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RESEARCH ARTICLE

Potential of ethyl acetate fractions of *Stryphnodendron adstringens* shells and fruit extracts of *Caesalpinia ferrea* to control bacterial leaf speck and on the potentiation of defense enzymes in tomato

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ABSTRACT

Considering the importance of bacterial leaf speck (*Pseudomonas syringae* pv. *tomato*) in reducing tomato yield and difficulties in disease control, this study investigated the effects of fractions of shells extract of *Stryphnodendron adstringens* (*Sa*) and fruit extract of *Caesalpinia ferrea* (*Cf*) compared to Acibenzolar-S-Methyl (ASM) on reducing bacterial leaf speck symptoms and on the potentiation of the activities of defense enzymes. The number of lesions per plant (NLP) was significantly lower in plants treated with the ethyl acetate fraction (EAF) of *S. adstringens* (*Sa*) and ASM compared to other treatments (EAF of *Cf*, *n*-butanol fractions of *Sa* and *Cf*, aqueous fractions of *Sa* and *Cf* and sterile distilled water). The bacteria were inhibited by the EAF of *Sa* and EAF of *Cf* and *n*-butanol fractions of *Sa* and *Cf* according to the bioautography assay. Saponins and tannins were the two major compounds found in these fractions based on the phytochemical analysis. Peroxidase (POX), polyphenoloxidase (PPO), *β*-1,3-glucanase (GLU) and phenylalanine ammonia-lyase (PAL) activities were determined on the leaves of plants treated with EAF *Sa*, ASM and sterile distilled water. Both POX and PAL activities were higher at 3 and 6 days after inoculation (dai), while the PPO and GLU activities were higher from 9 to 12 dai. It is suggested that saponins increased tomato resistance to *P. s.* pv. *tomato* because no antimicrobial activity against the bacteria was observed. In conclusion, the EAF *Sa* was very efficient in reducing bacterial leaf speck symptoms in conditions where the POX, PPO, PAL and GLU activities played a pivotal role in increasing tomato resistance to the disease.

Key words: *Pseudomonas syringae* pv. *tomato*, *Solanum lycopersicum*, induced host resistance, plant extracts.

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is one of the most consumed vegetables worldwide (Minami & Haag, 1989), and the occurrence of diseases can dramatically reduce its yield. Among the diseases that affect tomato plants, bacterial leaf speck, which is caused by *Pseudomonas syringae* pv. *tomato* (*Pst*) (Okabe, 1933; Young et al., 1978), is the most important (Silva & Lopes, 1995). Bacterial leaf speck can cause yield losses of up to 30% when tomato plants are grown in regions with high relative humidity and temperatures ranging from 20ºC to 25ºC (Silva & Lopes, 1995). Products containing copper and the inducer of resistance Acibenzolar-S-Methyl, in addition to the use of cultivars containing the resistant *Pto* gene, such as the cultivar BRS Tospodoro, are the current disease control methods recommended to growers (Lopes & Santos, 1994; Giordano et al., 2010). The tomato crop is one of the most dependent on the application of pesticides, which results in an increase in production costs and the selection of pathogen-resistant populations in addition to being toxic to growers and the environment (Mariconi, 1981).

In Brazil, there is considerable diversity among plant species capable of synthesizing a wide range of products that have antimicrobial activity against various microorganisms (Almeida, 1993). The compounds originating from the plants' secondary metabolism have no direct role in their growth but promote a better fitness in the environment (Taiz & Zeigher, 2004). Among the secondary metabolites, some compounds that exhibit antimicrobial activity are of special importance, such as essential oils, saponins, flavonoids, coumarins, phytoalexins, tannins and alkaloids (Taiz & Zeigher, 2004; Gurjar et al., 2012). The plant extracts that contain many of these metabolites have been investigated for their increased resistance against pathogens infection, such as the zimmu extract (*Allium sativum* L. x *Allium cepa* L.) in the control of *Xanthomonas campestris* pv. *malvacearum* on cotton (Satya et al., 2007) and *Lasiodiplodia theobromae* and *Colletotrichum musae* on banana (Sangeetha et al., 2013). Extracts of *Datura metel* increased rice resistance against *Rhizoctonia solani* and *X. oryzae* pv. *oryzae* (Kagale

et al., 2004), and the extract of *Hedera helix* reduced the symptoms of *Erwinia amylovora* on apple (Baysal & Zeller, 2004).

Induced resistance is a phenomenon that allows plants to enhance their level of basal resistance against pathogens attacks (Weller et al., 2012). According to Glazebrook (2005), Acibenzolar-S-Methyl (ASM), an analog of salicylic acid, is known to play a pivotal role in the signaling pathway that results in systemic acquired resistance (SAR). Diseases caused by *X. vesicatoria*, *P. syringae* pv. *tomato*, *Clavibacter michiganensis* subsp. *michiganensis* and *Ralstonia solanacearum* in tomato (Louws et al., 2001; Soylu et al., 2003, Ji et al., 2005; Cavalcanti et al., 2006) and *X. axonopodis* pv. *passiflorae* in passion fruit (Boro et al., 2011) showed decreased severities when the plants were sprayed with ASM. One of the plant defense mechanism against pathogens related to SAR is the activation of genes coding for chitinases, *β*-1,3-glucanases, phenylalanine ammonia-lyases, polyphenoloxidases and peroxidases (Van Loon, 1997).

The effects of fractions obtained from fruit extracts of *Caesalpinia ferrea* and shells of *Stryphnodendron adstringens* compared to the known inducer of resistance ASM were investigated as an alternative in reducing bacterial leaf speck symptoms and potentiating the activities of enzymes in tomato leaves.

MATERIALS AND METHODS

Plant materials

Stem bark samples were collected from "Barbatimão" (*Stryphnodendron adstringens*) tree trunks, and fruits were obtained from "Jucá" (*Caesalpinia ferrea*) trees in Boa Vista City, Roraima State, Brazil. Specimens obtained from these two plant species were deposited in the herbarium of the Universidade Federal de Roraima (Boa Vista, RO, Brazil) on January 2013 with the codes 3446 and 3445 for *S. adstringens* and *C. ferrea*, respectively. After collection, the stem bark and fruits were dried at room temperature (25°C) in a ventilated room for one week and stabilized in a circulating air oven at 40°C for 5 days. Next, the dried material was ground using a Thomas-Wiley mill and stored in glass vials protected from moisture and light using aluminum foil.

Extraction and fractionation of *S. adstringens* and *C. ferrea* components

Aqueous extracts of the stem bark and fruits were prepared by infusion at a ratio of 1:20 w/v (plant material: distilled water), boiled in water for 30 min with constant agitation, and then filtered through cotton. The aqueous extracts obtained were frozen at -80°C; the extracts were then freeze-dried and stored in a dry location in the dark. Subsequently, a total of 10 g of each lyophilized material was resuspended in water (200 mL), because the fractions were derived from aqueous extract. Afterward, the fractions were subjected to liquid-liquid serial and exhaustive partitioning in separator funnels with solvents of increasing polarity, resulting in the following fractions: ethyl acetate fraction of *S. adstringens* (EAF *Sa*), ethyl acetate fraction of *C. ferrea* (EAF *Cf*), *n*-butanol fraction of *S. adstringens* (BF *Sa*), *n*-butanol fraction of *C. ferrea* (BF *Cf*), aqueous fraction of *S. adstringens* (AF *Sa*) and aqueous fraction of *C. ferrea* (AF *Cf*). The ethyl acetate and *n*-butanol fractions were concentrated using a rotary evaporator at 50°C and 85°C, respectively. The vials were placed in a desiccator until the solvent was completely evaporated. The aqueous fractions were freeze-dried to remove the solvent.

Bioauthography

The bioautography was performed by Thin Layer Chromatography (TLC) employing TLC plates (SIL ALIGRAM g/UV 254, Merck) according to Wagner & Bladt (1996). A total of 10 ml of each fraction, re-suspended in methanol (5 mg/100 µl) and then eluted with mobile phase *n*-butanol/acetic glacial acid/water (45:10:35), was pipetted at each TLC plate. After elution, chromatographic plates were dried in a laminar flow in environment temperature for 5 days for complete removal of the solvent. Afterwards, they were sprayed with bacterial suspension. The bacterium was grown in liquid medium 523 (saccharose 10 g, casein acid hydrolysate 8 g, yeast extract 4 g, K_2HPO_4 2 g, $MgSO_4.7H_2O$ 0,3 g, agar 15 g, distilled water 1000 mL) (Kado & Heskett, 1970) for 48 h. Subsequently, 100 µL of the bacterial suspension at 1.0×10^8 CFU/mL were transferred to 20 ml of the medium 523 semi-solid at 50°C which was sprayed to completely cover the plates. Plates coated with layer of bacterial suspension were maintained at 28°C for 48 hours and then sprayed with triphenyltetrazolium chloride (TTC) a concentration of 20 mg/ml. The assay was performed in triplicate and the halos observed in the bioautograms were measured.

Phytochemical evaluation

Analysis of the chemical components was performed for different groups of secondary metabolites, such as anthraquinones, coumarins, flavonoids, cardiotonic heterosides, saponins, alkaloids and tannin using TLC according to Wagner & Bladt (1996). To characterize the chromatograms, specific reagents for each group of secondary metabolites were used as follow: anthraquinones (1,8 diidroxiantraquinona), coumarins (venalot), flavonoids (quercetina), cardiotonic (digitoxicina), saponins (18-*β*-glicirretínico), alkaloids (quinina) and tannins (pyrogallic acid). The eluent used for the separation of the compounds was a solution consisting of glacial acetic acid, *n*-butanol and water in the proportion of 45:10:35 (v/v/v). The fractions were solubilized in methanol (5 mg/100 µL) and deposited onto the plates using capillary glass. In some instances, they were detected under ultraviolet light (λ = from 254 to 365 nm) in a darkroom. The results obtained

using chromatography were compared with the respective standards.

Plant growth

Tomato seeds from the cultivar "Santa Clara" were sowed in plastic trays containing a substrate (Plantmax). Twenty-one days after sowing, the plants were transplanted into plastic pots containing 2 kg of substrate. Fifteen days after transplantation, the plants received 40 mL of a nutrient solution containing (in mg/L) 0.192 KCl, 0.104 K_2SO_4, 0.150 $MgSO_4.7H_2O$, 0.061 urea, 0.1 NH_4NO_3, 0.0003 $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.002 H_3BO_3, 0.007 $ZnSO_4$, 0.002 $CuSO_4.5H_2O$, 0.004 $MnCl_2.4H_2O$, 0.004 $FeSO_4.7H_2O$ and 0.006 disodium EDTA. The plants were maintained in a greenhouse (temperature of 25 ± 2ºC, humidity relative of 65 ± 5% and 12 hours light/12 hours dark) and irrigated as needed.

Inoculum of *Pst* and bacterial suspension preparation

The isolate of *Pst* was obtained from tomato plants with typical symptoms of bacterial leaf speck in Coimbra, Minas Gerais state, Brazil. The isolate (UFV-DFP 06) was preserved in 30% glycerol at -80ºC (Gonçalves et al., 2007), and its identity was confirmed using a pathogenicity test on tomato plants, LOPAT tests (Schaad, 1988) and sequencing analysis of the mitochondrial 16S ribosomal rRNA gene fragment. For use in the experiments, the *Pst* isolate was grown in 523 medium (Kado & Heskett, 1970) using the stretch mark method for 24 h at 28°C. Subsequently, the bacterial suspension was prepared in a 0.85% saline solution, and the bacterial concentration was adjusted to $absorbance_{540}$ = 0.20, which was approximately 1.0×10^8 CFU/mL.

Evaluation of the effect of fractions of the extracts against bacterial speck

The following treatments were used in this experiment: EAF *Cf*, BF *Cf*, AF *Cf*, EAF *Sa*, BF *Sa*, AF *Sa*, sterile distilled water (SDW) and ASM. The plants were sprayed with a solution of ASM at a concentration of 0.05 g/L (25 mL per plant). The extract fractions were diluted with water to a final concentration of 0.06 g/L, and 10 mL of each fraction was added to each plastic pot three days before inoculation with *Pst*. Tomato plants were inoculated at 15 days after transplant with a suspension of *Pst* at a concentration of 1.0×10^8 CFU mL^{-1} using a VL Airbrush atomizer (Paasche Airbrush Co.). Plants were maintained in a mist chamber for 24 h prior to inoculation and for an additional 24 h thereafter. After inoculation, the plants were placed in a growth chamber (temperature of 25 ± 2°C, relative humidity of 80 ± 5% and photoperiod of 12 h light) for 24 h. The number of lesions per plant (NLP) was evaluated at seven days after inoculation by counting the number of spots on all the leaves of each plant per replication of each treatment according to Schneider & Grogan (1977).

Determination of peroxidase (POX, EC1.11.1.7), polyphenoloxidase (PPO, EC 1.10.3.1), *β*-1,3-glucanase (GLU, EC 3.2.1.39) and phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) activities

In a separated experiment, leaf samples of plants from each replication and treatment were collected at 3, 6, 9 and 12 dai. Leaf samples from non-inoculated plants were collected at the same evaluation times. After sampling, leaf samples were stored individually in aluminum foil, rapidly frozen in liquid nitrogen and then stored in ultrafreezer at -80°C for further analysis.

To obtain the extracts used to determine the activities of POX, PPO, GLU and PAL, 0.3 g of leaf tissues was macerated with liquid nitrogen in a mortar with the addition of polyvinylpyrrolidone (PVP) 1% (w/v) to obtain a fine powder. The powder was homogenized in 2 mL of 50 mM sodium phosphate buffer (pH 6.5) containing 1 mM phenylmethylsulfonicfluoride (PMSF). The homogenized material was centrifuged at 20,000 × *g* for 25 min at 4°C and the supernatant was used to determine the enzymes activities. The POX and PPO activities were determined by the oxidation of pyrogallol according to the method of Kar & Mishra (1976). For POX activity, a mixture of 300 µL of distilled water, 250 µL of 100 mM potassium phosphate buffer (pH 6.8), 200 µL of 100 mM pyrogallol and 200 µL of 100 mM hydrogen peroxide was added to 50 µL of the extract. For PPO activity, the mixture was composed of 350 µL of distilled water, 350 µL of 100 mM potassium phosphate buffer (pH 6.8) and 200 µL of 100 mM pyrogallol, which was added to 100 µL of the extract. The absorbance was measured in a spectrophotometer (Evolution 60, Thermo Scientific) at 420 nm every 10 seconds for 1 min after addition of the extract to the mixture in a total of five readings. The molar extinction coefficient of 2.47 mM^{-1} cm^{-1} was used to calculate POX and PPO activities (Chance & Maehley, 1955), which were expressed in mM and µM of purpurogallin produced min^{-1} mg^{-1} of protein, respectively. Throughout the process, the microcentrifuge tubes were covered with aluminum foil to protect the mixture from oxidation by light. The GLU activity was determined as described by Lever (1972). The reaction was initiated by the addition of 20 µL aliquots of the supernatant to a mixture of 230 µL of 100 mM sodium acetate buffer (pH 5.0) and 250 µL of the substrate laminarin (Sigma-Aldrich) in a concentration of 4 mg/mL. The reaction mixture was incubated in a water bath for 30 min at 45°C. After the incubation period, the amount of reducing sugars was determined by adding 500 µL of dinitrosalicylic acid to the mixture and then incubating the resulting mixture in a water bath for 15 min at 100°C. The reaction was interrupted by cooling the samples in an ice bath. In the control samples, the reaction mixture was the same, except that the dinitrosalicylic acid was add at the same time as the extract. The absorbance of the product released by GLU was measured at 540 nm and the activity of GLU was expressed in absorbance units min^{-1} mg^{-1} of

protein. PAL activity was determined by adding 100 µL of the extract to a mixture containing 350 µL of 25 mM Tris-HCl buffer (pH 8.8) and 550 µL of 100 mM *L*-phenylalanine. The reaction mixture was incubated in a water bath at 30°C for 4 h. In the control samples, the *L*-phenylalanine was replaced with 1 mL of Tris-HCl buffer. The reaction was finalized by adding 60 µL of 6 N HCl. The absorbance of the *trans*-cinnamic acid derivatives was measured in a spectrophotometer at 290 nm and the molar extinction coefficient of 10^4 mM^{-1} cm^{-1} (Zucker, 1965) was used to calculate PAL activity, which was expressed in nM min^{-1} mg^{-1} of protein.

The protein concentration in each sample was determined according to the method of Bradford (1976).

Experimental design and statistical analysis

One experiment was performed in a completely randomized design with eight treatments and three replications to evaluate the NLP. This experiment was repeated once. The data were subjected to analysis of variance (ANOVA), and the treatment means were compared using Duncan's test at a probability of 5% and was calculated the standard deviation of the mean. Another experiment was performed in a completely randomized design with six treatments and four replications to obtain samples to determine the enzymes activities. This experiment was repeated once. The data were analyzed using ANOVA, and the treatment means were compared using confidence intervals at a probability of 5%. Statistical analyses were performed using SAS software (version 9.1, SAS Institute, Inc.).

RESULTS

Plant extracts activity

The bioautography was carried out in order to find out which compounds were responsible for the antimicrobial activity in the fractions studied. The *P. s.* pv. *tomato* growth was inhibited by fractions of *C. ferrea* and *S. adstringens* both in ethyl acetate and *n*-butanol. There was no significant differente between the zones of inhibition promoted by the two fractions. The aqueous fractions showed no activity against the bacteria. The phytochemical screening of the fractions of *C. ferrea* and *S. adstringens* in ethyl acetate and *n*-butanol revealed the presence of tannins and saponins, but the antimicrobial activity may have conferred by the former as evidenced by the formation of an inhibition zone in the bioauthography assay.

Number of bacterial leaf speck lesions

For Exp 1, NLP of bacterial leaf speck was reduced by 97%, 82% and 80% for the ASM, EAF *Sa* and BF *Sa* treatments, respectively, compared to the control treatment (Table 1). For Exp 2, NLP was reduced by 71%, 51% and 47% for the EAF *Sa*, ASM and BF *Cf* treatments, respectively, compared to the control treatment (Table 2).

TABLE 1 - NLP (number of lesions per plant) from the following treatments: ethyl acetate fraction of *S. adstringens* (EAF *Sa*), ethyl acetate of *C. ferrea* (EAF *Cf*), *n*-butanol fraction of *S. adstringens* (BF *Sa*), *n*- butanol fraction of *C. ferrea* (BF *Cf*), aqueous fraction of *S. adstringens* (AF *Sa*), aqueous fraction of *C. ferrea* (AF *Cf*), sterile distilled water (SDW) and Acibenzolar-S-Methyl (ASM) in Experiment 1. Means followed by the same letter are not significantly different ($P \leq 0.05$) by Duncan's test. SDM = standard deviation of means.

Treatments	NLP	SDM
SDW	998.3 a	146.1
BF *Cf*	646.0 b	30.0
EAF *Cf*	558.0 b	5.2
AF *Cf*	548.0 b	124.9
AF *Sa*	485.3 bc	33.5
BF *Sa*	198.0 cd	56.6
EAF *Sa*	183.0 cd	17.3
ASM	35.3 d	8.7

TABLE 2 - NLP (number of lesions per plant) from the following treatments: ethyl acetate fraction of *S. adstringens* (EAF *Sa*), ethyl acetate of *C. ferrea* (EAF *Cf*), *n*-butanol fraction of *S. adstringens* (BF *Sa*), *n*-butanol fraction of *C. ferrea* (BF *Cf*), aqueous fraction of *S. adstringens* (AF *Sa*), aqueous fraction of *C. ferrea* (AF *Cf*), sterile distilled water (SDW) and Acibenzolar-S-Methyl (ASM) on in Experiment 2. Means followed by the same letter are not significantly different ($P \leq 0.05$) by Duncan's test. SDM = standard deviation of means.

Treatments	NLP	SDM
AF *Sa*	2154.3 a	393.5
SDW	1760.0 a	322.2
EAF *Cf*	1225.0 ab	30.6
AF *Cf*	1194.0 ab	10.9
BF *Sa*	1182.3 ab	81.7
BF *Cf*	684.0 b	478.8
ASM	634.0 b	32.8
EAF *Sa*	373.0 b	13.3

Activities of defense enzymes

The treatment EAF *Sa*, that resulted in better protection against bacterial speck, was utilized for the experiment aiming to determine the activity of defense enzymes. For the non-inoculated plants, there was a significant difference between the ASM and control treatments at 3 dai and between the control treatment compared to the EAF Sa and ASM treatments at 12 dai (Figure 1A). In addition, POX activity was significantly greater for the inoculated plants treated with EAF *Sa* and ASM at 6 dai. At 9 and 12 dai, POX activity was significantly higher in plants sprayed with ASM compared to the EAF *Sa* and control treatments (Figure 1B). For the non-inoculated plants, there was no significant difference among the treatments for PPO activity (Figure 2A). PPO activity for the inoculated plants sprayed with ASM was significantly higher at 3 and 6 dai compared to the EAF *Sa* and control treatments. For PPO activity at 9 and 12 dai, there was a significant difference for the ASM

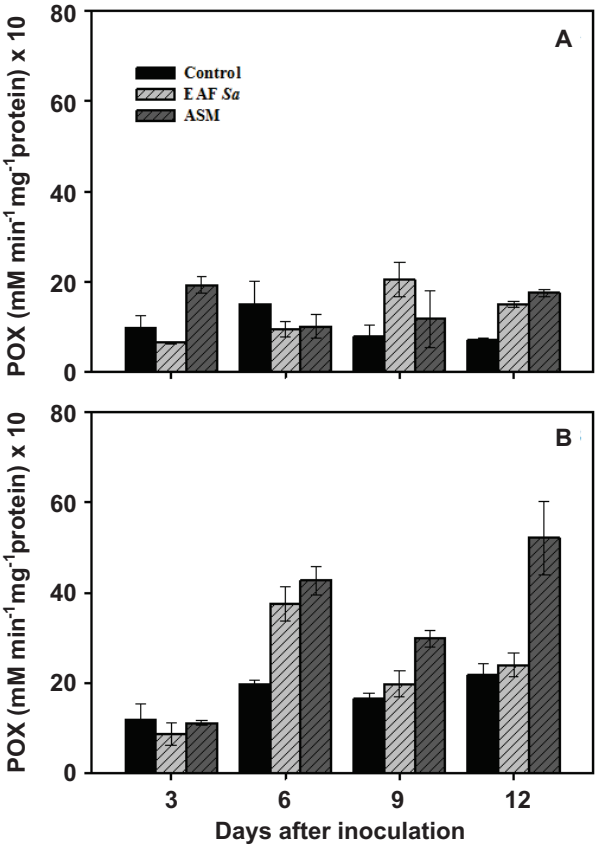

FIGURE 1 - Activity of peroxidases (POX) on the leaves of tomato plants non-inoculated (**A**) or inoculated (**B**) with *Pseudomonas syringae* pv. *tomato* and treated with the ethyl acetate fraction of *S. adstringens* (EAF *Sa*), sterile distilled water (control) and Acibenzolar-S-Methyl (ASM). The bars represent the confidence intervals at a probability of 5%.

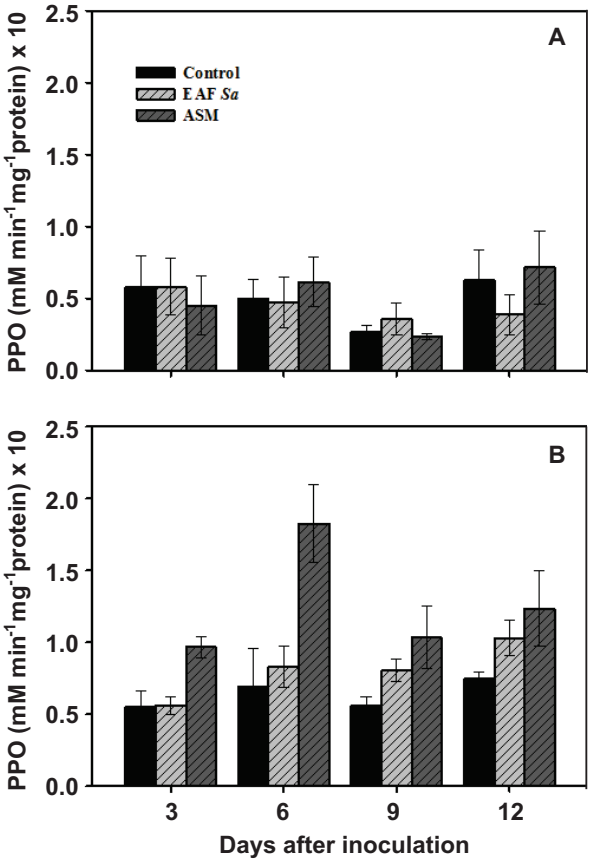

FIGURE 2 - Activity of polyphenoloxidases (PPO) on the leaves of tomato plants non-inoculated (A) or inoculated (B) with *Pseudomonas syringae* pv. *tomato* and treated with the ethyl acetate fraction of *S. adstringens* (EAF *Sa*), sterile distilled water (SDW) and Acibenzolar-S-Methyl (ASM). The bars represent the confidence intervals at a probability of 5%.

and EAF *Sa* treatments compared to the control treatment (Figure 2B). The GLU activity for non-inoculated plants was significantly higher at 9 and 12 days for the plants treated with EAF *Sa* and ASM compared to the control treatment (Figure 3A). The GLU activity was significantly higher for the inoculated plants and for plants sprayed with ASM at 3 and 6 dai compared to the EAF *Sa* and control treatments. At 9 dai, the GLU activity significantly increased in the inoculated plants treated with ASM or EAF *Sa*, but there was no significant difference between these two treatments. Moreover, GLU activity was higher at 12 dai for the inoculated plants treated with ASM and EAF *Sa* with a significant difference between these treatments as well as a significant difference compared to the control treatment (Figure 3B). For the non-inoculated plants treated with ASM or EAF *Sa*, PAL activity was significantly higher at 3 and 6 days compared to the control treatment. At 9 and 12 days, there was no significant difference between the ASM and EAF *Sa* treatments for GLU activity (Figure 4A). Furthermore, PAL activity in the inoculated plants treated with ASM and EAF *Sa* was significantly higher compared to the control treatment. At 3 dai, the PAL activity for plants treated with EAF *Sa* was significantly higher compared to the other treatments. At 6 dai, the PAL activity was significantly higher for the ASM and EAF *Sa* treatments compared to the control treatment, and at 9 dai, PAL activity was significantly higher for plants sprayed with ASM compared to the other treatments (Figure 4B).

DISCUSSION

To the best of our knowledge, this study is the first to demonstrate the potential of the extracts obtained from *S. adstringens* and *C. ferrea* in reducing bacterial leaf speck symptoms in tomato plants with a consequent increase in the activities of defense enzymes. The ethyl acetate fraction obtained from the fruits of *S. adstringens* contains substances that function as triggers of defense responses in tomato plants to protect against *Pst* infection. The phytochemical evaluation performed on the fractions in ethyl acetate indicated the presence of tannins and saponins. Probably the tannins were the compounds responsible for the

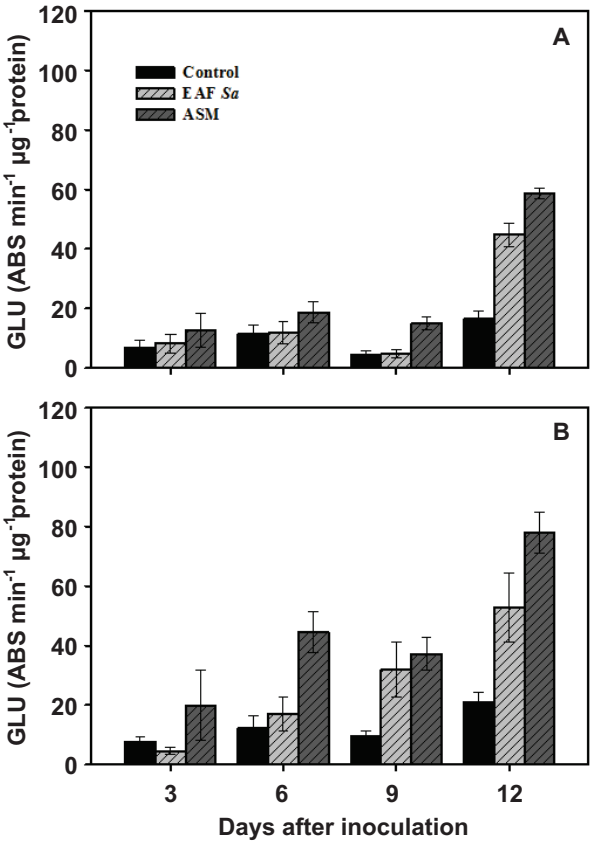

FIGURE 3 - Activity of β-1,3-glucanases (GLU) on the leaves of tomato plants non-inoculated (A) or inoculated (B) with *Pseudomonas syringae* pv. *tomato* and treated with the ethyl acetate fraction of *S. adstringens* (EAF *Sa*), sterile distilled water (control) and Acibenzolar-S-Methyl (ASM). The bars represent the confidence intervals at a probability of 5%.

antimicrobial activity against *P. s.* pv. *tomato* determined in the bioauthography assay. Tannins act by inhibiting bacterial and fungal enzymes and/or by complexing with enzyme substrates. In addition, tannins act on the cell membranes of microorganisms, thereby altering their metabolism via the inhibition of oxidative phosphorylation or by complexing metal ions, which reduces the availability of ions that are essential for microbial metabolism (Scalbert, 1991). It has been reported that avenacine and saponins are important compounds for oats resistance against pathogens (Osbourn, 1996). It is known that saponins act in the disruption of fungal cell membranes releasing cyanide and inhibiting cellular respiration (Osbourn, 1996). It is suggested that saponins were the compounds responsible for the increase in resistance of the tomato plants against the infection by *P. s.* pv. *tomato* due to their no antimicrobial activity against the bacteria.

Natural compounds can enhance the capacity of plants to acquire resistance to a broad spectrum of pathogens (Dubey et al., 2011; Conrath et al., 2002; Pastor et al., 2012). This process called priming of defense can provide long-lasting resistance based in host defense reaction upon pathogen infection and can be maintained long after the initial stimulus (Conrath, 2011; Pastor et al., 2012). The host defense mechanisms against pathogens involve metabolic changes that are linked to changes in the concentration of proteins, which are also known as pathogenesis-related proteins (PR-proteins). In the present study, POX activity was high in plants treated with ASM and EAF *Sa*, particularly in ASM-treated plants. Increased POX activity was also reported in tomato plants treated with ASM and citrus extract and in plants infected by *Xanthomonas vesicatoria* (Cavalcanti et al., 2006b). Furthermore, PPO and POX have phenolic compounds as substrates, and the products of their oxidation exhibit anti-microbial activity against pathogens (Mueller & Beckman, 1974). POX is also involved in the biosynthesis of ethylene, indole-3-acetic acid, lignin and suberin (Quiroga et al., 2000). These enzymes are important in tissue lignification, which acts as a physical barrier that prevents or reduces pathogen colonization and also decreases the diffusion of lytic enzymes and non-selective toxins (Nicholson & Hammerschimidt, 1992).

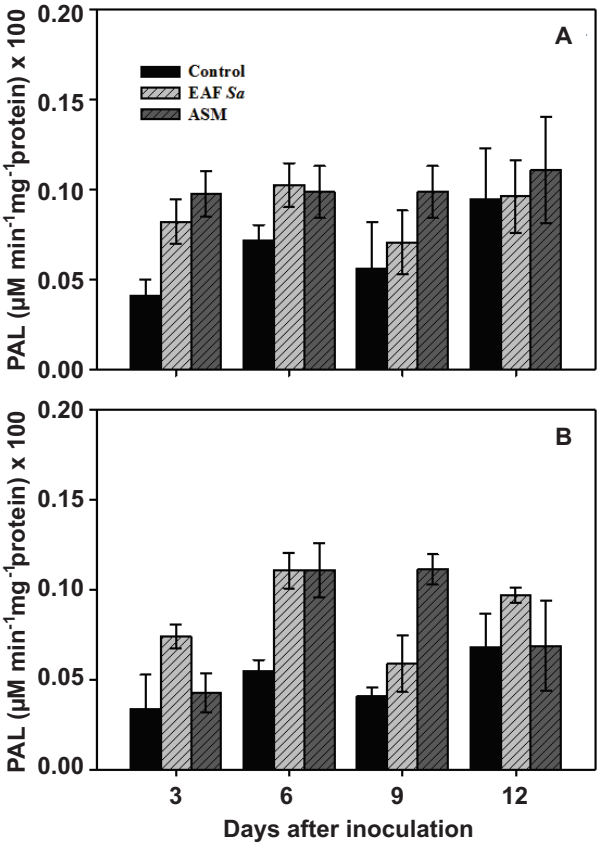

FIGURE 4 - Activity of phenylalanine ammonia-lyases (PAL) on the leaves of tomato plants non-inoculated (A) or inoculated (B) with *Pseudomonas syringae* pv. *tomato* and treated with the ethyl acetate fraction of *S. adstringens* (EAF *Sa*), sterile distilled water (control) and Acibenzolar-S-Methyl (ASM). The bars represent the confidence intervals at a probability of 5%.

The PPO activity was higher in plants treated with ASM and EAF *Sa*. PPO is capable of catalyzing the oxidation of phenolic compounds to quinones, which are highly toxic compounds against pathogens (Li & Steffens, 2002). Tomato plants overexpressing the PPO gene showed a greater capacity for oxidizing phenolic compounds and consequently a greater resistance against infection by *Pst* (Li & Steffens, 2002). Sprays of the leaf *Hedera helix* extract and ASM increased the resistance of apple fruits to infection by *Erwinia amylovora* due to an increase in POX activity (Baysal & Zeller, 2004).

Furthermore, GLU activity was high in plants sprayed with ASM prior to the appearance of bacterial leaf speck symptoms, which differed from plants treated with EAF *Sa*, in which its activity was higher at advanced stages of bacterial infection. The faster and stronger GLU activity in plants sprayed with ASM indicated that the plants were in a primed state induced by ASM. The highest GLU activity was important for tomato resistance against infection by *Erwinia amylovora* and *Xanthomonas campestris* pv. *vesicatoria* (Brisset et al., 2000; Cavalcanti et al., 2006a). GLU is a lytic enzyme capable of degrading polysaccharides found in the cell walls of pathogens (Van Loon et al., 2006); however, its role in the resistance against bacterial pathogens remains unclear (Cavalcanti et al., 2006a).

PAL activity was also high in plants treated with EAF *Sa* before the appearance of bacterial leaf speck symptoms, and its activity was higher in plants treated with EAF *Sa* and ASM when the first bacterial leaf speck lesions were observed on the tomato leaves. Thus, it is proposed that there was an increase in the production of phenolic compounds in response to *Pst* infection during this period because PAL is responsible for the deamination of the amino acid *L*-phenylalanine, which forms *trans*-cinnamic acid and many other phenolic compounds (Hammerchmidt & Nicholson, 1992; Assis et al., 2001). Cotton plants treated with the leaf extract of zimmu (*Allium sativum* L. × *Allium cepa* L.) showed higher activities of PAL, POX and PPO and high concentrations of phenolic compounds that contributed to an increase in resistance against *X. campestris* pv. *malvacearum* infection (Satya et al., 2007).

On the basis of these results, it can be concluded that the ethyl acetate fraction of *S. adstringens* was effective in reducing bacterial leaf speck symptoms on tomato leaves with the activities of the defense enzymes POX, PPO, PAL and GLU playing a pivotal role in the increase in host resistance.

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