SENSITIVITY OF XANTHOMONADS CAUSING TOMATO BACTERIAL SPOT TO COPPER AND STREPTOMYCIN AND *IN VIVO* INFRA-SPECIFIC COMPETITIVE ABILITY IN *XANTHOMONAS PERFORANS* RESISTANT AND SENSITIVE TO COPPER

E.R. Araújo[1], R.C. Pereira[1], M.A.S.V. Ferreira[1], A.M. Quezado-Duval[2] and A.C. Café-Filho[1]

[1] *Departamento de Fitopatologia, Universidade de Brasília, 70.910-900 Brasília, DF, Brazil*
[2] *Embrapa Hortaliças,70.359-970 Brasília, DF, Brazil*

SUMMARY

Failure to control tomato bacterial spot caused by *Xanthomonas* spp. with antibiotics and copper-based products is frequently reported. Also, in some pathosystems, isolates that have acquired resistance to one chemical agent may display altered fitness when compared to sensitive isolates. Therefore, the objectives of this study were (i) to evaluate the sensitivity of 94 isolates of *Xanthomonas* spp. to copper and streptomycin, and (ii) to compare the competitive ability of selected copper-sensitive and -resistant isolates of *X. perforans in planta*. Isolate reaction to each chemical was divided into four classes (S, sensitive; MS, moderately sensitive; R, resistant; and HR, highly resistant). While no isolates were recorded as HR to copper sulphate, 100% *X. gardneri* and 84.9% *X. perforans* were copper-R. Copper sensitive isolates were found only in *X. perforans* and *X. euvesicatoria*. Streptomycin-R and HR isolates predominated, except among *X. perforans* which had a majority sensitive or MS isolates. Six *X. perforans* isolates with contrasting responses to copper were inoculated to tomato plants, individually and in mixed inoculations for aggressiveness and competitiveness assessments. *In vivo* symptoms caused by S or MS isolates were significantly more severe than those caused by R isolates, indicating reduced fitness of the latter in the absence of copper sulphate. In one out of the three mixed inoculations the copper-R isolate was not recovered, further suggesting fitness impairment of copper-R isolates in the absence of selection pressure.

Key words: *Solanum lycopersicum*, *X. gardneri*, *X. perforans*, *X. vesicatoria, X. euvesicatoria,* chemical control, fitness.

Corresponding author: A.C. Café-Filho
Fax: +55.61.3107.2931
E-mail: cafefilh@unb.br

INTRODUCTION

Bacterial leaf spot of tomato, caused by four different species of *Xanthomonas*, namely *X. euvesicatoria*, *X. vesicatoria*, *X. perforans* and *X. gardneri* (Jones *et al*., 2004), is a limiting factor to tomato (*Solanum lycopersicum*) production worldwide. Both processing and fresh-market tomatoes may be severely affected when environmental conditions are conducive to the disease. No resistant cultivars are available and chemical control is frequently reported as ineffective (Quezado-Duval *et al*., 2003).

Although routinely employed in fresh and processing tomato crops in Brazil, copper fungicides and antibiotics registered for agricultural use often result in inefficient control. Indeed, the selection and persistence of isolates resistant to several of these chemicals has been hypothesized as one reason for the observed low efficiency of disease control. Resistance to streptomycin was first reported in Florida (USA) about half a century ago (Stall and Thayer, 1962). Later, again in Florida, the first report of resistance to copper was recorded (Marco and Stall, 1983). Bouzar *et al*. (1999) found *Xanthomonas* isolates resistant to both streptomycin and copper in the Caribbean and Central America. In Brazil, Carmo *et al*. (2001) reported poor efficiency of copper fungicides in sweet-pepper (*Capsicum annuum*) crops, an indication that resistant isolates may also be present in Brazil.

An early copper sensitivity study of *Xanthomonas* spp. causing bacterial spot on processing tomatoes in Brazil, found no resistant isolate at 200 µg ml^{-1} of copper sulphate, but evidenced differences in sensitivity at 50 µg ml^{-1}. All isolates of group C (presently *X. perforans*) were sensitive to streptomycin and 97% of them were sensitive to copper (Quezado-Duval *et al*., 2003).

Some studies have shown that plant pathogenic isolates that have acquired resistance to one chemical agent may have their competitiveness reduced when challenged by sensitive isolates in the absence of the product. Plant pathogenic fungal isolates sensitive to fungicides may produce larger amount of spores, display a higher mycelial growth rate *in vitro*, or cause larger number of lesions *in vivo* than the resistant isolates, sug-

Table 1. Origin, pathogenicity, biochemical characteristics and reaction to bactericides of the isolates studied.

Isolates	Origin/Collection year	Pathogenicity Tests		Race[a]	Starch Hydrolysis[b]	Response to copper/streptomycin	*Xanthomonas* species[c]
		cv. Bonny Best (Tomato)	cv. Early Wonder (Pepper)				
EH 2009-46	Uberaba-MG / 2009	+	+	T1	-	R/MS	*X. euvesicatoria*
EH 2009-47	Uberaba-MG / 2009	+	+	T1	-	S/HR	*X. euvesicatoria*
EH 2008-32	Luziânia-GO / 2008	+	-	T2	+	R/R	*X. vesicatoria*
EH 2008-167	Paty do Alferes-RJ / 2008	+	-	T2	+	R/HR	*X. vesicatoria*
EH 2008-168	Paty do Alferes-RJ / 2008	+	-	T2	+	R/R	*X. vesicatoria*
EH 2008-169	Paty do Alferes-RJ / 2008	+	-	T2	+	R/R	*X. vesicatoria*
EH 2008-170	Paty do Alferes-RJ / 2008	+	-	T2	+	R/R	*X. vesicatoria*
EH 2009-42	Uberaba-MG / 2009	+	-	T2	+	R/R	*X. vesicatoria*
EH 2005-11	São José dos Pinhais-PR / 2005	+	+	T2	-	R/R	*X. gardneri*
EH 2006-06	Mucugê-BA / 2006	+	+	T2	-	R/R	*X. gardneri*
EH 2006-08	Ibicoara -BA / 2006	+	+	T2	-	R/R	*X. gardneri*
EH 2007-12	Goianápolis-GO / 2007	+	+	T2	-	R/R	*X. gardneri*
EH 2007-13	Goianápolis-GO / 2007	+	+	T2	-	R/R	*X. gardneri*
EH 2007-17	Marilândia do Sul-PR / 2007	+	+	T2	-	R/R	*X. gardneri*
EH 2007-18	Marilândia do Sul-PR / 2007	+	+	T2	-	R/R	*X. gardneri*
EH 2007-19	Marilândia do Sul-PR / 2007	+	-	T2	-	R/R	*X. gardneri*
EH 2007-20	Marilândia do Sul-PR / 2007	+	+	T2	-	R/R	*X. gardneri*
EH 2007-22	Marilândia do Sul-PR / 2007	+	+	T2	-	R/R	*X. gardneri*
EH 2007-31	Caxias do Sul-PR / 2007	+	+	T2	-	R/R	*X. gardneri*
EH 2007-32	Caxias do Sul-PR / 2007	+	+	T2	-	R/R	*X. gardneri*
EH 2007-34	Caxias do Sul-PR / 2007	+	+	T2	-	R/R	*X. gardneri*
EH 2007-35	Caí-RS / 2007	+	+	T2	-	R/R	*X. gardneri*
EH 2007-37	Caí-RS / 2007	+	+	T2	-	R/R	*X. gardneri*
EH 2007-40	Caí-RS / 2007	+	+	T2	-	R/MS	*X. gardneri*
EH 2007-41	Caí-RS / 2007	+	+	T2	-	R/R	*X. gardneri*
EH 2007-42	Santa Lucia-RS / 2007	+	+	T2	-	R/R	*X. gardneri*
EH 2007-43	Santa Lucia-RS / 2007	+	+	T2	-	R/R	*X. gardneri*
EH 2007-44	Santa Lucia-RS / 2007	+	+	T2	-	R/R	*X. gardneri*
EH 2007-45	Santa Lucia-RS / 2007	+	+	T2	-	R/R	*X. gardneri*
EH 2008-22	Espírito Santo-ES / 2008	+	+	T2	-	R/HR	*X. gardneri*
EH 2008-35	São Paulo-SP / 2008	+	+	T2	-	R/R	*X. gardneri*
EH 2008-171	São Sebastião do Alto-RJ / 2008	+	+	T2	-	R/R	*X. gardneri*
EH 2008-179	Espírito Santo-ES/ 2008	+	+	T2	-	R/R	*X. gardneri*
EH 2009-22	Goianápolis-GO / 2009	+	+	T2	-	R/R	*X. gardneri*
EH 2009-30	Araguari-MG / 2009	+	+	T2	-	R/HR	*X. gardneri*
EH 2009-33	Uberaba-MG / 2009	+	-	T2	-	R/R	*X. gardneri*
EH 2009-37	Uberaba-MG / 2009	+	-	T2	-	R/HR	*X. gardneri*
EH 2009-40	Uberaba-MG / 2009	+	+	T2	-	R/R	*X. gardneri*
EH 2009-45	Uberaba-MG / 2009	+	+	T2	-	R/MS	*X. gardneri*
EH 2009-49	Araguari-MG / 2009	+	+	T2	-	R/S	*X. gardneri*
EH 2009-183	Teresópolis-RJ / 2009	+	+	T2	-	R/R	*X. gardneri*
EH 2005-30*	Vicentinópolis-GO / 2005	+	nd	T3	+	MS/S	*X. perforans*
EH 2005-54*	Goiânia-GO / 2005	+	-	T3	+	MS/S	*X. perforans*
EH 2005-60	Patos de Minas-MG / 2005	+	nd	T3	+	MS/S	*X. perforans*
EH 2006-10	Ibicoara -BA / 2006	+	nd	T3	+	R/S	*X. perforans*
EH 2006-44*	Itaberaí-GO / 2006	+	-	T3	+	MS/S	*X. perforans*
EH 2007-09	Itaberaí-GO / 2007	+	nd	T3	+	MS/S	*X. perforans*
EH 2007-26*	Joviânia-GO / 2007	+	-	T3	+	MS/S	*X. perforans*
EH 2008-13*	Rio Verde-GO / 2008	+	-	T3	+	S/S	*X. perforans*
EH 2008-14	Rio Verde-GO / 2008	+	nd	T3	+	R/R	*X. perforans*
EH 2008-16*	Goiânia-GO / 2008	+	-	T3	+	S/R	*X. perforans*
EH 2008-19	Goiânia-GO / 2008	+	nd	T3	+	R/R	*X. perforans*
EH 2008-20	Goiânia-GO / 2008	+	nd	T3	+	MS/S	*X. perforans*
EH 2008-28	Luziânia-GO / 2008	+	nd	T3	+	R/R	*X. perforans*
EH 2008-36	Catalão-GO / 2008	+	-	T3	+	R/R	*X. perforans*
EH 2008-172	São Sebastião do Alto-RJ / 2008	+	-	T3	+	R/R	*X. perforans*
EH 2008-173	São Sebastião do Alto-RJ / 2008	+	-	T3	+	R/R	*X. perforans*
EH 2008-174	São Sebastião do Alto-RJ / 2008	+	-	T3	+	R/R	*X. perforans*
EH 2008-175	São Sebastião do Alto-RJ / 2008	+	-	T3	+	R/R	*X. perforans*
EH 2008-176	Miracema-RJ / 2008	+	-	T3	+	R/S	*X. perforans*
EH 2008-177	Miracema-RJ / 2008	+	-	T3	+	R/R	*X. perforans*
EH 2009-05	Camocim São Félix-PE / 2009	+	-	T3	+	MS/S	*X. perforans*
EH 2009-10	Anápolis-GO/ 2009	+	nd	T3	+	MS/S	*X. perforans*
EH 2009-12	Anápolis-GO / 2009	+	-	T3	+	R/MS	*X. perforans*
EH 2009-13	Anápolis-GO / 2009	+	-	T3	+	R/MS	*X. perforans*
EH 2009-14	Anápolis-GO / 2009	+	-	T3	+	R/R	*X. perforans*
EH 2009-15	Anápolis-GO / 2009	+	-	T3	+	R/R	*X. perforans*
EH 2009-20	Goianápolis-GO / 2009	+	-	T3	+	R/R	*X. perforans*
EH 2009-21	Goianápolis-GO / 2009	+	-	T3	+	R/R	*X. perforans*
EH 2009-23	Goianápolis-GO / 2009	+	-	T3	+	R/R	*X. perforans*
EH 2009-24	Goianápolis-GO / 2009	+	-	T3	+	R/R	*X. perforans*
EH 2009-26	Goianápolis-GO / 2009	+	-	T3	+	R/R	*X. perforans*
EH 2009-31	Uberaba-MG / 2009	+	-	T3	+	R/S	*X. perforans*
EH 2009-32	Uberaba-MG / 2009	+	-	T3	+	R/MS	*X. perforans*
EH 2009-34	Uberaba-MG / 2009	+	-	T3	+	R/S	*X. perforans*
EH 2009-38	Uberaba-MG / 2009	+	-	T3	+	R/S	*X. perforans*
EH 2009-39	Uberaba-MG / 2009	+	-	T3	+	R/R	*X. perforans*
EH 2009-44	Uberaba-MG / 2009	+	-	T3	+	R/HR	*X. perforans*
EH 2009-48	Araguari-MG / 2009	+	-	T3	+	R/S	*X. perforans*
EH 2009-55	Araguari-MG / 2009	+	-	T3	+	R/S	*X. perforans*
EH 2009-56	Araguari-MG / 2009	+	-	T3	+	R/S	*X. perforans*
EH 2009-57	Araguari-MG / 2009	+	-	T3	+	R/R	*X. perforans*
EH 2009-58	Araguari-MG / 2009	+	-	T3	+	R/R	*X. perforans*
EH 2009-59	Araguari-MG / 2009	+	-	T3	+	R/R	*X. perforans*

EH 2009-61	Araguari-MG / 2009	+	-	T3	+	R/R	*X. perforans*
EH 2009-78	Guaraciaba do Norte-CE / 2009	+	-	T3	+	R/R	*X. perforans*
EH 2009-79	Guaraciaba do Norte-CE / 2009	+	-	T3	+	R/S	*X. perforans*
EH 2009-80	Guaraciaba do Norte-CE / 2009	+	-	T3	+	R/R	*X. perforans*
EH 2009-95	Vicosa do Ceará-CE / 2009	+	-	T3	+	R/HR	*X. perforans*
EH 2009-97	Vicosa do Ceará-CE / 2009	+	-	T3	+	R/R	*X. perforans*
EH 2009-177	Tangará da Serra-MT / 2009	+	-	T3	+	R/S	*X. perforans*
EH 2009-179	Tangará da Serra-MT / 2009	+	-	T3	+	R/S	*X. perforans*
EH 2009-181	Tangará da Serra-MT / 2009	+	-	T3	+	R/R	*X. perforans*
EH 2009-182	Tangará da Serra-MT / 2009	+	-	T3	+	R/R	*X. perforans*

a. race identification according to Jones *et al.* (1995).
b. biochemical test for differentiation of *Xanthomonas* species causing tomato bacterial spot
c. according to biochemical tests, pathogenicity assays, rep-PCR and specific primers
S: Sensitive reaction; MS: Moderate sensitive reaction; R: Resistant reaction; HR: Highly resistant reaction; nd. Not determined.
* X. *perforans* isolates selected for the *in vivo* competitiveness assays.
Numbers designate code from the Plant Pathogenic Bacterial Collection at Embrapa Hortaliças, Brazil

gesting loss of aggressiveness and competitive fitness of the resistant strains in the absence of selection pressure (e.g. Webber, 1988; Sanoamuang and Gaunt, 1995).

However, in other pathosystems, the acquisition of resistance has not been associated with reduction of competitive potential. Among oomycetes, resistant isolates may present equal or superior performance in biological parameters such as latent period and growth rates, and no appreciable loss in aggressiveness compared to sensitive ones (e.g. Café-Filho and Ristaino, 2008; Hu *et al.*, 2008; Corio-Costet *et al.*, 2011).

Fitness costs in prokaryotes are mostly studied among bacteria of medical or environmental concern. Kang and Park (2010) showed that an antibiotic-resistant strain of *Acinetobacter* sp. displayed significant phenotypic and physiological changes. Such alterations have been characterized as biological fitness costs (Andersson, 2006). On the other hand, Luciani *et al.* (2009) demonstrated that the overall fitness of antibiotic-resistant strains of *Mycobacterium tuberculosis* was comparable with that of sensitive strains. Among plant pathogenic bacteria, Sundin and Bender (1993) and Cazorla *et al.* (2002), among others, studied copper and streptomycin resistance in *Pseudomonas syringae*, determining that resistance is encoded by conjugative plasmids. Still within *P. syringae*, Hwang *et al.* (2005) showed that resistance to copper is widespread, while naturally-occurring streptomycin resistance was rare. While fewer such studies were published on *Xanthomonas*, resistance to copper among xanthomonads and pseudomonads has been argued to be of similar nature (Voloudakis *et al.*, 1993).

Although the occurrence of resistance to copper among *Capsicum* strains of *Xanthomonas campestris* pv. *vesicatoria* has been reported (*e.g.* Marco and Stall, 1983; Adaskaveg and Hine, 1985), the assessment of the biological costs of copper or antibiotic resistance in *Xanthomonas* species associated with tomato bacterial spot has not yet been done. The only study found in the literature regarding competition among the *Xanthomonas* complex associated with tomato bacterial spot focuses on bacteriocin production, not copper or streptomycin resistance (Hert *et al.*, 2005). That study determined that bacteriocins provided *X. perforans* strains with a competitive advantage over the wild-type *X. perforans* strain and *X. euvesicatoria*. It was further reported that a virulence-attenuated strain of *X. perforans* could be deployed as a biological control agent against *X. euvesicatoria* (Hert *et al.*, 2009).

The characterization of the response of each *Xanthomonas* species causing bacterial spot to streptomycin and copper is essential for dosage recommendation and disease management advice. Therefore, the objectives of this study were (i) to characterize a collection of *Xanthomonas* isolates from tomato-producing regions in Brazil as to their *in vitro* reaction to copper and to streptomycin sulphates, and (ii) to assess the infra-specific competitive ability of *X. perforans* isolates with contrasting reactions to copper sulphate, on the basis of their aggressiveness to tomato and population recovery from diseased tissue.

MATERIALS AND METHODS

Reaction of *Xanthomonas* spp. to copper and streptomycin. A collection of 94 *Xanthomonas* spp. (53 *X. perforans,* 33 *X. gardneri,* 6 *X. vesicatoria* and 2 *X. euvesicatoria*), deposited at the Plant Pathogenic Bacterial Collection of Embrapa-Hortaliças, Brasília, Brazil, was tested *in vitro* for reaction to copper and streptomycin (Table 1). Isolates were collected between 2005 and 2009 from fresh-market and processing tomato fields in the north-eastern, mid-western, south-eastern and southern Brazilian regions. Lower numbers of *X. euvesicatoria* and *X. vesicatoria* in this collection reflect that these species were less prevalent in Brazilian tomato fields (Pereira *et al.*, 2011). Isolates had been previously identified as *Xanthomonas* according to the following diagnostic tests (Schaad *et al.*, 2001): Gram reaction (-); aerobic growth (+); fluorescence on King`s B (-); yellow colonies on NA (+); growth at 33°C on YDC (+); catalase activity (+) (Pereira *et al.*, 2011). *Xanthomonas* species were determined by comparison of their rep-PCR (BOX and/or REP) fingerprints (Louws *et al.*, 1995) to those of reference strains obtained from the

Instituto Biológico de Campinas, São Paulo, Brazil (*X. euvesicatoria* IBSBF 2363, *X. vesicatoria* IBSBF 2364, *X. perforans* IBSBF 2370, and *X. gardneri* IBSBF 2373). Representative haplotypes of each species were further confirmed by specific primers (Koenraadt *et al*., 2009). Pathogenicity was confirmed by inoculations on tomato and pepper plants in the greenhouse (average temperature of 28.7°C). The collection was kept in phosphate buffer (K_2HPO_4, KH_2PO_4, pH 7.0) at room temperature and at – 80°C on sterile 30% glycerol solution. For sensitivity assessments, isolates were initially incubated on NA medium for 48 h at 28°C and a 5×10^8 CFU ml^{-1} (OD_{600} = 0.3) bacterial suspension was prepared in 1 ml of 10 mMol l^{-1} magnesium sulphate ($MgSO_4.7H_2O$, Sigma Cell Culture). Ten microlitres of this suspension were then transferred to CYE medium (Zevenhuizen *et al*., 1979), containing casitone, 1.7 g l^{-1}; yeast extract, 0.35 g l^{-1}; glycerol, 2.0 g l^{-1}; and agar, 15 g l^{-1} and to NA medium for the sensitivity assays to copper and streptomycin, respectively. CYE agar has been previously used to assess *Pseudomonas cichorii* (Pohronezny *et al*., 1994) and *Xanthomonas* spp. (Pernezny *et al*., 2008) sensitivity to copper. CYE agar has a low capacity to complex with copper ions and was supplemented with copper sulphate ($CuSO_4.5H_2O$, Merck) in the following concentrations: 50 μg ml^{-1} (Quezado-Duval *et al*., 2003), 100 and 200 μg ml^{-1} (Obradovic *et al*., 2004). These concentrations correspond to 0.2, 0.4 and 0.8 mM $CuSO_4$, respectively.

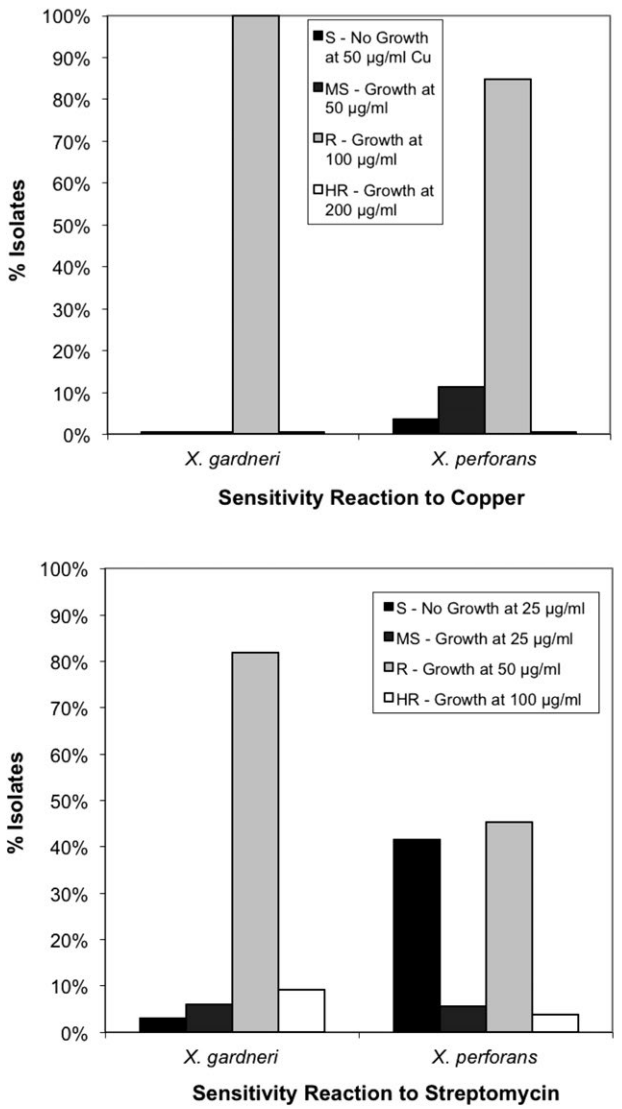

Fig. 1. Reaction to copper and streptomycin of *Xanthomonas gardneri* (n = 33) and *X. perforans* (n = 53) causing bacterial leaf spot of tomato. Resistance was characterized by confluent growth in CYE agar, amended with 50, 100 or 200 μg ml^{-1} of copper sulphate, or 25, 50 or 100 μg ml^{-1} streptomycin, 72 h after incubation at 28°C. S: Sensitive reaction; MS: Moderate sensitive reaction; R: Resistant reaction; HR: Highly resistant reaction.

For the streptomycin sensitivity assay, NA medium was supplemented with streptomycin sulphate (Sigma-Aldrich, USA) at 25 μg ml^{-1} (Quezado-Duval *et al*., 2003), 50 and 100 μg ml^{-1} (Obradovic *et al*., 2004). Isolates were tested in three replicates, and confluent growth, equivalent to growth in NA control plates, was recorded after 72 h of incubation at 28°C for resistance assessments. Isolates were considered sensitive (S) when they did not grow at 50 μg ml^{-1} or 25 μg ml^{-1} of copper sulphate or streptomycin sulphate, respectively, and highly resistant (HR) if they grew confluently at 200 μg ml^{-1} (0.8 mM $CuSO_4$) or 100 μg ml^{-1} of each respective chemical. Moderately sensitive (MS) isolates grew at 50, but not at 100 μg ml^{-1} of copper sulphate or at 25, but not at 50 μg ml^{-1} of streptomycin sulphate, and resistant (R) isolates grew at 100, but not at 200 μg ml^{-1} of copper sulphate, and at 50, but not at 100 μg ml^{-1} of streptomycin sulphate. Control cultures of all isolates were incubated in similar conditions in non-amended NA and CYE for comparison purposes and only colonies that presented confluent growth equivalent to that of the controls in all three replicates were considered positive for each respective treatment.

Evaluation of competitive ability of *Xanthomonas perforans* isolates with contrasting reactions to copper sulphate. The isolates used in this assay were collected in processing tomato fields in the state of Goiás (Brazil). Six *X. perforans* isolates, with contrasting *in vitro* reactions to copper according to the tests described above were selected (Table 1) for inoculation to the susceptible tomato cv. Yuba (CNPH 851). A preliminary assay showed that there were no significant differences in growth among the isolates when cultivated for 48 h on CYE agar medium. Plants were inoculated at the 5th-6th leaf stage with a manual sprayer, with a suspension of 5×10^8 CFU ml^{-1} of each isolate, as in Astua-Monge *et al*. (2000), separately or co-inoculated with a 1:1 mixture of resistant (R) and sensitive/moderately sensitive (S/MS) isolates in three combinations, as in Shenge *et al*. (2008). The bacterial suspensions were distributed

homogeneously on the leaves up to saturation and plants were subsequently covered with humid plastic bags for 48 h and kept in pots in the greenhouse with natural light and mean temperature of 27±4°C. A total of nine treatments, *viz.* six single inoculations and three mixed inoculations combining one MS, two S and three R isolates were tested in a completely randomized block design. Experimental units were represented by five plants and the experiment was replicated twice. Competitiveness ability was assessed by measuring disease severity and bacterial recovery from inoculated plants as described below.

Disease severity assessment. Disease severity, measured as the percentage of diseased leaf area, was used to estimate isolate aggressiveness, defined in Andrivon (1993) as the quantity of disease induced by a pathogenic strain on a susceptible host. Twelve days after inoculations five leaflets from each treatment were collected for the video-image scanning of diseased leaf area, using the QUANT software v. 1.0, which differentiates diseased and healthy tissue by artificial colouring (Vale *et al*., 2001). Chlorotic leaf areas surrounding lesions were not computed as diseased areas in this study.

Bacterial recovery from tomato plants inoculated singly or simultaneously with two strains of *X. perforans*. Reisolation from plants inoculated with single or mixed bacterial suspensions was attempted 12 days after inoculation, on CYE medium amended or non-amended with 100 μg ml^{-1} copper sulphate (for recovery of resistant or all isolates, respectively). Two samples, composed of three symptomatic leaflets, were collected from each treatment replicate. Eight-mm leaflet sections of half diseased and half healthy tissue were dipped in ethanol 70% for 10 sec, followed by a 30 sec dip in sodium hypochlorite 10%, and a triple wash in magnesium sulphate 10 mMol l^{-1} to avoid growth of non target microorganisms, since a *X. perforans* selective medium was not available. Samples were then macerated in sterilized water and diluted up to 10^{-5}, to allow for colony counts. Aliquots (100 μl) of these suspensions were then transferred to copper-amended and non-amended CYE plates. Colonies were counted on each medium after a 72 h incubation at 28°C. Each treatment was assessed in three replicates. Analysis of variance and mean separation (Fisher´s LSD) for the aggressiveness test and the competitive assay were carried out with the statistical program SAS (SAS Institute, 2002).

RESULTS

Reaction of *Xanthomonas* spp. to copper and streptomycin. Isolates representative of all four species were, to some degree, insensitive to copper or streptomycin (Table 1). Frequency of copper and streptomycin reaction of the two most representative species in our sampling, namely *X. gardneri* and *X. perforans* are represented in Fig. 1. Although none of the isolates grew in CYE at a copper concentration of 200 μg ml^{-1} (cop-HR reaction), 90.4% were resistant at the 100 μg ml^{-1} concentration (cop-R reaction), and a further 6.4% were insensitive at the 50 μg ml^{-1} concentration (cop-MS reaction). Only 3.2% of the isolates of all species combined were cop-S. Within the two most prevalent species, it is worth noticing that 100% of *X. gardneri* (n = 33) isolates were classified as cop-R, and 84.9% of *X. perforans* (n = 53) were cop-R, while only eight were cop-S or cop-MS (Fig. 1). Among the two least prevalent species, all *X. vesicatoria* isolates (n = 6) were cop-R, while one of the two representatives of *X. euvesicatoria* was cop-S and the other was cop-R (Table 1).

Overall frequency of HR to streptomycin (strep-HR) among all four species was 7.4% (growth at 100 μg ml^{-1}), while 62.8% were classified as strep-R (growth at 50 μg ml^{-1}) and a further 4.3% were strep-MS (growth at 25 μg ml^{-1}). The remaining 25.5% of the isolates was strep-S. Within the two prevalent species, *X. gardneri* was predominantly strep-R (82%) or strep-HR (9%) while most strep-S isolates were found in *X. perforans* (21 out of 22 strep-S isolates) (Fig. 1). Among the two least prevalent species, no strep-S isolates were found. Five *X. vesicatoria* isolates were strep-R and one was strep-HR. Of the two representatives of *X. euvesicatoria*, one was strep-MS and the other was strep-HR (Table 1).

Evaluation of competitive ability of *Xanthomonas perforans* isolates with contrasting reactions to copper sulphate. Copper-S or MS isolates caused up to 13.8% and 7.6% diseased area, respectively, while cop-R isolates caused between 4.5% and 6.2% diseased leaf area, 12 days after inoculation. In single inoculations, significantly ($P \leq 0.05$) more severe symptoms were found with isolate 2008-13 and 2008-16 (both cop-S). Copper-R isolates were consistently the least aggressive (Fig. 2). In the mixed inoculations disease levels were variable.

No additive or synergistic effects on disease severity were evident when two *X. perforans* isolates with contrasting reactions to copper were inoculated simultaneously onto the same tomato plant. Conversely, competition between mixed isolates may be inferred in one case: when cop-S isolate 2008-13 was inoculated singly, it produced a larger diseased area than when it was inoculated jointly with cop-R isolate 2006-44 (Fig. 2).

Bacterial recovery from tomato plants inoculated singly or simultaneously with two strains of *X. perforans*. Colonies of *X. perforans* were reisolated in non-amended CYE from all mixed inoculations, in similar numbers (Fig. 3). More interestingly, in one out of the

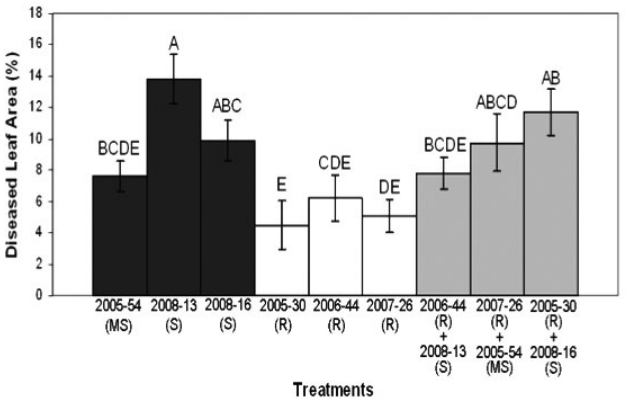

Fig. 2. Percentage of diseased tomato leaf area by copper-resistant or sensitive *Xanthomonas perforans*, 12 days after single or combined inoculations. Bars with same letter are not significantly different (LSD, $p \leq 0.05$). R = isolate resistant at 100 μg ml^{-1} of copper sulphate. R = isolate resistant to 100 μg ml^{-1} of copper sulphate; MS = isolate resistant to 50 μg ml^{-1} and sensitive to 100 μg ml^{-1} of copper sulphate; S = isolate sensitive to copper sulphate at 50 μg ml^{-1}.

three mixed inoculations [isolate 2007-26 (cop-R) + 2005-54 (cop-MS)], recovery of the copper-resistant isolate was unsuccessful in all three replicates, strongly suggesting fitness impairment under no pressure for copper resistance (Fig. 3).

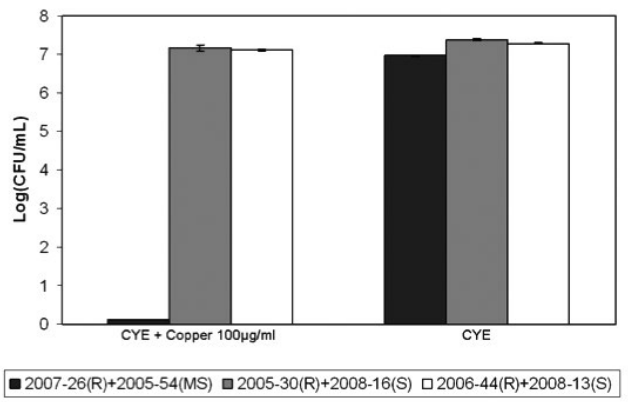

Fig. 3. Recovery of isolates of copper-resistant and sensitive *Xanthomonas perforans* in non-amended or copper-amended CYE, 12 days after joint inoculations in tomato leaves. R = isolate resistant to 100 μg ml^{-1} of copper sulphate; MS = isolate resistant to 50 μg ml^{-1} and sensitive to 100 μg ml^{-1} of copper sulphate; S = isolate sensitive to copper sulphate at 50 μg ml^{-1}.

DISCUSSION

All studies published so far concerning xanthomonads causing bacterial spot and resistance to bactericides have not reported isolate response for each of the four bacterial species that currently form this disease complex. In the present work we showed that there is a significant variation in copper and streptomycin sensitivity among the two most prevalent species in Brazil, *viz. X. perforans* and *X. gardneri*. Variation was also recorded among the two least prevalent ones, *X. vesicatoria* and *X. euvesicatoria*. Furthermore, and more importantly, the distribution of sensitivity to those antimicrobial compounds among isolates seems to be remarkably related to the species.

Copper resistant phenotype is widespread among plant pathogenic bacteria, such as *Pseudomonas syringae* (Hwang *et al.*, 2005) and *Xanthomonas* spp. (Marques *et al.*, 2009; Teixeira *et al.*, 2008). Copper resistance among xanthomonads causing bacterial spot, mainly in peppers, has also been reported in the US (Marco and Stall, 1983; Adaskaveg and Hine, 1985; Ritchie and Dittapongpitch, 1991), Brazil (Aguiar *et al.*, 2003) and Serbia (Obradovic *et al.*, 2004). In the US and Canada, disease management of bacterial spot based on copper products has not resulted in effective control (Abbasi *et al.*, 2002). Likewise, cupric compounds registered for agricultural use in Brazil, often do not achieve efficient disease control (Carmo *et al.*, 2001). The occurrence of resistant strains may be one of the causes of this low efficiency.

Adaskaveg and Hine (1985) found that the frequency of pepper cop-R isolates among populations of *X. campestris* pv. *vesicatoria* of different regions depended on the frequency of copper bactericides employment by growers. Ritchie and Dittapongpitch (1991) reported that 63% of a collection of mostly pepper isolates of *X. campestris* pv. *vesicatoria* were resistant to copper at 200 μg ml^{-1} in North Carolina (USA). In contrast, Quezado-Duval *et al.* (2003) found no isolates resistant to copper at 200 μg ml^{-1} in Brazilian processing tomato fields. Here we analyzed a different set of isolates, which also included isolates from fresh market tomato fields and again no cop-HR isolates were found. Thus, it seems that the level of copper resistance currently found naturally in tomato fields in Brazil is somewhat lower than those recorded in the US. Nevertheless, in this study cop-R (growth at 100 μg ml^{-1}) was prevalent (90.4% of all isolates tested), which still poses a significant threat to disease management.

Regarding *X. perforans*, Quezado-Duval *et al.* (2003) reported that 97% of group C isolates (=*X. perforans*) were sensitive at 50 μg ml^{-1} (cop-S) and all were sensitive to streptomycin. Since then, the distribution of resistant isolates in the field population of *X. perforans* seems to have shifted significantly, as our results with isolates collected from 2005 to 2009 show that 84.9% of the isolates are now cop-R and 52.8% are strep-R or HR (Fig. 1). Indeed, there may be a shift in course towards increasing resistance to chemicals in the Brazilian populations of xanthomonads, especially within *X. perforans*. Sundin and Bender (1993) detected a similar

state of flux towards copper and streptomycin resistance in natural populations of *P. syringae* pv. *syringae*. However, they did not assess fitness parameters of those isolates. The observed increase in the prevalence of copper or streptomycin resistance is probably due to the widespread and continuous use of related antimicrobial control products.

Results of the selection pressure towards copper resistance are quite evident in *X. gardneri* and *X. vesicatoria,* but comparatively less clear in *X. perforans* and *X. euvesicatoria.* Recent studies showed that these latter two species are closely related based on AFLP markers and evolutionary genome divergence analysis (Hamza *et al*., 2010), and phylogenetic comparisons of *gyrB* sequences (Parkinson *et al*., 2009). This could partially explain their similarities when responding to selection pressure towards resistance in nature.

With reference to streptomycin resistance, the distribution pattern among classes was conspicuously dependent on the species. *X. perforans* presented a remarkable distinct sensitivity pattern, compared to the other species. Of the 53 *X. perforans* isolates tested, 41.5% were strep-S (absence of growth at 25 μg ml^{-1}). Among the other three species, only one additional isolate of *X. gardneri* was also sensitive. Conversely, *X. gardneri* and *X. vesicatoria* isolates were mostly strep-R or HR. Although only two isolates of *X. euvesicatoria* were included in this study, due to the low prevalence of this species in tomato fields in Brazil in recent years, one strep-HR was found. One possible reason for the discrepancy between *X. perforans* and the other species is that it may be a recently introduced species and consequently has not been exposed as long as the others to the antibiotic under field conditions. The reported low level of diversity found in *X. perforans* in Brazil (Quezado-Duval *et al*., 2005) agrees with this. In addition, it can be speculated that the biological fitness cost of resistance in *X. perforans* is higher than in the other bacterial spot pathogens and this could prevent the appearance of more strep-R or HR isolates within this species.

Among *X. perforans* isolates differing in sensitivity to copper, cop-R isolates were consistently the least aggressive ones. Several factors may affect competitive ability in bacteria. For example, other studies in xanthomonads causing bacterial spot have shown that the production of bacteriocins may provide a competitive advantage for *X. perforans* causing displacement of *X. euvesicatoria* (Hert *et al*., 2005). So far no study has focused on the effect of copper resistance on infraspecific competitive ability within each species that form this complex. The lower percentage of diseased leaf area caused by cop-R isolates, when compared to cop-S or MS isolates, suggests a negative correlation between copper resistance and the ability of *X. perforans* to cause disease on tomato, thus characterizing a measurable biological fitness cost. It is well known that the acquisition of resistance to antimicrobial compounds may reduce biological fitness, but there are also examples of low biological cost or even cost-free variants in several species, from widely different habitats (Kang and Park, 2010; Luciani *et al*., 2009; Lilley and Bailey, 1997). In oomycetes (Chromista) and also among true fungi the two scenarios have been recorded. In competition assays, resistant *Phytophthora* isolates grown in the absence of the control products (metalaxyl or mefenoxam), were as competitive or even more aggressive than the sensitive ones, measured by spore production, mycelial growth and ability to cause damage to their hosts (Hu *et al*., 2008; Café-Filho and Ristaino, 2008; Kadish *et al*., 1990). Also, in *Plasmopara viticola*, no evident cost was detected for quinone outside-inhibitor (QoI) fungicide-resistant isolates (Corio-Costet *et al*., 2010). However, in other pathosystems, the reverse situation has been observed. When selection pressure is removed, sensitive isolates have competitive advantages over the resistant ones of the same species (Sanoamuang and Gaunt, 1995; Webber, 1988).

Excess copper in bacterial cells may be toxic, simultaneously affecting several metabolic functions. In order to survive in an environment with high concentrations of this metal, bacteria must activate regulatory mechanisms related to the acquisition of plasmid-borne resistance genes (Teixeira *et al*., 2008; Rensing and Grass, 2003). It is possible that this additional metabolic cost for those isolates that acquired resistance genes results in loss of competitiveness in the absence of cupric compounds in the environment. The competitiveness among resistant and sensitive *X. perforans* isolates, when estimated by the success of isolate recovery from mixed inoculations of cop-R and cop-MS depended on the isolate combination. However, in one of the mixed inoculations (Fig. 3), re-isolation of the cop-R isolate was unsuccessful in all three replicates, strongly supporting the hypothesis of fitness impairment in the absence of selection pressure. Furthermore, the three copper sensitive or cop-MS isolates were seemingly more competitive than the three cop-R isolates, as they always caused greater percentage of diseased leaf area in absence of selection pressure for copper resistance as well (Fig. 2). This requires a more careful use of copper products for bacterial spot control, in a manner similar to that recommended by Wolf (1981), i.e. by avoiding routine, prophylactic use of chemicals, applying antimicrobial compounds parsimoniously in the field, and integrating their use with other control measures. These measures may prevent the prevalence of resistant individuals in a population, and might even revert dominance of cop-R isolates by interrupting copper sprays, as the lack of selection pressure will presumably favor the sensitive and more competitive isolates, therefore reinstating the effectiveness of control.

ACKNOWLEDGEMENTS

Alice Maria Quezado-Duval acknowledges National Research Council (CNPq) financial support (grant # 578.775/2008-5); Edivânio Araújo and Roberta Pereira were recipients of CNPq graduate scholarships during the course of this study; and Adalberto Café-Filho (grant # 301.095/2009-4) and Marisa Ferreira (grant # 306.584/2009-3) are CNPq research fellows.

REFERENCES

Abbasi P.A., Soltani N., Cuppels D.A., Lazarovits G., 2002. Reduction of bacterial spot disease severity on tomato and pepper plants with foliar applications of ammonium lignosulfonate and potassium phosphate. *Plant Disease* **86**: 1232-1236.

Adaskaveg J.E., Hine R.B., 1985. Copper tolerance and zinc sensitivity of Mexican strains of *Xanthomonas campestris* pv. *vesicatoria*, causal agent of bacterial spot of pepper. *Plant Disease* **69**: 993-996.

Aguiar L.A., Kimura O., Castilho A.M.C., Castilho K.S.C., Ribeiro R.L.D., Akiba F., Carmo M.G.F., 2003. Efeito de formulações cúpricas e cuprorgânicas na severidade da mancha-bacteriana e na população residente de *Xanthomonas campestris* pv. *vesicatoria* em pimentão. *Horticultura Brasileira* **21**: 44-50.

Andersson D.I., 2006. The biological cost of mutational antibiotic resistance: any practical conclusions? *Current Opinion in Microbiology* **9**: 461-465.

Andrivon D., 1993. Nomenclature for pathogenicity and virulence: the need for precision. *Phytopathology* **83**: 889-890.

Astua-Monge G., Minsavage G.V., Stall R.E., Vallejos C.E., Davis M.J., Jones J.B., 2000. *Xv4-vrxv4*: A new gene-for-gene interaction identified between *Xanthomonas campestris* pv. *vesicatoria* race T3 and the wild tomato relative *Lycopersicon pennellii*. *Molecular Plant-Microbe Interactions* **13**: 1346-1355.

Bouzar H., Jones J.B., Stall R.E., Louws F.J., Schneider M., Rademaker J.L.W., Bruijn F.J., Jackson L.E., 1999. Multiphasic analysis of *Xanthomonas* causing bacterial spot disease on tomato and pepper in the Caribbean and Central America: Evidence for common lineages within and between countries. *Phytopathology* **89**: 328-335.

Café-Filho A.C., Ristaino J.B., 2008. Fitness of isolates of *Phytophthora capsici* resistant to mefenoxam from squash and pepper fields in North Carolina. *Plant Disease* **92**: 1439-1443.

Carmo M.G.F., Macagnan D., Carvalho A.O., 2001. Progresso da mancha-bacteriana do pimentão a partir de diferentes níveis iniciais de inóculo e do emprego ou não do controle com oxicloreto de cobre. *Horticultura Brasileira* **19**: 342-347.

Cazorla F.M., Arrebola E., Sesma A., Pérez-García A., Codina J.C., Murillo J., de Vicente A., 2002. Copper resistance in *Pseudomonas syringae* strains isolated from mango is encoded mainly by plasmids. *Phytopathology* **92**: 909-916.

Corio-Costet M.F., Dufour M.C., Cigna J., Abadie P., Chen W.J., 2011. Diversity and fitness of *Plasmopara viticola* isolates resistant to QoI fungicides. *European Journal of Plant Pathology* **129**: 315-329.

Hamza A.A., Robène-Soustrade I., Jouen E., Gagnevin L., Lefeuvre P., Chiroleu F., Pruvost O., 2010. Genetic and pathological diversity among *Xanthomonas* strains responsible for bacterial spot on tomato and pepper in the Southwest Indian Ocean Region. *Plant Disease* **94**: 993-999.

Hert A.P., Roberts P.D., Momol M.T., Minsavage G.V., Tudor-Nelson S.M., Jones J.B., 2005. Relative importance of bacteriocin-like genes in antagonism of *Xanthomonas perforans* tomato race 3 to *X. euvesicatoria* tomato race 1 strains. *Applied and Environmental Microbiology* **71**: 3581-3588.

Hert A.P., Marutani M., Momol M.T., Roberts P.D., Olson S.M., Jones J.B., 2009. Suppression of the bacterial spot pathogen *Xanthomonas euvesicatoria* on tomato leaves by an attenuated mutant of *Xanthomonas perforans*. *Applied and Environmental Microbiology* **75**: 3323-3330.

Hu J.H., Hong C.X., Stromberg E.L., Moorman G.W., 2008. Mefenoxam sensitivity and fitness analysis of *Phytophthora nicotianae* isolates from nurseries in Virginia, USA. *Plant Pathology* **57**: 728-736.

Hwang M.S.H., Morgan R.L., Sarkar S.F., Wang P.W., Guttman D.S., 2005. Phylogenetic characterization of virulence and resistance phenotypes of *Pseudomonas syringae*. *Applied and Environmental Microbiology* **71**: 5182-5191.

Jones J.B., Stall R.E., Scott J.W., Somodi G.C., Bouzar H., Hodge N.C., 1995. A third tomato race of *Xanthomonas campestris* pv. *vesicatoria*. *Plant Disease* **79**: 395-398.

Jones J.B., Lacy G.H., Bouzar H., Stall R.E., Schaad N.W., 2004. Reclassification of the xanthomonads associated with bacterial spot disease of tomato and pepper. *Systematic and Applied Microbiology* **27**: 755-762.

Kadish D., Grinberger M., Cohen Y., 1990. Fitness of metalaxyl-sensitive and metalaxyl-resistant isolates of *Phytophthora infestans* on susceptible and resistant potato cultivars. *Phytopathology* **80**: 200-205.

Kang Y.S., Park W., 2010. Trade-off between antibiotic resistance and biological fitness in *Acinetobacter* sp. strain DR1. *Environmental Microbiology* **12**: 1304-1318.

Koenraadt H., van Betteray B., Germain R., Hiddink G., Jones J.B., Oosterhof J., Rijlaarsdam A., Rooda P., Wouldt B., 2009. Development of specific primers for the molecular detection of bacterial spot of pepper and tomato. *Acta Horticulturae* **808**: 99-102.

Lilley A.K., Bailey M.J., 1997. Impact of plasmid pQBR103 acquisition and carriage on the phytosphere fitness of *Pseudomonas fluorescens* SBW25: burden and benefit. *Applied and Environmental Microbiology* **63**: 1584-1587.

Louws F.J., Fulbright D.W., Stephens C.T., de Bruijn F.J., 1995. Differentiation of genomic structure by rep-PCR fingerprinting to rapidly classify *Xanthomonas campestris* pv. *vesicatoria*. *Phytopathology* **85**: 528-536.

Luciani F., Sisson S.A., Jiang H., Francis A.R., Tanaka M.M., 2009. The epidemiological fitness cost of drug resistance in *Mycobacterium tuberculosis*. *Proceedings of the National Academy of Sciences USA* **106**: 14711-14715.

Marco G.M., Stall R.E., 1983. Control of bacterial spot of pepper initiated by strains of *Xanthomonas campestris* pv. *vesicatoria* that differ in sensitivity to copper. *Plant Disease* **67**: 779-781.

Marques E., Uesugi C.H., Ferreira M.A.S.V., 2009. Sensitivity to copper in *Xanthomonas campestris* pv. *viticola*. *Tropical Plant Pathology* **34**: 406-411.

Obradovic A., Mavridis A., Rudolph K., Janse J.D., Arsenijevic M., Jones J.B., Minsavage G.V., Wang J.F., 2004. Characterization and PCR-based typing of *Xanthomonas campestris* pv. *vesicatoria* from peppers and tomatoes in Serbia. *European Journal of Plant Pathology* **110**: 285-292.

Parkinson N., Cowie C., Heeney J., Stead D., 2009. Phylogenetic structure of *Xanthomonas* determined by comparison of *gyrB* sequences. *International Journal of Systematic and Evolutionary Microbiology* **59**: 264-274.

Pereira R.C., Araújo E.R., Quezado-Duval A.M., Ferreira M.A.S.V., 2011. Occurrence of *Xanthomonas* species causing bacterial spot in fresh market tomato. *Acta Horticulturae* **914**: 61-64.

Pernezny K., Nagata R., Havranek N., Sanchez J., 2008. Comparison of two culture media for determination of the copper resistance of *Xanthomonas* strains and their usefulness for prediction of control with copper bactericides. *Crop Protection* **27**: 256-262.

Pohronezny K., Sommerfeld M., Raid R.N., 1994. Streptomycin resistance and copper tolerance among strain of *Pseudomonas cichorii* in commercial celery seedbeds. *Plant Disease* **84**: 150-153.

Quezado-Duval A.M., Gazzoto Filho A., Leite Júnior R.P., Camargo L.E.A., 2003. Sensibilidade a cobre, estreptomicina e oxitetraciclina em *Xanthomonas* spp. associadas à mancha-bacteriana do tomate para processamento industrial. *Horticultura Brasileira* **21**: 672-677.

Quezado-Duval A.M., Lopes C.A., Leite Júnior R.P., Lima M.F., Camargo L.E.A., 2005. Diversity of *Xanthomonas* spp. associated with bacterial spot of processing tomatoes in Brazil. *Acta Horticulturae* **695**: 101-108.

Rensing C., Grass G., 2003. *Escherichia coli* mechanisms of copper homeostasis in a changing environment. *Microbiology Reviews* **27**: 197-213.

Ritchie D.F., Dittapongpitch V., 1991. Copper-and streptomycin-resistant strains and host differentiated races of *Xanthomonas campestris* pv. *vesicatoria*. *Plant Disease* **75**: 733-736.

Sanoamuang N., Gaunt R.E., 1995. Persistence and fitness of carbendazim- and dicarboximide-resistant isolates of *Monilinia fructicola* (Wint.) Honey in flowers, shoots and fruits of stone fruit. *Plant Pathology* **44**: 448-457.

SAS Institute, 2002. SAS System Version 9 for Microsoft Windows, Cary, NC, USA.

Schaad N.W., Jones J.B., Chun W., 2001. Laboratory Guide for Identification of Plant Pathogenic Bacteria. APS Press, St. Paul, MN, USA.

Shenge K.C., Mabagala R.B., Mortensen C.N., 2008. Coexistence between neighbours: *Pseudomonas syringae* pv. *tomato* and *Xanthomonas campestris* pv. *vesicatoria*, incitants of bacterial speck and spot diseases of tomato. *Archives of Phytopathology and Plant Protection* **41**: 559-571.

Stall R.E., Thayer P.L., 1962. Streptomycin resistance of the bacterial spot pathogen and control with streptomycin. *Plant Disease Reporter* **46**: 389-392.

Sundin G.W., Bender C.L., 1993. Ecological and genetic analysis of copper and streptomycin resistance in *Pseudomonas syringae* pv. *syringae*. *Applied and Environmental Microbiology* **59**: 1018-1024.

Teixeira E.C., Oliveira J.C.F., Novo M.T.M., Bertolini M.C., 2008. The copper resistance operon *copAB* from *Xanthomonas axonopodis* pathovar *citri*: gene inactivation results in copper sensitivity. *Microbiology* **154**: 402-412.

Vale F.X.R., Fernandes Filho E.I., Liberato J.R., Zambolim L., 2001. Quant – a software to quantify plant disease severity. *VIII International workshop on Plant Disease Epidemiology, Ouro Preto, Brazil*: 160.

Voloudakis A.E., Bender C.L., Cooksey D.A., 1993. Similarity between copper resistance genes from *Xanthomonas campestris* and *Pseudomonas syringae*. *Applied and Environmental Microbiology* **59**: 1627-1634.

Webber J.F., 1988. Effect of MBC fungicide tolerance on the fitness of *Ophiostoma ulmi*. *Plant Pathology* **37**: 217-224.

Wolf M.S., 1981. Integrated use of fungicides and host resistance for stable disease control. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* **295**: 175-184.

Zevenhuizen L.P.T.M., Dolfing J., Eshuis E.J., Scholtern-Koerselman J., 1979. Inhibitory effects of cooper on bacteria related to the free ion concentration. *Microbial Ecology* **5**: 139-146.

Received August 11, 2011
Accepted September 27, 2011