et al., 2020). Similarly, an ASM application produced an increase in defense-related enzyme activity levels, including GLU and POX in cabbage against black rot pathogen (Amaral et al., 2019). Additionally, GLU acts as a cell wall degrading enzyme that can degrade the cell wall components of fungi (Felipini et al., 2015). Furthermore, Narasimhamurthy et al. (2019) demonstrated that POX activity accumulated in tomato plants treated with SA against bacterial wilt pathogen. Additionally, the enhancement of POX activity in rice leaf tissues as a result of SA stimulation was shown to confer resistance against bacterial blight (Shasmita et al., 2019). Similarly, exogenous SA significantly enhanced the activities of POX and other antioxidative enzymes in rubber leaves, subsequently reducing *P. palmivora* invasion in the host plant (Deenamo et al., 2018). Additionally, POX is involved in the reinforcement of plant cell walls for lignification and suberization for protecting plants against pathogens (Diniz et al., 2019). The SA pathway is normally activated in the host plant against biotrophs (Achuo et al., 2004; Wang et al., 2018; Yu et al., 2019), but the current experiment demonstrated that SA treatment could enhance resistance to necrotrophic pathogen. This may suggest that the pathogen growth in plant tissues can be suppressed by SA-responsive gene products or restrict the growth via hypersensitive reaction (Murphy et al., 2000)

In conclusion, based on morphological and molecular studies of the SIH1 isolate, one of the pathogens causing banana leaf spot was identified as *C. eragrostidis*. Additionally, both the activators tested were effective at minimizing the incidence of leaf spot disease caused by *C. eragrostidis* on banana cv. "Hom Thong." Both SA and ASM play a role in either the suppression of pathogen growth or induction of disease resistance in banana plants, as evidenced by the accumulation of endogenous SA, TPCs, and defense-related enzymes. Thus, both chemicals may be further used as potential alternative control agents for leaf spot disease management in banana crops.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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