



Resistance of *Xanthomonas euvesicatoria* strains from Brazilian pepper to copper and zinc sulfates

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Manuscript received on July 5, 2016; accepted for publication on April 9, 2017

ABSTRACT

Bacterial spot, caused by *Xanthomonas* spp., is one of the major bacterial diseases in pepper (*Capsicum annuum* L.). The infection results in reduced crop yield, particularly during periods of high rainfall and temperature, due to the low efficiency of chemical control with copper bactericides. This study evaluated the copper and zinc sulfate sensitivity of 59 pathogenic strains of *Xanthomonas euvesicatoria* isolated from pepper plants produced in various regions throughout Brazil. Both the respective sulfates and a mixture thereof was evaluated at 50, 100, 200 and 400 $\mu\text{g.mL}^{-1}$. All the evaluated strains were found to be resistant to zinc sulfate (100 $\mu\text{g.mL}^{-1}$) and 86.4% were resistant to copper sulfate (200 $\mu\text{g.mL}^{-1}$). The mixture of copper (200 $\mu\text{g.mL}^{-1}$) and zinc (200 $\mu\text{g.mL}^{-1}$) sulfates inhibited the growth of all strains of *X. euvesicatoria*. To our knowledge this is the first study to report the resistance of *X. euvesicatoria* strains from pepper plants to copper and zinc sulfates in Brazil.

Key words: Bacterial spot, *Capsicum annuum*, chemical sensitivity, control.

INTRODUCTION

Pepper plants (*Capsicum annuum* L.) are cultivated around the world and contain compounds that add flavor and aroma to foods, in addition to being a valuable source of vitamins (Fontes 2005). In the 2013 season, 472,526 tons of peppers were produced worldwide, and Brazil was responsible for the production of 42,312 tons (FAOSTAT 2016).

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* Contribution to the centenary of the Brazilian Academy of Sciences.

Bacterial spot disease, caused by *Xanthomonas euvesicatoria*, *X. vesicatoria*, *X. perforans* and *X. gardneri*, is detrimental to pepper and tomato crops in several regions of the world, particularly during seasons of high rainfall and temperature (Quezado-Durval and Lopes 2010, Osdaghi et al. 2016, Potnis et al. 2015). *X. euvesicatoria* (Areas et al. 2015) and *X. perforans* (Araújo et al. 2017) are the major etiological agents causing bacterial spot in pepper and tomato crops, respectively, in Brazil.

Bacterial spot is very difficult to control due to the high pathogen variability and low efficiency of

chemicals. Also, the efficiency of chemical control varies with the region, season and the intensity and frequency of spraying (Carmo et al. 2001). In Brazil, copper hydroxide and copper sulfate are currently registered for use as chemical agents to control bacterial spot in pepper crops (Agrofit 2016).

The first report of copper resistance in *Xanthomonas* spp. from pepper was reported in Florida, USA (Marco and Stall 1983). In Brazil, Maringoni and Kimati (1987a) reported a low *in vitro* sensitivity of *Xanthomonas* spp. from pepper and tomato plants to copper sulfate, copper oxychloride and copper hydroxide at 100 µg.mL⁻¹. However, these bacterial strains were sensitive to mixtures of copper and dithiocarbamate fungicides, suggesting a synergism between these chemicals.

Carmo et al. (2001) evaluated the effect of weekly spraying with copper oxychloride on the progress of bacterial spot infection in pepper plants in Brazil and observed varying efficiency. In most instances, the chemicals were found to be inefficient, particularly in environmental conditions favorable for disease development. Another study from Brazil by Aguiar et al. (2003), evaluated the effect of spraying copper sulfate, copper oxychloride, cuprous oxide and a mixture of cuprous oxide and mancozeb, on the control of bacterial spot infection in pepper plants. They reported that spraying with either copper oxide alone or in combination with mancozeb, reduced the epiphytic population of *Xanthomonas* spp., and controlled the disease to a satisfactory level.

Martin and Hamilton (2004) reported *Xanthomonas* spp. strains that were resistant to copper sulfate in pepper crops in Australia. More recently, Araújo et al. (2012) compared their results with those of Ritchie and Dittapongpitch (1991), and Quezado-Duval et al. (2003) finding that the level of copper sulfate-resistant *Xanthomonas* spp. in Brazilian tomato strains was lower than that detected in the USA.

Resistance to copper fungicides occurs due to continuous spraying of crops with copper chemicals, which results in the selective pressure of resistant bacteria (Martin and Hamilton 2004). *Xanthomonas* spp. from pepper plants may possess copper-resistant genes, which can be located in plasmids and transferred via conjugation to sensitive strains, resulting in these strains becoming resistant (Stall et al. 1986).

This study aimed to evaluate the *in vitro* sensitivity of 59 *X. euvesicatoria* strains obtained from peppers produced throughout several regions of Brazil, to copper sulfate, zinc sulfate and mixtures thereof. The results presented herein will be important for improving the management of bacterial spot infection in pepper crops in Brazil.

MATERIALS AND METHODS

STRAINS OF *Xanthomonas euvesicatoria*

We used 59 *X. euvesicatoria* strains isolated from the leaves of pepper plants showing symptoms of bacterial spot from several locations in Brazil (Table I). These bacterial strains are the same as those used by Areas et al. (2015) that were identified by PCR and physiological features by these authors.

PATHOGENICITY TEST

Pepper plants (cv. Early California Wonder) were grown in 3 L plastic pots with substrate (sand, ravine clay soil and the commercial substrate Bioplant® at a ratio of 1:1:1) under greenhouse conditions (20–30°C and 70–90% RH). The plants were inoculated with *X. euvesicatoria* six weeks after sowing. All bacterial strains used for the inoculation had been grown in NSA medium (Dinghra and Sinclair 1995). The colonies were suspended in sterile distilled water and the bacterial suspensions were standardized by turbidity (OD_{600nm} = 0.1) to 10⁸CFU.mL⁻¹. Four pepper plants were inoculated per bacterial strain, using the syringe infiltration method (two leaves per pepper plant).

TABLE I
***Xanthomonas euvesicatoria* strains from pepper evaluated in this study.**

Location/State	Strain
Apodi/Rio Grande do Norte (RN)*	Xcv 255/Xcv 256/Xcv 257/Xcv 258/Xcv 259
Bacuriti/ Sao Paulo (SP)*	Xcv 214/Xcv 215/Xcv 216/Xcv 218/Xcv 219
Belém/Para (PA)*	Xcv 190/Xcv 191/Xcv 198/Xcv 199/Xcv 201/Xcv 202/Xcv 207/Xcv 209
Bertioga/Sao Paulo (SP)*	Xcv 81
Botucatu/ Sao Paulo (SP)*	Xcv 01
Bragança Paulista/ Sao Paulo (SP)*	Xcv 154/Xcv 157/Xcv 239
Brasília/Distrito Federal (DF)**	P-7
Campinas/ Sao Paulo (SP)*	Xcv 237
Cardeal/Sao Paulo (SP)*	Xcv 250/Xcv 251/Xcv 252/Xcv 253/Xcv 274
Elias Fausto/ Sao Paulo (SP)*	Xcv 93/Xcv 188/Xcv 238/Xcv 240/P-3
Ibiúna/ Sao Paulo (SP)*	Xcv 236
Lins/ Sao Paulo (SP)*	Xcv 02/Xcv 53/Xcv 54/Xcv 62/Xcv 70
Mogi das Cruzes/ Sao Paulo (SP)*	Xcv 96/Xcv 97/Xcv 98
Nova Friburgo/Rio de Janeiro (RJ)**	P-1/P-13
Piracicaba/ Sao Paulo (SP)**	P-14
Pouso Alegre/Minas Gerais (MG)*	Xcv 231/Xcv 232/Xcv 233/Xcv 242/Xcv 249
São Miguel Aracanjó/ Sao Paulo (SP)*	Xcv 248/Xcv 261
Socorro/ Sao Paulo (SP)*	Xcv 263
Tinguá/Ceara (CE)*	Xcv 266/Xcv 287/Xcv 288/Xcv 289

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Bacterial spot symptoms were assessed ten days after inoculation (Maringoni and Kimati 1987b).

In vitro ASSAY

The growth of all the evaluated strains was assessed for sensitivity to copper sulfate (Sigma-Aldrich Co., USA), zinc sulfate (Ecibra Co., Brazil), and mixtures thereof (50% each) at 0, 50, 100, 200 and 400 $\mu\text{g.mL}^{-1}$. The evaluated concentrations of sulfates were similar to those used by Ritchie and Dittapongpitch (1991), Gore and O'Garro (1999), Costa et al. (2012) and Osdaghi et al. (2016). The copper and zinc sulfates were dissolved in distilled and sterilized water and then added to the sterilized PSA medium (Dinghra and Sinclair 1995) in a melted state at 50°C, to obtain the desired concentrations. PSA medium without added

sulfates was used as a control. PSA medium was selected as it has been used previously in assays developed by Ritchie and Dittapongpitch (1991), Gore and O'Garro (1999) and Mirik et al. (2007), for the same purpose.

All the studied bacterial strains were cultivated on NSA at 28°C for 48 h. The bacterial suspensions were prepared in sterile distilled water and standardized by turbidity ($\text{OD}_{600\text{nm}} = 0.1$) to 10^8 CFU.mL⁻¹. Aliquots (10 μL) were then seeded onto four equidistant points on the surface of the PSA medium. The plates were incubated at 28°C for 24 h, and presence (insensitive) or absence (sensitive) of bacterial growth was recorded. Five replicates were performed for each strain and at each chemical concentration.

RESULTS AND DISCUSSION

All the evaluated *X. euvesicatoria* strains were pathogenic to pepper and insensitive to copper or zinc sulfates at 50 or 100 $\mu\text{g.mL}^{-1}$. At 200 $\mu\text{g.mL}^{-1}$ copper sulfate, 13.6% of the strains did not grow. Furthermore, the growth of 11.9% of the strains was inhibited at 200 $\mu\text{g.mL}^{-1}$ zinc sulfate. At 400 $\mu\text{g.mL}^{-1}$, the growth of 35.6 and 42.4% of the *X. euvesicatoria* strains was inhibited for copper and zinc sulfate, respectively. Treatment of the strains with a copper and zinc sulfates mixture showed increased sensitivity, with 3.4, 49.2 and 100% of the strains inhibited at 100, 200 and 400 $\mu\text{g.mL}^{-1}$, respectively (Figure 1).

Xanthomonas spp. from tomato and pepper plants have been shown to be resistant to copper and zinc sulfates when grown in a PSA medium containing 200 and 100 $\mu\text{g.mL}^{-1}$ of these chemicals, respectively (Ritchie and Dittapongpitch 1991, Ward and O'Garro 1992, Gore and O'Garro 1999). The strains evaluated in this study were predominantly resistant to zinc sulfate (100%) and copper sulfate (86.4%). All strains collected from Apodi (RN), Tinguá (CE), Nova Friburgo (RJ) and Brasília (DF) were resistant to both copper and zinc sulfates, while over 80% of the strains from São Paulo (SP), Minas Gerais (MG) and Pará (PA) were resistant to copper sulfate (Table II). All the strains were resistant to zinc sulfate (Table II).

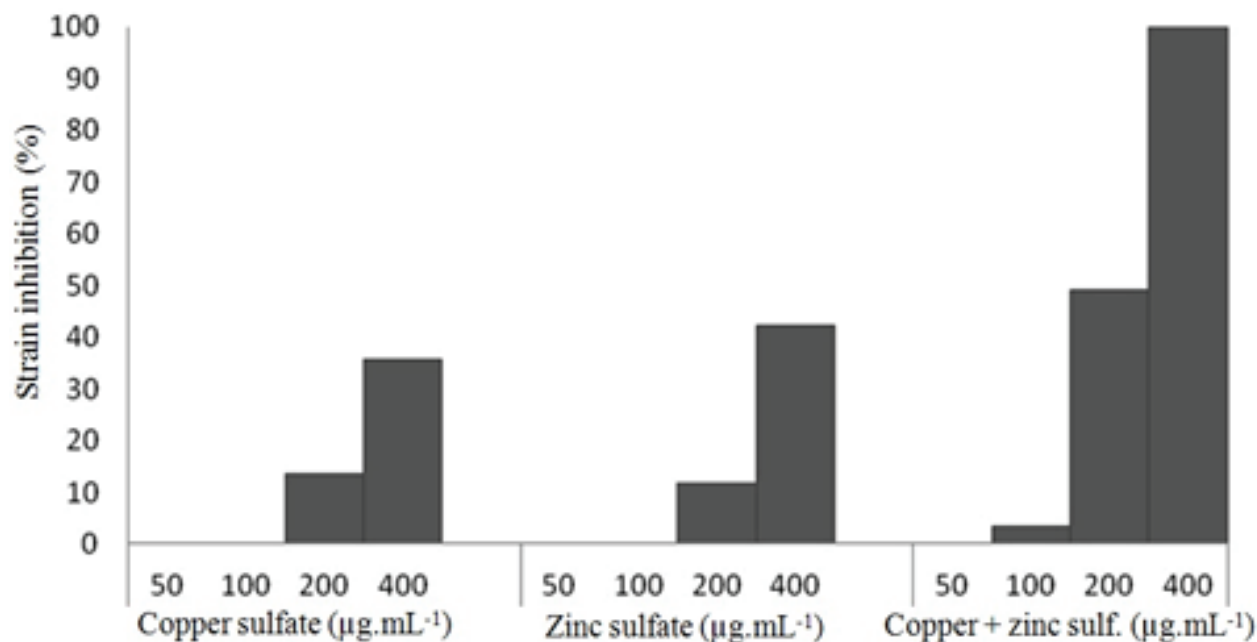


Figure 1 - *In vitro* sensitivity of *Xanthomonas euvesicatoria* strains from pepper to copper sulfate, zinc sulfate and a mixture of copper and zinc sulfates.

TABLE II
***In vitro* inhibition of *Xanthomonas euvesicatoria* strains from several locations in Brazil to copper sulfate, zinc sulfate and a mixture of copper and zinc sulfates.**

Product	Concentration ($\mu\text{g.mL}^{-1}$)	Strain/location						
		State of Sao Paulo (34) ^{a,b}	Belem/PA(8) ^a	Pouso Alegre/MG (5) ^a	Apodi/RN (5) ^a	Tingua/CE (4) ^a	Nova Friburgo/RJ (2) ^a	Brasília/DF (1) ^a
Copper sulfate	50	0 ^c	0	0	0	0	0	0
	100	0	0	0	0	0	0	0
	200	17.65	12.25	12.5	0	0	0	0
	400	41.18	25	40	0	50	50	0
Zinc sulfate	50	0	0	0	0	0	0	0
	100	0	0	0	0	0	0	0
	200	11.76	12.5	12.5	0	25	0	0
	400	38.24	25	80	40	75	0	0
Copper + zinc sulfates ^d	50	0	0	0	0	0	0	0
	100	5.88	0	0	0	0	0	0
	200	35.29	50	60	40	50	0	0
	400	100	100	100	100	100	100	100

^aNumber of strains analyzed; ^bSeveral localities; ^cPercentage inhibition; ^dMixture containing 50% of copper and 50% of zinc sulfates.

The high percentage of *X. euvesicatoria* strains resistant to copper sulfate is due to the intensive use of copper fungicides for the control of bacterial spot over many years, which has resulted in the selection of strains resistant to copper (Ritchie and Dittapongpitch 1991, Ward and O'Garro 1992, Gore and O'Garro 1999, Aguiar et al. 2000).

Several studies have previously investigated the *in vitro* sensitivity and resistance, or both, of *Xanthomonas* spp. from tomato and pepper plants to copper and zinc sulfates. Ward and O'Garro (1992) observed that 61 and 64% of *Xanthomonas* spp. from tomato and pepper plants were resistant to copper (200 $\mu\text{g.mL}^{-1}$) and zinc sulfates (100 $\mu\text{g.mL}^{-1}$), respectively. Similar results were obtained by Ritchie and Dittapongpitch (1991), Gore and O'Garro (1999) and Costa et al. (2012), who observed that over 60% of *Xanthomonas* spp. from tomato and pepper plants were resistant to treatment with 200 $\mu\text{g.mL}^{-1}$ copper sulfate.

X. euvesicatoria strains evaluated in our study were more sensitive to treatment with the copper

and zinc sulfates mixture, than either chemical alone, due to synergism between these chemicals. Similar results were obtained by Maringoni and Kimati (1987a), using a mixture of cupric and thiocarbamate fungicides, which was found to inhibit the *in vitro* growth of *Xanthomonas* spp. obtained from pepper and tomato plants. Spraying of crops with mixtures of copper oxide and mancozeb fungicides has shown a good level of bacterial spot control in pepper plants (Aguiar et al. 2003). Thus, the use of fungicides containing both copper and thiocarbamates have been suggested for the management of bacterial spot on pepper plants in Brazil. To our knowledge this is the first study to investigate the resistance of *X. euvesicatoria* strains to copper and zinc sulfates obtained from pepper plants produced in various areas throughout Brazil.

ACKNOWLEDGMENTS

The authors thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

(CAPES) for the granting of the Master's scholarship for the first author and Sakata Seed Sudamérica for the financial support and donation of *Xanthomonas euvesicatoria* strains.

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