

Chemical products induce resistance to *Xanthomonas perforans* in tomato

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Submitted: January 21, 2014; Approved: November 28, 2014.

Abstract

The bacterial spot of tomato, caused by *Xanthomonas* spp., is a very important disease, especially in the hot and humid periods of the year. The chemical control of the disease has not been very effective for a number of reasons. This study aimed to evaluate, under greenhouse conditions, the efficacy of leaf-spraying chemicals (acibenzolar-S-methyl (ASM) (0.025 g.L⁻¹), fluazinam (0.25 g.L⁻¹), pyraclostrobin (0.08 g.L⁻¹), pyraclostrobin + methiran (0.02 g.L⁻¹ + 2.2 g.L⁻¹), copper oxychloride (1.50 g.L⁻¹), mancozeb + copper oxychloride (0.88 g.L⁻¹ + 0.60 g.L⁻¹), and oxytetracycline (0.40 g.L⁻¹) on control of bacterial spot. Tomatoes Santa Clara and Gisele cultivars were pulverized 3 days before inoculation with *Xanthomonas perforans*. The production of enzymes associated with resistance induction (peroxidase, polyphenol oxidase, phenylalanine ammonia-lyase, β-1,3-glucanase, and protease) was quantified from leaf samples collected 24 hours before and 24 hours after chemical spraying and at 1, 2, 4, 6, and 8 days after bacterial inoculation. All products tested controlled bacterial spot, but only ASM, pyraclostrobin, and pyraclostrobin + metiram increased the production of peroxidase in the leaves of the two tomato cultivars, and increased the production of polyphenol oxidase and β-1,3-glucanase in the Santa Clara cultivar.

Key words: *Solanum lycopersicum*, bacterial spot, control, enzymes.

Introduction

The tomato can be considered one of the main fruits produced and consumed in Brazil, and tomatoes are grown in several regions of the country, especially the south, southeast and mid-west (Camargo and Camargo Filho, 2008). There are several factors that decrease tomato production, including diseases caused by fungi, bacteria, and viruses (Lopes and Avila, 2005).

A disease known as bacterial spot, caused by *Xanthomonas euvesicatoria*, *X. vesicatoria*, *X. perforans*, and *X. gardneri* (Jones *et al.*, 2004), attacks tomatoes in Brazil (Quezado-Duval *et al.*, 2005; Pereira *et al.*, 2011). Field management of the disease is difficult due to high temperatures and intense rainfall, and preventive measures are adopted for the management of bacterial spot, especially crop rotation, the use of healthy seeds and seedlings,

balanced fertilization, and spraying of chemical products (Lopes and Avila, 2005).

In tomato fields, the use of copper fungicides, antibiotics, and copper carbamate mixtures do not always provide satisfactory control of the disease (Maringoni *et al.*, 1986) and can cause the selection of resistant bacterial isolates (Marco and Stall, 1983; Maringoni and Kimati 1987; Quezado-Duval *et al.*, 2003). Previous research has demonstrated the effectiveness of applying acibenzolar-S-methyl (ASM) to control bacterial spot (Louws *et al.*, 2001; Cavalcanti *et al.*, 2005a, b). ASM induces resistance to the disease in tomato plants (Cavalcanti *et al.*, 2005a, b) without directly affecting the bacteria (Itako *et al.*, 2012a).

In Brazil, several fungicides are used to control fungal diseases in tomato including pyraclostrobin (Agrofit, 2012). Besides controlling early blight and late blight in tomatoes, it also activates the synthesis of proteins associated

with the induction of resistance in plants (Herms *et al.*, 2002; Vigo *et al.*, 2012). Studies conducted by Itako *et al.* (2012b) showed that ASM and pyraclostrobin help control the bacterial spot on the tomato hybrid AP 529, and that these products affect the activity of peroxidase, polyphenol oxidase, and β -1,3-glucanase.

This study aimed to evaluate, under greenhouse conditions, the efficacy of leaf spraying with various chemicals (ASM, fluzinam, pyraclostrobin, pyraclostrobin + metiram, copper oxychloride, copper oxychloride + mancozeb, and oxytetracycline) on the control of bacterial spot on Santa Clara and Gisele tomato cultivars. We also evaluated whether these chemicals induce the production of peroxidase, polyphenol oxidase, β -1,3-glucanase, phenylalanine ammonia-lyase, and protease, which are associated with resistance to bacterial spot of tomato (Cavalcanti *et al.*, 2005a, b; Itako *et al.*, 2012b; Luiz *et al.*, 2012).

Materials and Methods

Effects of fungicides and antibiotic on bacterial spot

In order to evaluate the effect of different products on the control of bacterial spot of the tomato cultivars Santa Clara and Gisele, two sets of these plants were transplanted into pots 25 days after emergence. Pots contained 3 L of substrates consisting of soil, manure, and thick sand (3:2:1 (v/v)), plus 50 g of dolomite lime and 100 g of 4-14-8 fertilizer per 100 L of mixture. The plants were kept in a greenhouse. Fifteen days after transplanting, they were sprayed with acibenzolar-S-methyl (0.025 g.L⁻¹), fluazinam (0.25 g.L⁻¹), pyraclostrobin (0.08 g.L⁻¹), pyraclostrobin + methiran (0.02 g + 2.2 g.L⁻¹), copper oxychloride (1.50 g.L⁻¹), mancozeb + copper oxychloride (0.88 g.L⁻¹ + 0.60 g.L⁻¹), and oxytetracycline (0.40 g.L⁻¹). Three days after spraying, leaves were inoculated with a suspension of *X. perforans* strain (10⁶ cfu.mL⁻¹) by spraying. The control treatment was represented by tomato plants inoculated or not inoculated and sprayed with water. Twenty-four hours before and 24 hours after inoculation, the plants were placed in a moist chamber.

Bacterial spot severity was assessed using a diagrammatic scale that considers the leaf area, according to Mello *et al.* (1997). Disease evaluations were done 8, 11, 14, 17, and 20 days after inoculation (DAI). The severity values obtained were used to determine the area under the disease progression curve (AUDPC), according to Campbell and Madden (1990).

The experimental design was randomized, with nine treatments (seven chemicals, control inoculated, and control non-inoculated) and seven replicates. Each experimental plot consisted of one pot containing two plants. The AUDPC values were subjected to analysis of variance using SISVAR software (Ferreira, 2011) and means were compared by the Scott-Knott test at 5% probability.

Activation of the enzymes associated with resistance induction in tomato plants pulverized with fungicides and antibiotic, and inoculated with *X. perforans*

Two trials, each with one cultivar (Santa Clara or Gisele), were conducted in the greenhouse. The plants were sprayed with chemicals and inoculated with *X. perforans*, using the same methodology and the same experimental design as described in effect of fungicides and antibiotic on bacterial spot, with five replicates.

Samples for foliar enzymatic determinations were collected 24 h before and 24 h after spraying with fungicides and antibiotic, and 1, 2, 4, 6, and 8 days after inoculation. The samples were packed in plastic bags and placed immediately in an expanded polystyrene box containing ice and transported without delay to the laboratory for weighing and processing, and subsequent freezer storage.

Leaf samples were homogenized mechanically in 4 mL of 100 mM sodium acetate buffer (pH 5.0). The homogenate was centrifuged at 20,000 g for 25 min, at 4 °C, and the supernatant was stored at -20 °C for determination of the activity of peroxidase, polyphenol oxidase, β -1,3-glucanase, phenylalanine ammonia-lyase, and protease, and for protein content determination.

Peroxidase activity was determined using a direct spectrophotometric method by measuring the conversion of guaiacol into tetraguaiacol at 470 nm (Lusso and Pascholati, 1999). Peroxidase activity results are expressed as specific activity ($\Delta_{\text{abs}} 470 \text{ nm} \cdot \text{min}^{-1} \cdot \mu\text{g}^{-1} \text{ protein}$).

Polyphenol oxidase activity was obtained by the method of Duangmal and Apentem (1999). The reaction was prepared by mixing 900 μL of substrate and 100 μL of enzymatic extract. The substrate was composed of catechol at a concentration of 20 mM in 100 mM sodium phosphate buffer (pH 6.0). Readings were performed directly for a period of 3 min. The results are expressed as $\Delta_{\text{abs}} 420 \text{ nm} \cdot \text{min}^{-1} \cdot \mu\text{g}^{-1} \text{ protein}$.

β -1,3-glucanase activity was determined by colorimetric quantification of the glucose released from laminarin, through the use of hydrazide *p*-hydroxybenzoic acid (PAHBAH) (Lever, 1972). The reaction mixture containing 150 μL of enzymatic extract and laminarin (2.0 mg.mL⁻¹) was incubated at 37 °C for 1 h (Abeles and Foence, 1970). After this period, 1.5 mL of PAHBAH solution was added (0.5 g dissolved in 20 mL of 0.5 M HCl plus 80 mL of 0.5 M NaOH), and then the mixture was heated to 100 °C for 10 min. After cooling to 25 °C, the sample absorbances were determined at 410 nm against the extraction buffer.

Phenylalanine ammonia-lyase activity was determined by colorimetric quantification of trans-cinnamic acid released from the phenylalanine substrate (Umesha, 2006). The absorbance of the samples was determined at 290 nm against the extraction buffer, and the value of the control was subtracted from each sample (this control cor-

responded to a mixture of 100 μL protein extract and 900 μL of 25 mM Tris HCl buffer, pH 8.8). The absorbance readings were plotted on the standard curve for the trans-cinnamic acid and the enzymatic activity was expressed in μg of trans-cinnamic per min per mg protein ($\mu\text{g}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$).

Protease activity was obtained according to Fialho (2004). A total of 0.5 g of casein was dissolved in 40 mL distilled water and allowed to stir for 20 min. Then, 1 mL of NaOH (1 M) and 2.5 mL of Tris (1 M) were added. After a period of agitation, with the casein completely dissolved, the pH was adjusted to 7.8 with 85% phosphoric acid. For the reaction, 500 μL of substrate was incubated with 200 μL of sample at 30 °C for 30 min. The reaction was stopped by adding 650 μL of 10% (m/v) trichloroacetic acid (TCA), followed by centrifugation at 10,000 g for 15 min. The absorbance reading was conducted at 280 nm and the results are expressed as absorbance units per mg protein ($\text{AU}\cdot\text{mg}^{-1}$).

Total protein in the extracts was determined according to Bradford (1976). To each 0.8 mL sample, 0.2 mL of Bradford concentrate reagent was added while stirring. The protein concentration, expressed in terms of μg equivalent of bovine serum albumin (BSA) in 1 mL of sample (μg protein. mL^{-1}) was determined using a standard curve of BSA concentrations, ranging from 0 to 20 $\mu\text{g}\cdot\text{mL}^{-1}$.

The area under the enzyme progression curve was calculated as a function of time (samples), as in Soares *et al.* (2004), and the data was subjected to analysis of variance, using the statistical program SISVAR software (Ferreira, 2011). Means were compared by the Scott-Knott test at 5% probability

Results

Effects of fungicides and antibiotic on bacterial spot

Values of the AUDPC for the tomato cultivars Santa Clara and Gisele sprayed with different chemicals and inoculated with *X. perforans* are shown in Table 1. In both experiments, each with a different tomato cultivar, the AUDPC of the inoculated control differed from that of the chemical treatments ($p \leq 0.05$). The lowest values of AUDPC was obtained with acibenzolar-S-methyl in both cultivars. The tomato varieties differed in their responses to the chemicals evaluated. After the acibenzolar-S-methyl, the best treatments for the Gisele cultivar were copper + mancozeb, fluazinam, and oxytetracycline, and the best treatments for the Santa Clara cultivar were pyraclostrobin + metiram, fluazinam, and oxytetracycline. The AUDPC value for control inoculated treatment of the two tomato cultivars also differed, being lower for Santa Clara cultivar than for Gisele cultivar.

Table 1 - Area under the disease progress curve (AUDPC) of the bacterial spot for two tomatoes cultivars sprayed with fungicides and antibiotic.

Treatment	Santa Clara	Gisele
Acibenzolar-S-methyl	12.83 a*	55.50 a
Pyraclostrobin + metiram	38.04 b	210.26 c
Pyraclostrobin	58.42 c	194.41 c
Copper oxychloride	66.00 d	178.40 c
Copper oxychloride + mancozeb	51.75 c	120.72 b
Fluazinam	31.96 b	128.52 b
Oxytetracycline	42.00 b	131.03 b
Control inoculated	104.85 e	436.03 d
Control non-inoculated	0.0	0.0
C.V. (%)	17.0	13.2

*Means followed by the same letter do not differ by Scott-Knott test at 5% probability.

Evaluation of the production of enzymes associated with plant resistance induction

Increased production of peroxidase, polyphenol oxidase (Table 2), and β -1,3-glucanase (Table 3) were observed in leaves of the Santa Clara cultivar ($p \leq 0.05$), whereas only the peroxidase increased significantly ($p \leq 0.05$) in the leaves of the Gisele cultivar when ASM, pyraclostrobin + metiram, and pyraclostrobin were sprayed (Table 2). There were no significant differences ($p > 0.05$) in the production of phenylalanine ammonia-lyase or protease in the leaves of either tomato cultivar (Table 3) sprayed with the different chemicals.

Discussion

The effectiveness of chemicals on the control of bacterial spot of tomato is variable, since it depends, in part, on how vulnerable the bacteria is to the chemicals used. In our study, a reduction in AUDPC when ASM was sprayed was associated with the induction of biochemical mechanisms of resistance (Kessmann *et al.*, 1994), as previously reported in several studies (Calvacanti *et al.*, 2006a, b; Ishida *et al.*, 2008; Itako *et al.*, 2012b). The results observed here for the effectiveness of ASM on control of the disease are consistent with those observed by Silva *et al.* (2003), Cavalcanti (2006a, b) and Itako *et al.* (2012b).

The copper-based products, fluazinam and oxytetracycline, satisfactorily reduced the AUDPC. With the exception of fluazinam, these products are traditionally used in the control of bacterial leaf spot and other bacterial diseases of tomato (Louws *et al.*, 2001). The efficacy of these products, however, appears to depend on the intrinsic sensitivity of the bacterial strains prevalent in the field, since low sensitivity and/or resistance *in vitro* has been reported, not only to copper-based products, but also to some antibiotics (Marco and Stall, 1983; Maringoni and Kimati, 1987; Bou-

Table 2 - Area under the enzyme progression curve of peroxidase and polyphenol oxidase in leaves of two tomatoes cultivars sprayed with fungicides and antibiotic, after inoculation with *Xanthomonas perforans*.

Treatment	Santa Clara		Gisele	
	Peroxidase	Polyphenol oxidase	Peroxidase	Polyphenol oxidase
Acibenzolar-S-methyl	28.18 a*	8.67 a	44.88 a	15.95 a
Piraclostrobin + metiram	24.12 a	7.32 a	39.47 a	11.62 b
Piraclostrobin	20.61 b	8.40 a	42.66 a	11.91 b
Copper oxychloride	8.06 d	5.34b	21.38 c	9.76 c
Cop. oxych. + mancozeb	8.66 d	4.25 b	22.14 c	9.14 c
Fluazinam	14.13 c	5.25 b	31.56 b	18.06 a
Oxitetracliline	9.59 d	5.38 b	23.39 c	4.98 c
Control inoculated	11.77 c	5.61 b	16.39 c	6.10 c
Control non-inoculated	12.47 c	6.30 b	31.09 b	14.52 a
C.V. (%)	25.7	16.6	18.2	29.6

*Means followed by the same letter do not differ by Scott-Knott test at 5% probability.

Table 3 - Area under the enzyme progression curve of β -1,3 glucanase, phenylalanine ammonia-lyase and protease in leaves of two tomatoes cultivars sprayed with fungicides and antibiotic, after inoculation with *Xanthomonas perforans*.

Treatment	Santa Clara			Gisele		
	β -1,3-glucanase	Phenylalanine ammonia-lyase	Protease	β -1,3-glucanase	Phenylalanine ammonia-lyase	Protease
Acibenzolar-S-mehtyl	312.86 a	6.47 a	9.96 a	332.33 a	6.24 a	7.45 a
Piraclostrobin + metiram	310.47 a	6.50 a	10.45 a	226.57 a	7.40 a	6.98 a
Piraclostrobin	229.86 b	6.75 a	10.75 a	277.94 a	7.31 a	6.91 a
Copper oxychloride	154.22 c	6.92 a	11.64 a	155.01 b	8.97 a	8.06 a
Cop. oxych. + mancozeb	154.22 c	7.16 a	12.26 a	271.98 a	9.51 a	8.60 a
Fluazinam	190.24 c	8.35 a	12.46 a	160.37 b	6.28 a	7.59 a
Oxitetracliline	112.06 c	8.83 a	13.21 a	142.04 b	6.10 a	7.89 a
Control inoculated	144.95 c	9.13 a	14.28 a	145.06 b	6.67 a	6.41 a
Control non-inoculated	148.78 c	11.27 a	17.69 a	256.23 a	6.59 a	10.20 a
C.V. (%)	17.43	17.43	27.35	39.37	36.48	22.03

*Means followed by the same letter do not differ by Scott-Knott test at 5% probability.

zar *et al.*, 1999; Aguiar *et al.*, 2000; Quezado-Duval *et al.*, 2003).

According to Marco and Stall (1983), the use of ethylenebis (dithiocarbamate) fungicides mixed with copper compounds has been effective on control of copper-resistant or semi-resistant strains of *Xanthomonas* spp. Maringoni and Kimati (1987) observed some strains of *Xanthomonas* spp. were more sensitive *in vitro* to a mixture of copper with mancozeb or zineb than to copper alone.

Piraclostrobin, pyraclostrobin + metiram, and fluazinam are recommended for the control of fungal diseases on the leaves of tomatoes, but are not indexed as controlling bacterial spot (Agrofit, 2012). However, under the conditions tested in this study, these products have shown promise for control of bacterial spot of tomato.

The increased activity of peroxidase, polyphenol oxidase, and β -1,3-glucanase in leaves on the Santa Clara cultivar and peroxidase in leaves of the Gisele cultivar may be associated with less disease severity, especially when leaves are sprayed with ASM, pyraclostrobin, and pyraclostrobin + metiram (Table 4), since ASM and pyraclostrobin are not known to have a direct action on the bacterium *in vitro*, whereas the other products evaluated have bactericidal and/or bacteriostatic action (Itako *et al.*, 2012a).

Peroxidases, besides strengthening the cell wall via increased production of lignin and suberin, polysaccharides, and glycoproteins rich in hydroxyproline, stimulate the production of free oxygen, which has an antimicrobial effect and induces the production of phytoalexin (Bolwell *et al.*, 1995; Wojtaszek, 1997; Kristensen *et al.*, 1999). The polyphenol oxidase catalyzes the oxidation of phenols to

quinones in the presence of oxygen. The expression of polyphenol oxidase may add an additional line of defense by protecting plants from pathogens and insects (Thipyapong and Steffens, 1997). According to Cutt and Klessig (1992), β -1,3-glucanases cause the release of glycosidic fragments not only from the pathogen itself but also from the walls of the plant cells, and these glycosidic fragments can elicit the host defense.

Research involving ASM and Ecolife® showed the effectiveness of these products on the control of bacterial spot, caused by *X. vesicatoria*, on the Santa Cruz Kada tomato cultivar, and also in the activation of peroxidase, polyphenol oxidase, and β -1,3-glucanase. The increased activity of these enzymes was correlated with induced resistance of leaves of this tomato cultivar (Cavalcanti *et al.*, 2006a, b). Similar results were obtained by Itako *et al.* (2012b), who reported a reduction in disease symptoms and increased production of peroxidase, polyphenol oxidase, and β -1,3-glucanase on leaves of tomato AP 529 hybrid sprayed with ASM and pyraclostrobin. According to Luiz *et al.* (2012), there was a reduction in the severity of bacterial spot caused by *X. gardneri*, and increased production of peroxidase, polyphenol oxidase, and glucanase in tomato leaves of Santa Cruz Kada sprayed with polysaccharides extracted from leaves of *Aloe barbadensis*.

Research using pyraclostrobin in plant defense against some pathogens has been reported by Herms *et al.* (2002), who found an increased resistance of the tobacco plant to infection by the tobacco mosaic virus and *Pseudomonas syringae* pv. *tabaci*. In their data, salicylic acid and PR-1 proteins accumulated in the tissues of the treated plants, indicating that the pyraclostrobin activated defense mechanisms in the plants, in addition to its fungicidal action. Vigo *et al.* (2012), on the other hand, found that ASM and pyraclostrobin caused increased levels of polyphenol oxidase, peroxidase, and total soluble proteins on the leaves of snap beans Bragança cultivar, suggesting their participation in the mechanisms of induction of resistance to common bacterial blight. These results were similar to those observed here for the tomato, and thus is an indication that these products, particularly the pyraclostrobin, induced mechanisms of resistance, since these mechanisms are well known for ASM. Thus, the action of ASM and pyraclostrobin on control of bacterial spot caused by *X. perforans* on leaves of the tomato Santa Clara and Gisele cultivars is associated with the production of enzymes related with resistance induction.

Acknowledgments

This research was supported by FAPESP (The State of Sao Paulo Research Foundation), grant 2010/52702-0, and National Council for the Improvement of Higher Education (CAPES).

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Associate Editor: Raquel Silva Peixoto

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