Tropical Plant Pathology, vol. 36, 2, 074-080 (2011)

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RESEARCH ARTICLE / *ARTIGO*

Induced resistance against *Xanthomonas axonopodis* pv. *passiflorae* in passion fruit plants

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ABSTRACT

Control of bacterial leaf spot of yellow passion fruit using the abiotic resistance inducer, acibenzolar-S-methyl (ASM), and the biotic agents, harpin protein and glycoproteins extracted from two *Xanthomonas* species, was evaluated. The inducers were applied by spraying the leaves 72 h before the inoculation with *Xanthomonas axonopodis* pv. *passiflorae*. The inducers were also applied by seed immersion and the inoculation was performed when the seedlings had four true leaves. The results showed that ASM conferred a protection up to 70% at the concentration of 12.5 µg a.i. mL^{-1}, while harpin led to an increase in bacterial symptoms. The glycoproteins from *Xanthomonas* spp. conferred up to 72% protection in plants against the bacterium. ASM or harpin provided up to 90% and 47% protection, respectively, in yellow passion fruit seedlings raised from treated seeds. Thus, leaf treatment with ASM or the glycoproteins from *Xanthomonas* spp. and seed treatment with ASM or harpin are potent inducers of resistance in passion fruit plants against *X. axonopodis* pv. *passiflorae*.
Key words: *Passiflora edulis,* acibenzolar-S-methyl, glycoproteins, harpin protein, plant resistance inducers, seed treatment.

RESUMO

Resistência induzida contra *Xanthomonas axonopodis* pv. *passiflorae* em maracujazeiro

Esta pesquisa avaliou a possibilidade de controle da mancha bacteriana do maracujazeiro amarelo através da avaliação do indutor de resistência abiótico acibenzolar-S-metil (ASM) e dos agentes bióticos, proteína harpina e glicoproteínas extraídas de duas espécies de *Xanthomonas*. Os indutores foram aplicados por aspersão nas folhas 72 h antes da inoculação com *Xanthomonas axonopodis* pv. *passiflorae*. Os indutores foram também aplicados em sementes por imersão e a inoculação foi realizada quando as plântulas apresentaram quatro folhas verdadeiras. Os resultados mostraram que o ASM conferiu proteção de até 70% na concentração de 12,5 µg i.a. mL^{-1}, enquanto que a proteína harpina proporcionou um aumento dos sintomas da bacteriose. As glicoproteínas de *Xanthomonas* spp. conferiram proteção de até 72% nas plantas contra a bactéria. Nas plântulas provenientes de sementes tratadas com ASM ou proteína harpina houve proteção de até 90% e 47%, respectivamente, contra a mancha bacteriana. ASM ou glicoproteínas de *Xanthomonas* spp., quando aplicadas por aspersão em folhas, e ASM ou proteína harpina, quando aplicados em sementes, são potenciais indutores de resistência em maracujazeiro amarelo a *X. axonopodis* pv. *passiflorae*.
Palavras-chave: *Passiflora edulis*, acibenzolar-S-metil, glicoproteínas, indução de resistência em plantas, proteína harpina, tratamento de sementes.

INTRODUCTION

In Brazil one of the most important species of passion fruit is *Passiflora edulis* Sims f. *flavicarpa* Deg. (yellow passion fruit). However, the crop yield is affected by many factors including plant pathogens. Among those, *Xanthomonas axonopodis* pv. *passiflorae*, the causal agent of bacterial spot disease, is one of the most important pathogens of the passion fruit vine, decreasing its period of commercial exploitation. The bacterium is present in the areas where this passifloraceae is cultivated, infecting all commercially grown passion fruit species (Beriam & Malavolta, 2006).

Although bacterial diseases are currently very difficult to control, plants can be induced to develop enhanced resistance to pathogen infection by treatment with a variety of abiotic and biotic inducers (Walters et al., 2005). Resistance induced by such agents is referred to as Systemic Acquired Resistance (SAR) or Induced Systemic Resistance (ISR), and has a broad spectrum and a long lasting effect that were verified in several plant-pathogen interactions (Hammerschmidt et al., 2001). The observed protecting effect was dependent on light, temperature, inducer and inoculum concentrations and the period between inducer application and pathogen inoculation (Sticher et al., 1997).

Part of the Master Thesis of the first author. Instituto Biológico. São Paulo SP. 2009.

The synthetic compound acibenzolar-S-methyl (ASM) has been described as one of the most promising products that can induce SAR in plants and in seeds against different pathogens (Latunde-Dada & Lucas, 2001; Iriti & Faoro, 2003; Guzzo et al., 2004; Ishida et al., 2008). This compound has been marketed as a plant activator with the trade name of Bion® (Syngenta, Europe) and Actigard® (Syngenta, USA) and is also used commercially in Brazil on tomato to control bacterial diseases. Studies have shown that pre-harvest application of ASM in strawberry orchards delayed the development of *Botrytis cinerea* in fruits (Terry & Joyce, 2000). Pre-harvest treatment of tomato seedlings with ASM showed a reduction in the severity of the bacterial canker caused by *Clavibacter michiganensis* subsp. *michiganensis* (Baysal et al., 2003). Seedlings of a susceptible cultivar of *Vigna unguiculata* (cowpea) grown from seeds previously treated with ASM were protected against anthracnose caused by *Colletotrichum destructivum* (Latunde-Dada & Lucas, 2001).

Harpin, a protein of bacterial origin from *Erwinia amylovora*, has also been used by several researchers as a resistance inducer and was first commercialized with the trade name of Messenger® by EDEN Bioscience Corporation until 2008 (Jones, 2001) and afterwards with the trade name ProAct™ by PLANT HEALTH CARE. Capdeville et al. (2002, 2003) showed a reduction of the blue mold symptoms caused by *Penicillium expansum* in apples treated with harpin. Post-harvest treatment of peaches with two commercial products containing harpin at 80 mg L^{-1} of active ingredient (a.i.) reduced the development of *Monilinia fructicola* (Danner et al., 2008). Graham & Leite (2004) showed a reduction up to 55% at the concentration of 60 mg L^{-1} a.i. of harpin in symptoms of citrus bacterial spot, caused by *X. axonopodis pv. citrumelo* in citrumelo 'Swingle'.

Glycoproteins can also activate defense responses in some plant-pathogen interactions. Grapevines treated with proteins extracted from *Phytophthora oligandrum* were protected against *B. cinerea* by 75% (Mohamed et al., 2007). According to Romeiro & Kimura (1997), glycoproteins isolated from *Xanthomonas campestris* pv. *vesicatoria* induced resistance to this pathogen when applied to pepper plants 72 h before challenge inoculation. Based on these reports, the objective of this study was to evaluate the control of *X. axonopodis* pv. *passiflorae* using acibenzolar-S-methyl as well as harpin and glycoproteins extracted from *X. axonopodis* pv. *passiflorae* and *X. campestris* pv. *campestris*, applied to leaves or seeds of yellow passion fruit.

MATERIALS AND METHODS

Yellow passion fruit (*Passiflora edulis* f. *flavicarpa*) seeds obtained from Campinas, São Paulo State, were sown in plastic bags filled with a mixture of soil, sand and dried cow manure (1:1:1) supplemented with simple superphosphate and KCl, in a greenhouse at a temperature range of 25-30ºC and automatic irrigation. The pathovar reference strain IBSBF 1343 of *X. axonopodis* pv. *passiflorae* used in this study was obtained from the Phytobacteria Culture Collection of Instituto Biológico (Campinas, Brazil). The bacterium was isolated from *P. edulis* plants (Araraquara, São Paulo, Brazil) and preserved in peptone glycerol at -80°C. The strain was recovered in nutrient agar (NA) culture medium and was kept in a sterile aqueous suspension at room temperature.

Aqueous suspensions prepared from the commercial product Bion® 500WG, which contains 50% of the active ingredient (a.i.), were used at the concentrations of 12.5 and 25 µg a.i. mL^{-1}. Aqueous suspensions prepared from the commercial product Messenger® containing 3% of harpin were used at the concentrations of 3; 7.5; 15 and 30 µg a.i. mL^{-1}. The extraction of glycoproteins (GPs) from *X. axonopodis* pv. *passiflorae* and *X. campestris* pv. *campestris* was carried out according to Digat & Cambra (1976). After extraction, the protein concentrations of bacteria filtrates were estimated by the method of Bradford (Bradford, 1976) and expressed as micrograms bovine serum albumin (BSA) equivalent per milliliter of filtrate (µg BSA Eq mL^{-1}). The GPs extracted from both bacteria were used at the concentration of 97.93 µg BSA Eq mL^{-1}.

Leaves of yellow passion fruit plants (approximately 3 months old) were sprayed with the abiotic and biotic agents, at the concentrations previously described, 72 hours before challenge inoculation. Control plants were sprayed with deionized water instead of the agents. Plants were inoculated by spraying the same previously treated leaves with a bacterial suspension at the concentration of $3x10^8$ colony forming unit (CFU) mL^{-1} through leaf wounds, performed by pricking leaves with a needle. Following inoculation, plants were placed for 72 h at 100% relative humidity in a chamber under a 12 h-photoperiod at a temperature range of 23-26ºC. The plants were observed daily until the emergence of symptoms and the evaluation was performed 10 days after the inoculation with the bacterium. All plants were evaluated on the same day.

A second experiment was performed to evaluate the systemic effect of the agents in the reduction of bacterial leaf spot symptoms. The agents were sprayed on the first four basal leaves of yellow passion fruit plants, 72 h before inoculation, while control plants received only deionized water. Plants were then challenge-inoculated with the bacteria in the four basal leaves pretreated with the inducers and in the two non-induced top leaves. Inoculation was carried out as previously described. The assays were conducted in a completely randomized design, using groups of 5 to 10 plants per treatment.

In a third experiment, yellow passion fruit seeds were treated with the resistance inducers ASM, at the concentrations 12.5 and 25 µg a.i. mL^{-1}, or harpin, at the concentrations of 3 and 7.5 µg a.i. mL^{-1}. Seeds were soaked for 24 h in each one of the solutions. Treated seeds were

sown in pots filled with the same substrate previously described and maintained in a greenhouse at a temperature of 25-30ºC and automatic irrigation. Control seedlings were raised from seeds soaked in sterile deionized water. Five pots were used for each treatment, with five seeds per pot, totalizing 25 plants. The experimental design was completely randomized and 25 plants in each treatment group were used for each experiment (25 replicates). After 30 days, when the seedlings had developed a pair of true leaves, ten plants of each treatment were boosted by foliar spray with the resistance inducers. In this case, the first pairs of true leaves were treated with one application of the inducer. The remaining 15 plants did not receive this additional treatment. When the seedlings of all plants, treated once with the resistance inducers (only the seeds were treated before sowing) or twice (the seeds were treated before sowing and the first true leaves of seedlings were sprayed once with the inducer), presented two pair of true leaves, inoculations with the bacterial suspension were carried out as previously described.

In all experiments the evaluation of the symptoms was carried out with the Quant V.1.0 - Software for Quantification of Plants Diseases (Vale et al., 2003) to determine the disease severity, expressed as percentage of infected foliar area. The protection was calculated as reduction of the disease severity and expressed as percentage of the control. In the experiments performed to evaluate the systemic effect of the inducers in the reduction of bacterial leaf spot symptoms, the percentage of protection against the disease was calculated by comparison between corresponding leaves in the control and treated plants. The data obtained in all experiments were subjected to ANOVA variance analysis and the means were compared by Tukey's test ($P \leq 0.05$) to determine statistical significance. All the experiments were repeated at least three times.

RESULTS

Plants of yellow passion fruit treated with ASM at the concentration of 12.5 µg a.i. mL^{-1} showed a decrease in bacterial leaf spot symptoms, in relation to the control (Table 1, Figure 1). Furthermore, ASM did not show any effect on the *in vitro* growth of *X. axonopodis* pv. *passiflorae* (data not shown). No significant effect was observed regarding

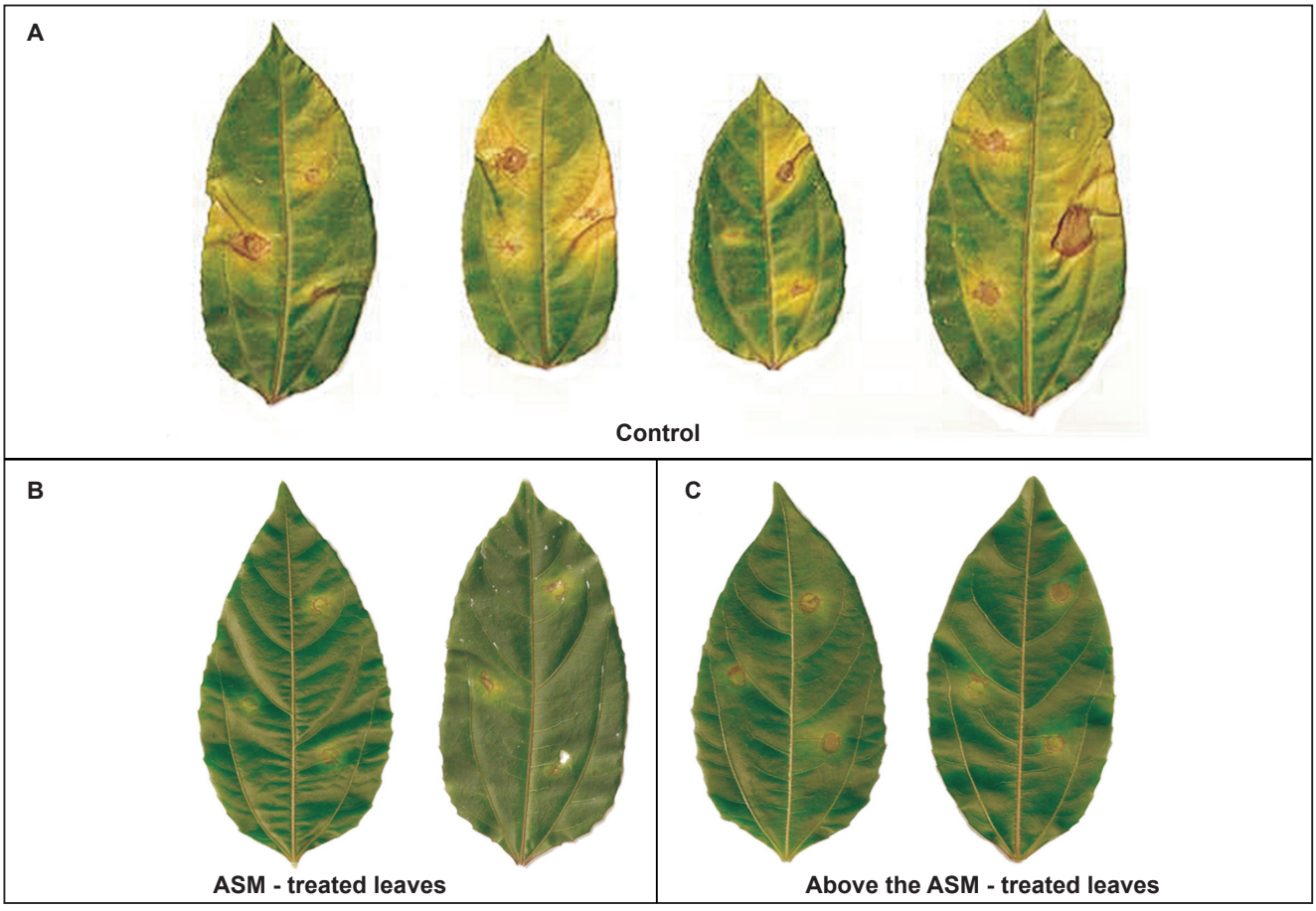

FIGURE 1 - A. Effect of foliar treatment with acibenzolar-S-methyl (ASM) on the development of bacterial leaf spot symptoms caused by *Xanthomonas axonopodis* pv. *passiflorae* on yellow passion fruit plants; **B.** Leaves treated with deionized water; **C.** ASM-treated leaves at a concentration of 12.5 µg a.i mL^{-1}. Non-induced leaves above the ASM-treated leaves at a concentration of 12.5 µg a.i. mL^{-1}.

TABLE 1 - Effect of foliar treatment with acibenzolar-S-methyl (ASM) on the development of bacterial leaf spot symptoms caused by *Xanthomonas axonopodis* pv. *passiflorae* on yellow passion fruit plants

Treatments	Foliar infected area (%)[1]	Protection (%)
ASM-treated leaves (12.5 µg a.i. mL^{-1})	8.0 ± 1.7 b	67.4
ASM-treated leaves (25 µg a.i. mL^{-1})	15.3 ± 4.6 ab	38.0
Control	24.6 ± 6.4 a	----
Non-induced leaves above the ASM-treated leaves (12.5 µg a.i. mL^{-1})	8.0 ± 2.6 b	70.2
Non-induced leaves above the ASM-treated leaves (25 µg a.i. mL^{-1})	15.1 ± 5.5 ab	43.7
Control	26.8 ± 7.0 a	----

[1]Means and standard errors followed by different letters are significantly different, Tukey´s test, $P \leq 0.05$.

disease severity reduction when ASM was applied at the concentration of 25 µg a.i. mL^{-1} on the yellow passion fruit leaves (Table 1). Harpin did not induce local resistance in yellow passion fruit leaves (Table 2). All harpin concentrations led to an increase in bacterial leaf symptoms, varying from 88.6 to 108.6%. The results obtained using glycoprotein extracts from both *X. axonopodis* pv. *passiflorae* and *X. campestris* pv. *campestris* showed local reductions in disease severity in yellow passion fruit plants (Table 3). However, untreated leaves of plants treated with GPs did not show significant reduction in the percentage of foliar infected area compared to the corresponding controls.

The treatments of yellow passion fruit seeds with ASM (12.5 and 25 µg a.i. mL^{-1}) and harpin (3 and 7.5 µg a.i. mL^{-1}) showed that both inducers, in the two applied concentrations, were able to reduce the bacterial leaf spot symptoms in seedlings further inoculated with *X. axonopodis* pv. *passiflorae* (Table 4), providing a systemic protection in yellow passion fruit against the bacterial disease. The protection was enhanced when seedlings grown from seeds treated with the abiotic resistance inducer were boosted with a foliar treatment with ASM at both concentrations (Table 5). However, when harpin was applied by foliar spray to the seedlings grown from harpin-treated seeds, a significant reduction in bacterial leaf spot relative to controls could only be detected at the concentration of 3 µg a.i. mL^{-1}.

DISCUSSION

The decreases in disease severity observed in ASM-treated leaves at the concentration of 12.5 µg a.i. mL^{-1} and in untreated systemic leaves above treated leaves, suggest that both local and systemic resistance induction occurred. Several compounds have been reported to induce SAR in a variety of plants against a wide range of microbial pathogens without possessing direct antimicrobial activity (Barilli et al., 2010). The results are in agreement with data from the literature describing several plant-pathogen interactions that showed the ability of ASM to induce resistance in plants (Iriti & Faoro, 2003; Guzzo et al., 2004; Ishida et al., 2008; Faize et al., 2009). However, when ASM at the concentration of 25 µg a.i. mL^{-1} was applied to leaves of the yellow passion fruit, no significant effect was observed on disease severity. According to Kuhn (2007), the induction of resistance in bean plants against *Xanthomonas axonopodis* pv. *phaseoli*, causal agent of bacterial blight, with the use of this SAR inducer was achieved only when low concentrations of ASM were applied. On the other hand, the fact that only the lowest concentration of ASM was active when directly sprayed on leaves in the pathosystem under study, confirms the statement of Kuć (1995) that the protecting effect observed in plants is dependent on several factors, including the concentration of the inducer.

TABLE 2 - Effect of foliar treatment with harpin on the development of bacterial leaf spot symptoms caused by *Xanthomonas axonopodis* pv. *passiflorae* on yellow passion fruit plants

Treatments	Foliar infected area (%)[1]	Disease increase (%)
Harpin 3 µg a.i. mL^{-1}	50.2 ± 4.8 a	103.7
Harpin 7.5 µg a.i. mL^{-1}	46.8 ± 4.0 a	90.1
Harpin 15 µg a.i. mL^{-1}	51.4 ± 3.7 a	108.6
Harpin 30 µg a.i. mL^{-1}	46.5 ± 3.4 a	88.6
Control	24.6 ± 3.2 b	----

[1]Means and standard errors followed by different letters are significantly different, Tukey´s test, $P \leq 0.05$.

TABLE 3 - Effect of foliar treatments with the glycoproteins isolated from the *Xanthomonas axonopodis* pv. *passiflorae* (Xa 2267) and *Xanthomonas campestris* pv. *campestris* (Xa 2150) on the development of bacterial leaf spot symptoms caused by *Xanthomonas axonopodis* pv. *passiflorae* on yellow passion fruit plants

Treatments	Foliar infected area (%)[1]	Protection (%)
Xa 2150-treated leaves	6.4 + 1.0 a	72.5
Xa 2267-treated leaves	10.9 ± 1.3 a	53.0
Control	23.3 ± 2.9 b	----
Non-induced leaves above the Xa 2150-treated leaves	14.2 + 6.3 a	0
Non-induced leaves above the Xa 2267-treated leaves	9.1 ± 2.4 a	20.1
Control	11.4 ± 2.5 a	----

Means and standard errors followed by different letters are significantly different, Tukey´s test, $P \leq 0.05$.

TABLE 4 - Effects of seed treatments with different concentrations of acibenzolar-S-methyl (ASM) and harpin on the development of bacterial leaf spot symptoms in yellow passion fruit seedlings, caused by *Xanthomonas axonopodis* pv. *passiflorae*

Treatments	Foliar infected area (%)[1]	Protection (%)
ASM (12.5 µg a.i. mL^{-1})	3.7 ± 0.9 c	81.2
ASM (25 µg a.i. mL^{-1})	7.7 ± 1.5 bc	61.4
Harpin (3 µg a.i. mL^{-1})	11.0 ± 2.7 bc	44.7
Harpin (7.5 µg a.i. mL^{-1})	11.1 ± 2.2 b	44.0
Control	19.8 ± 2.4 a	----

[1]Means and standard errors followed by different letters are significantly different, Tukey´s test, $P \leq 0.05$.

TABLE 5 - Effects of acibenzolar-S-methyl (ASM) and harpin foliar treatments on seedlings grown from treated seeds on the development of bacterial leaf spot symptoms caused by *Xanthomonas axonopodis* pv. *passiflorae* on yellow passion fruit

Treatments[1]	Foliar infected area (%)[2]	Protection (%)
ASM (12.5 µg a.i. mL^{-1})	2.1 ± 0.4 c	89.5
ASM (25 µg a.i mL^{-1})	2.0 ± 0.4 c	90.0
Harpin (3 µg a.i. mL^{-1})	10.6 ± 1.5 b	46.4
Harpin (7.5 µg a.i. mL^{-1})	15.1 ± 2.3 ab	23.8
Control	19.8 ± 2.4 a	----

[1]Treatments performed on leaves of seedlings grown from seeds treated with ASM or harpin.
[2]Means and standard errors followed by different letters are significantly different, Tukey´s test, $P \leq 0.05$.

According to Ouchi (1983), the treatment of plants with potential resistance inducers can promote an occasional suppression resulting in more susceptibility of the hosts to the pathogen. This phenomenon is called "induced susceptibility". In the present experiment of resistance induction with the harpin sprayed directly on leaves of yellow passion fruit plants this situation was observed, since an increase in bacterial leaf spot symptoms was detected in treated plants. However, there is also the possibility that the enhanced susceptibility observed in harpin-treated plants resulted from a direct toxic effect of the product on the physiology of the plant, as already observed by Kuhn et al. (2006) in the *Manihot esculenta*-*Xanthomonas axonopodis* pv. *manihotis* pathosystem with *Curcuma longa* extract treatment. Another possibility is that harpin stimulated pathogen growth as observed by Galdeano et al. (2010), where the highest concentrations of harpin increased germination of *Cercospora coffeicola* and, consequently, increased symptoms in coffee plants treated with harpin. The results obtained in the present study contrast with those reported in the literature that indicate the ability of harpin to induce resistance in plants (Capdeville et al., 2002, 2003; Graham & Leite, 2004; Yang et al., 2005; Danner et al., 2008).

The results obtained using glycoprotein extracts from both *X. axonopodis* pv. *passiflorae* and *X. campestris* pv. *campestris* indicate induction of local protection in yellow passion fruit plants against *X. axonopodis* pv. *passiflorae*. However, systemic resistance was not observed. These results are in agreement with those obtained by Romeiro & Kimura (1997) for the interaction *Capsicum annuum* - *X. campestris* pv. *vesicatoria*, where glycoproteins obtained from cells of this bacterial pathogen were infiltrated into pepper leaves conferring protection against a compatible isolate. Our results still corroborate the reports of Di Piero et al. (2006) and Mohamed et al. (2007) in which protein fractions obtained from *Lentinula edodes* basidiocarps and *Pythium oligandrum* were capable of eliciting defense responses and disease protection in cucumber seedlings against *Colletotrichum lagenarium* and grapevine leaves against *Botrytis cinerea*, respectively.

The results obtained with seed treatments of yellow passion fruit with ASM (12.5 and 25 µg a.i. mL^{-1}) and harpin (3 and 7.5 µg a.i. mL^{-1}) were particularly important, bearing in mind that control measures for bacterial diseases are generic and usually adopted in a preventive manner, and that they can contribute to integrated management to control this disease. Both inducers promoted a systemic protection at the applied concentrations in yellow passion fruit against the bacterial disease.

The protection was enhanced when seedlings grown from seeds treated with the abiotic resistance inducer were boosted with a foliar treatment with ASM at both concentrations. Induction of systemic resistance has also been shown when leaves of yellow passion fruit plants were sprayed with ASM inducer (Table 1). The observed protection in seedlings obtained from seeds treated with ASM is in agreement with the results obtained by Latunde-Dada & Lucas (2001). Cowpea seedlings, raised from seeds treated with ASM, were protected against anthracnose caused by *C. destructivum* when further inoculated with the pathogen. Other compounds can also afford protection when applied in seeds. For instance, Benhamou et al. (1994) showed that tomato seedlings were protected against *Fusarium oxysporum* f. sp. *radicis-lycopersici*, when seeds were treated with chitosan combined with a modified substrate. When harpin was applied by foliar spray to the seedlings grown from harpin-treated seeds, a reduction in bacterial leaf spot could be detected only at the concentration of 3 µg a.i. mL^{-1}. Other studies also showed that higher concentrations of resistance inducers were not effective in reducing disease symptoms or induced phytotoxicity in treated plants (Latunde-Dada & Lucas, 2001). The results of this study demonstrated the potential of the tested abiotic and biotic agents in the control of yellow passion fruit bacterial leaf spot caused by *X. axonopodis* pv *passiflorae*.

ACKNOWLEDGMENTS

We thank Irene Maria Gatti de Almeida, from Instituto Biológico, Campinas SP for reviewing the manuscript and for helpful suggestions.

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TPP 167 - Received 16 August 2010 - Accepted 27 April 2011
Section Editor: Marisa A.S.V. Ferreira