

Control of Bacterial Spot of Tomato and Activation of Enzymes Related to Resistance by Chemicals Under Field Conditions

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Abstract

The control of tomato bacterial spot by the application of chemicals was evaluated on field condition during two trials. Thirty days after transplanting and every 14 days the tomato plants PA-529 hybrid were sprayed with acibenzolar-S-methyl (2.5 g·100 L⁻¹), fluazinam (25 g·100 L⁻¹), pyraclostrobin (8 g·100 L⁻¹), pyraclostrobin + metiram (20 g·100 L⁻¹ + 220 g·100 L⁻¹), copper oxychloride (25.20 g·100 L⁻¹), mancozeb + copper oxychloride (88 g·100 L⁻¹ + 60 g·100 L⁻¹), oxytetracycline (40 g·100 L⁻¹) and water (control), totaling five applications during the crop cycle. Forty days after transplanting, the plants were inoculated with a *Xanthomonas perforans* isolate (10⁷ UFC·mL⁻¹), disease severity was assessed (10, 19, 29, and 36 days after inoculation), and the area under the disease progress curve was calculated. All chemicals controlled the disease, but better results for total mass and number of fruit was obtained for fluazinam, pyraclostrobin, pyraclostrobin + metiram, copper oxychloride, and mancozeb + copper oxychloride in the first trial, and pyraclostrobin and pyraclostrobin + metiram in the second trial. The average fruit weight, °Brix, pH and total acidity were not affected by the application of the chemicals. The enzymatic activity of the polyphenol oxidase and peroxidase was higher in the first trial, but in the second, only the polyphenoloxidase had the highest activity in the treatments with acibenzolar-S-methyl, pyraclostrobin, and pyraclostrobin + metiram on tomato leaves. Products containing pyraclostrobin increased the activity of some enzymes without reducing the productivity.

Keywords: *Solanum lycopersicum*, chemical control, enzyme, *Xanthomonas perforans*

1. Introduction

The bacterial spot is considered one of the major diseases of tomatoes. It causes a reduction in productivity by damaging the leaves, causing flowers and fruit to fall prematurely, and by increasing sunburnt fruit (Gitaitis et al., 1992; Vale et al., 2004; Lopes & Quezado-Duval, 2005). Four species of *Xanthomonas* are implicated as causal agents of tomato spot: *X. euvesicatoria*, *X. vesicatoria*, *X. perforans*, and *X. gardneri* (Jones et al., 2004). These species are known to occur in tomatoes in Brazil (Quezado-Duval et al., 2005b; Pereira et al., 2011).

Several preventive strategies are employed in the control of bacterial spot, such as use of healthy seedlings and seed and crop rotation (Lopes & Quezado-Duval, 2005; Kurozawa & Pavan, 2005; Silva-Lobo et al., 2005). The chemical control of tomato bacterial spot has been performed with various products, and its success can be attributed to the effectiveness of the products used, the periods and frequency of spraying, and, especially, the sensitivity of the population of *Xanthomonas* spp. to them (Stall, 1993). Some studies have shown that antibiotics and copper products do not work very well as control agents, and the possible cause of this inefficiency is the selection of resistant strains of *Xanthomonas* spp. (Marco & Stall, 1983; Bouzar et al., 1999; Aguiar et al., 2000; Quezado-Duval et al., 2003). Cupric fungicides, antibiotics, and acibenzolar-S-methyl can control tomato bacterial spot on tomato, with the latter being able to induce resistance in plants (Louws et al., 2001; Cavalcanti et al., 2006). However, some reports have shown that production is reduced when acibenzolar-S-methyl is used (Louws et al., 200; Quezado-Duval et al., 2005) due to energy expenditure (Kunh

& Pascholati, 2010). Resistance induction, which increases the plant's defenses against a wide spectrum of pathogenic organisms, can be induced by biotic or abiotic agents (Hammerschmidt et al., 1982).

Evidence has been presented on the direct influence of strobilurin on plant physiology (Herms et al., 2002; Köehle et al., 2002). The physiological changes can be positive (Venancio et al., 2005) as they activate biochemical mechanisms that increase plant resistance to pathogens (Herms et al., 2002; Venancio et al., 2003; Venancio et al., 2005; Vigo et al., 2012).

The search for other options to control bacterial spot of tomato has proven a challenge due to the limited availability of effective products. The integration of existing control methods and research involving induction of resistance may contribute to the increased efficiency of strategies to control bacterial spot of tomatoes. The objective of this study was to evaluate the effectiveness of fungicides and one antibiotic to control bacterial spot and to study the induction of enzymes associated with resistance. In order to test the substances, we sprayed them onto tomato hybrid AP-529 in experiments conducted in the field in 2009–2010 and 2010–2011.

2. Materials and Methods

2.1 Field Experiments

Two experiments were conducted from October to January, one in 2009–2010 and another in 2010–2011. The experiments were conducted in a field located in Botucatu, State of São Paulo, Brazil (latitude: 22°57'S, longitude 48°22'W). The Figure 1 show the second experiment. We used PA-529 hybrid, which is characterized by a growth cycle of 110–120 days and pear-type fruit of 80–90 g. Seeds were planted in polystyrene trays filled with commercial substrate, and seedlings were transplanted into the experimental field 30 days after planting.

Each plot was represented by three rows, each 3.00 m long. The space between the rows was 1.0 m, totaling 9 m². For the evaluations, we used the center-line of the parcel, which had a working area of 3 m² and contained 10 plants. The tomato seedlings were transplanted with a line spacing of 0.3 m. The experimental design was a randomized block design with four replications.

Fertilization was performed 7 days before transplanting of the seedlings and subsequently fertilizer was made according Gomes et al. (1999). Other aspects of the cultivation were standard for tomatoes in the region. Sprinkler irrigation was used to meet the water needs of the tomato plants.



Figure 1. Field experiment conducted from October 2010 to January 2012 with tomato PA-529 hybrid (A), tomato plants on control treatment (B), and typical bacterial spot symptoms on leaflets (C)

2.2 Chemicals Evaluated in the Control of Bacterial Spot

The tomato plants were sprayed with acibenzolar-S-methyl (2.5 g·100 L⁻¹), pyraclostrobin + metiram (20 g·100 L⁻¹ + 220 g·100 L⁻¹), pyraclostrobin (8 g·100 L⁻¹), copper oxychloride (25.20 g·100 L⁻¹), mancozeb + copper

oxychloride (88 g·100 L⁻¹ + 60 g·100 L⁻¹), fluazinam (25 g·100 L⁻¹) and oxytetracycline (40 g·100 L⁻¹) 30 days after transplanting. The chemical application was performed using a backpack sprayer with a constant CO₂ pressure source. The spray volume used varied according to how much was needed to completely wet the aerial portions of the plants.

The products were sprayed every 14 days, totaling five applications during the crop cycle, in addition to weekly spraying with chlorothalonil (150 g·100 L⁻¹) and other insecticides required for pest control (Maringoni et al., 1986). The control treatment involved spraying with chlorothalonil and insecticides only.

2.3 Assessment of Bacterial Spot

Isolate Tom-3051 of *X. perforans* (Collection of Plant Pathogenic Bacteria from Plant Protection Department, College of Agricultural Sciences, Paulista State University, Botucatu, SP, Brazil) was used in the study. The bacteria were cultivated in 500 ml nutrient broth (5 g peptone and 3 g meat extract in 1000 mL of water) for 24 h at 28°C. After this period, the suspension was diluted in the proportion 1:10 in distilled water reaching a final concentration of approximately 10⁷ UFC·mL⁻¹. The spray inoculation was done 40 days after transplanting of the plants, 10 days after the first application of the chemicals.

The disease assessment was carried out on the middle-third of the ten plants at the center-line using the diagrammatic scale developed to evaluate foliar lesions of bacterial spot (Mello et al., 1997). The evaluations were performed at 10, 19, 29, and 36 days after inoculation (DAI). Using the severity values obtained, the area under the disease progress curve (AUDPC) was calculated as in Campbell and Madden (1990). The AUDPC values were subjected to analysis of variance and their means were compared using the Scott-Knott test at 5% probability.

2.4 Harvest and Chemical Analyses of Fruit

In order to evaluate the tomato yield, fruit were harvested manually, and their total mass (Kg), the number of fruit, and the average weight (g) of each fruit for each plot were determined. The harvest was performed in two stages, the first 90 days after transplanting, and the second up to 105 days after transplanting.

Five fruit were randomly taken from each plot and subjected to chemical analyses (pH, titratable acidity (TA) and soluble solids (°Brix)) as in the standards of Institute Adolfo Lutz (2008). The results obtained in the evaluations were subjected to analysis of variance, and the means were compared by the Scott-Knott test at 5% probability.

2.5 Enzymatic Activity of the Leaves Treated and Inoculated with *X. perforans*

In order to evaluate the activity of peroxidase, polyphenol oxidase, β-1,3-glucanase, phenylalanine ammonia lyase, and protease, leaf samples were collected 5 days before inoculation and 6, 15, and 25 days after inoculation. The leaves were cooled on ice during transport to the laboratory, where they were stored at -20°C.

The leaf samples were mechanically homogenized in 4 ml of 100 mM sodium acetate buffer (pH 5.0). The homogenate was centrifuged at 10 000 g for 25 min at 4°C, and the obtained supernatant was considered to be the enzyme extract.

The enzymatic activity of the peroxidase was determined at 30°C using a direct spectrophotometric method by measuring the conversion of guaiacol in tetraguaiacol at 470 nm (Lusso & Pascholati, 1999). The peroxidase activity results are expressed as specific activity (Δ_{abs} 470 nm·min⁻¹·μg⁻¹ protein).

The polyphenoloxidase activity was obtained by the method of Duangmal & Apentem (1999), and the results are expressed as Δ_{abs}420 nm min⁻¹ μg⁻¹ protein.

The β-1,3-glucanase activity was determined by the colorimetric quantification of the glucose released from laminarin, through the use of hydrazide p-hydroxybenzoic acid (PAHBAH) (Lever, 1972). The absorbance of the samples was determined at 410 nm against the extraction buffer.

The activity of the phenylalanine ammonia-lyase was determined by colorimetric quantification of trans-cinnamic acid released from the substrate phenylalanine (Umesha, 2006). The absorbance readings were plotted on the standard curve for the trans-cinnamic acid and enzymatic activity expressed in μg of trans-cinnamic per min per mg protein (g·min⁻¹·mg⁻¹).

The protease activity was obtained in accordance with the methodology used by Fialho (2004). The reading of absorbance was carried out at 280 nm and the results are expressed as absorbance units per mg of protein (AU·mg⁻¹).

The quantification of the total proteins present in the extracts was determined using the Bradford method (1976).

With the results obtained at different times of collection of leaf samples, the area under the progress curve of the production of the compounds was evaluated (enzymes) as a function of time (samples) (Soares et al., 2004). The data were subjected to analysis of variance, using the statistical program SISVAR (Ferreira, 2000) and the means were compared by the Scott-Knott test at 5% probability.

3. Results

3.1. Severity of Bacterial Spot and Productivity

The results of the AUDPC obtained in the two tests are shown in Table 1. All products controlled the disease since the values of AUDPC differed and were lower than those observed in the control treatment.

Table 1. Area under the disease progress curve (AUDPC) for the assays conducted in the agricultural years of 2009–2010 and 2010–2011 on tomato hybrid AP-529 inoculated with *Xanthomonas perforans*

Treatment	AUDPC	
	2009-2010	2010-2011
Acibenzolar-S-methyl	405.54 b*	383.45 b
Pyraclostrobin + metiram	440.18 b	366.36 b
Pyraclostrobin	447.24 b	404.09 b
Copper oxychloride	499.28 b	409.42 b
Mancozeb + copper oxychloride	432.79 b	386.13 b
Fluazinam	412.48 b	435.91 b
Oxytetracyclin	453.71 b	431.24 b
Control	815.87 a	716,89 a
CV %	8.17	11.84

*Means followed by the same letters in the column do not differ, based on the Scott-Knott test at 5% probability.

The values of the total mass of the fruit, fruit number, and average weight of fruit in the two tests are shown in Table 2 and Table 3. In the first trial (Table 2), the products that differed from the control in the total mass and number of fruit were fluazinam, pyraclostrobin, pyraclostrobin + metiram, copper oxychloride, and mancozeb copper oxychloride. In the second trial, for these parameters, only pyraclostrobin, and pyraclostrobin + metiram differed from the control. The average fruit weight was not affected by the application of the chemicals evaluated (Tables 2 and 3).

Table 2. Production (Kg/parcel), number, and mean mass (g) of fruit of tomato hybrid AP-529 sprayed with chemical products and inoculated with *Xanthomonas perforans* in 2009-2010

Treatment	Production (Kg/parcel)	Number of fruit	Mean weight of fruit (g)
Acibenzolar-S-methyl	6.27 b	87.20 b	73.75 a
Pyraclostrobin + methiram	10.76 a	144.70 a	74.00 a
Pyraclostrobin	8.36 a	127.00 a	67.50 a
Copper oxychloride	9.52 a	123.70 a	77.50 a
Mancozeb + copper oxychloride	9.57 a	133.00 a	72.50 a
Fluazinam	10.71 a	135.70 a	81.00 a
Oxytetracyclin	5.37 b	69.50 b	78.25 a
Control	4.30 b	54.20 b	80.75 a
CV	27.37	25.98	10.49

*Means followed by the same letters in the column do not differ, based on the Scott-Knott test at 5% probability.

Table 3. Production, number and mean mass of fruit of tomato hybrid AP-529 sprayed with chemical products and inoculated with *Xanthomonas perforans* in 2010–2011

Treatment	Production (Kg/parcel)	Number of fruits	Mean weight of fruit (g)
Acibenzolar-S-methyl	12.10 b*	260.00 b	46.20 a
Pyraclostrobin + methiram	16.70 a	364.00 a	46.30 a
Pyraclostrobin	15.70 a	341.70 a	45.50 a
Copper oxychloride	11.80 b	286.00 b	41.50 a
Mancozeb + copper oxychloride	11.10 b	237.50 b	47.20 a
Fluazinam	10.90 b	243.70 b	47.80 a
Oxytetracyclin	10.20 b	237.70 b	42.70 a
Control	10.30 b	220.50 b	47.00 a
CV	24.63	24.00	11.22

*Means followed by the same letters in the column do not differ, based on the Scott-Knott test at 5% probability.

3.2 Chemical Analyses of the Fruit

The results regarding the analysis of °Brix, pH, and total acidity of the fruit in the two assays are shown in Table 4. These parameters did not differ among treatments, except for the °Brix test conducted in 2009–2010, for which the control differed from the other treatments. It should be noted that the coefficient of variation (CV) observed in this analysis was very low (1.04%) compared to the value obtained in the experiment conducted in 2010–2011, which was 11.42%.

Table 4. Soluble solids (°Brix), pH, and total acidity of tomato hybrid AP-529 sprayed with chemical products and inoculated with *Xanthomonas perforans* in 2009-2010 and 2010-2011

Treatment	2009-2010			2010-2011		
	°Brix	pH	Total acidity (mg citric acid /100g)	°Brix	pH	Total acidity (mg citric acid /100g)
Acibenzolar-S-methyl	4.14 a*	4.33 b	397.05 a	5.07 a	4.04 a	380.40 a
Pyraclostrobin + methiram	4.14 a	4.33 b	366.31 a	5.23 a	3.92 a	391.33 a
Pyraclostrobin	4.21 a	4.33 b	393.27 a	5.14 a	4.01 a	398.04 a
Copper oxychloride	4.11 a	4.34 b	382.96 a	5.33 a	3.90 a	305.90 a
Mancozeb + copper oxychloride	4.11 a	4.29 b	353.88 a	5.11 a	3.89 a	412.85 a
Fluazinam	4.15 a	4.34 b	381.68 a	5.33 a	3.67 a	379.97 a
Oxytetracyclin	4.08 a	4.33 b	368.81 a	5.10 a	4.35 a	382.53 a
Control	3.84 a	4.51 a	338.98 a	4.85 a	4.58 a	347.09 a
CV	7.09	1.04	15.05	4.13	11.42	18.09

*Means followed by the same letters in the column do not differ, based on the Scott-Knott test at 5% probability.

3.3 Enzyme Activity

The results of enzyme activity are shown in Table 5. The leaves of tomato hybrid AP-529 sprayed with acibenzolar-S-methyl, pyraclostrobin, and pyraclostrobin + metiram, produced more peroxidase in the test of 2009–2010 and polyphenol oxidase in tests of both 2009–2010 and 2010–2011, thus differing from other treatments. There was no increase in the production of phenylalanine ammonia-lyase, β -1,3-glucanase or protease in the leaves in any treatment evaluated in the two trials.

Table 5. Enzymatic activity expressed as the area under the progress curve of the production of the compounds evaluated in leaves of hybrid tomato AP-529 sprayed with chemicals and inoculated with *Xanthomonas perforans* in 2009–2010 and 2010–2011

Treatment	2009-2010				
	Peroxidase	Polyphenol oxidase	Phenylalanine ammonia-lyase	β -1,3 glucanase	Protease
Acibenzolar-S-methyl	170.71 a*	56.71 a	8.81 a	142.76 a	23.41 a
Pyraclostrobin + methiram	179.61 a	68.19 a	9.26 a	137.60 a	38.19 a
Pyraclostrobin	179.61 a	66.84 a	10.15 a	147.88 a	48.19 a
Copper oxychloride	125.90 b	29.38 b	10.32 a	165.27 a	27.67 a
Mancozeb + copper oxychloride	117.33 b	31.09 b	11.22 a	183.4 5 a	18.77 a
Fluazinam	148.19 b	46.33 b	13.07 a	191.11 a	26.52 a
Oxytetracyclin	121.90 b	31.56 b	12.70 a	213.35 a	31.56 a
Control	118.20 b	33.89 b	9.11 a	220.90 a	28.89 a
CV	15.71	26.93	21.86	39.40	40.13
Treatment	2010-2011				
	Peroxidase	Polyphenol oxidase	Phenylalanine ammonia-lyase	β -1,3 glucanase	Protease
Acibenzolar-S-methyl	114.90 a*	33.31 a	8.20 a	204.56 a	48.45 a
Pyraclostrobin + methiram	110.40 a	31.37 a	7.84 a	193.23 a	49.51 a
Pyraclostrobin	114.11 a	30.63 a	10.72 a	144.50 a	30.89 a
Copper oxychloride	117.22 a	21.96 b	10.94 a	136.99 a	41.63 a
Mancozeb + copper oxychloride	131.11 a	19.28 b	8.26 a	166.78 a	37.99 a
Fluazinam	121.88 a	21.79 b	10.32 a	220.22 a	45.25 a
Oxytetracyclin	133.21 a	21.75 b	10.15 a	182.55 a	34.60 a
Control	130.50 a	20.89 b	13.94 ^a a	145.69 a	41.58 a
CV	15.19	26.71	25.5	41.50	32.43

* Means followed by the same letters in the column do not differ, based on the Scott-Knott test at 5% probability.

4. Discussion

In Brazil, bacterial spot of tomato is a serious problem because the control of the disease in the field is often not satisfactory. Among the reasons why chemical control is not always effective are the weather, which is favorable to the disease, and the fact that not all populations of *Xanthomonas* spp. are sensitive to the products used. Acibenzolar-S-methyl, however, is known to be useful in controlling the disease (Louws et al., 2001; Paradela Filho et al., 2001; Quezado-Duval et al., 2005a; Cavalcanti et al., 2006), which was confirmed in the present study.

Unfortunately, the control of bacterial spot with acibenzolar-S-methyl or other products does not always result in higher productivity. This was demonstrated in the two tests conducted here using acibenzolar-S-methyl and oxytetracycline. Our results are consistent with those of Paradela et al. (2001) and Quezado-Duval et al. (2005a) for tomato, and those of Romero et al. (2001) for chili pepper. There is evidence that acibenzolar-S-methyl controls the disease by inducing plant resistance, but the product is implicated in reducing plant productivity (Godard et al., 1999; Dietrich et al., 2005). Plants that allocate their resources to defense against pathogens are

less productive since the metabolic changes that lead to resistance have a fitness cost associated with them (Iriti & Faoro, 2003).

The low productivity observed in some treatments is directly reflected in the number of the fruit (Tables 2 and 3). This can be explained by the fact that, in addition to foliar necrosis, which exposes the fruit to sun-burn, the disease can cause stains and decay fruit, as well as causing flowers to fall down (Vale et al., 2004).

The copper-based products and antibiotics are still being regularly used in the field to control bacterial spot. Bacterial sensitivities to antibiotics and/or to copper have been reported in some publications (Marco & Stall, 1983; Aguiar et al., 2000; Quezado-Duval et al., 2005a). Unlike the results obtained in the present study, Maringoni et al. (1986) tested several copper-based fungicides and antibiotics to control bacterial spot in field conditions using tomato cultivar Agrocica 16. According to their data, the fungicides and antibiotics at the concentrations and applications used did not satisfactorily control the disease. Other parameters were evaluated, such as: number of fruit with symptoms of the disease, number of fruit with sunburn, and total output. They concluded that spraying with copper fungicides did not reduce the leaf area affected by the disease nor did it increase fruit production.

McCarter (1992) obtained satisfactory control of bacterial spot on sweet pepper and increased production with weekly sprays of copper hydroxide + mancozeb. In contrast, the experiments of Carmo et al. (2001) resulted in variable levels of control of bacterial spot on pepper using copper oxychloride.

The success or failure of attempts to chemically control the disease can be partly attributed to a greater or lesser effectiveness of the active ingredients applied, general care, and time of treatments, and especially to the sensitivity of the pathogen population to the products employed (Stall, 1993). Regardless, chemicals continue to be used, firstly because of the lack of alternatives, and, secondly, because they also protect the plants against other pathogens, even when they are not very effective in controlling bacterial spot.

Although the use of fluazinam to control bacterial diseases in plants had not been recorded in the literature, this product reduced the severity of the bacterial spot pathogen on tomato leaves (Table 1) and had a variable effect on fruit production (Tables 2 and 3). Under *in vitro* conditions, this product had a bactericidal action against *X. perforans* (Itako et al., 2012).

Research on pyraclostrobin-based products was conducted by Nascimento (2009), who evaluated the effect of various chemicals, pulverized on seedlings and in the field, on bacterial spot of the hybrid tomato Heinz 9992 and found that the application of pyraclostrobin + metiram resulted in a good yield.

Regarding the chemical analyses of fruit, the desirable pH of tomatoes to prevent the proliferation of microorganisms is below 4.5. When the pH is higher than this, longer periods of sterilization of the raw material through thermal processing are necessary, resulting in higher energy consumption and higher processing costs (Monteiro et al., 2008). In both experiments conducted in the present study, we obtained a pH value lower than 4.5 in the fruit after the application of chemicals, which provides further evidence on the interference of the disease on this parameter of the fruit (Table 4). Regarding the °Brix and total acidity, the application of different chemicals did not affect the control of this fruit parameter (Table 4).

The balance between acidity and sugar is extremely important from the sensory standpoint, because these compounds are mainly responsible for the characteristic flavor of tomato. The sugars of tomato include mainly glucose and fructose (Gould, 1992). The higher the content of soluble solids (°Brix), the greater the yield when it comes to industrial tomatoes and the lower is the energy expended in the pulp concentration processing (Silva & Giordano, 2000). The average values of °Brix in raw material received by the industries in Brazil have been around 4.5 °Brix (Raupp et al., 2009). The soluble solids in the fruit, besides being a genetic characteristic of the cultivar, are influenced by fertilization, irrigation, and temperature. In tomatoes aimed for industrial processing, bacterial spot can cause a reduction in soluble solids by increased exposure of the fruit to the sun (Lopes & Quezado-Duval, 2005).

Given the results obtained regarding enzymatic activities, it appears that acibenzolar-S-methyl, pyraclostrobin, and pyraclostrobin + metiram induced the production of peroxidase (Table 5) and polyphenoloxidase (Tables 5 and 6) and these may be involved in the reduction of disease susceptibility (Table 2) as acibenzolar-S-methyl and pyraclostrobin did not inhibit the *in vitro* growth of *X. perforans* (Itako et al., 2012). No influence on the production of phenylalanine ammonia-lyase, β -1,3-glucanase, or protease was observed when the other chemicals evaluated were used. Our results partially agree with those of Vigo et al. (2012) on snap-bean plants where spraying acibenzolar-S-methyl and pyraclostrobin resulted in higher levels of polyphenol oxidase,

peroxidase, and total soluble proteins, suggesting their involvement in the mechanisms of induction of resistance on the common bacterial blight caused by *Xanthomonas axonopodis* pv. *phaseoli*.

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