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SHORT COMMUNICATION / *COMUNICAÇÃO*

Sensitivity to copper in *Xanthomonas campestris* pv. *viticola*

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ABSTRACT

Bacterial canker, caused by *Xanthomonas campestris* pv. *viticola*, affects grapevines in the irrigated areas of the São Francisco river valley, in the states of Pernambuco and Bahia. Several practices for disease management have been adopted including copper sprays. This is the only available chemical control method and most frequently used in the areas affected by the disease. The objective of this work was to determine the sensitivity to copper of strains of *X. campestris* pv. *viticola* collected in different locations and over a period of years, from 1998 to 2006. Variation in sensitivity to copper oxychloride and copper sulfate was observed among the 21 strains tested. The minimum inhibitory concentration (MIC) varied from 10 to 60 µg/mL Cu^{2+}, for both compounds. A general increase in copper tolerance over the years was also observed, with the Brazilian strains being more tolerant than the type-strain, collected in 1972 in India. The differences observed in copper sensitivity may lead to the selection and dominance of the more tolerant strains in the bacterial population as copper compounds continue to be used in the region.
Keywords: *Vitis vinifera,* copper tolerance, grapevine bacterial canker.

RESUMO

Sensibilidade ao cobre em *Xanthomonas campestris* pv. *viticola*

O cancro bacteriano, causado por *Xanthomonas campestris* pv. *viticola,* afeta o cultivo irrigado de videira no Vale do Submédio São Francisco, nos estados de Pernambuco e Bahia. O manejo da doença tem sido realizado através de um conjunto de práticas que inclui a aplicação de cúpricos. Este tem sido o único método de controle químico disponível e o mais utilizado nas áreas de ocorrência da doença. O objetivo deste estudo foi avaliar a sensibilidade ao cobre de estirpes de *X. campestris* pv. *viticola* coletadas em diferentes localidades, entre os anos de 1998 a 2006. Detectou-se variabilidade na sensibilidade ao oxicloreto de cobre e ao sulfato de cobre entre as 21 estirpes testadas. A concentração mínima inibitória variou entre 10 e 60 µg/mL Cu^{2+}, para os dois produtos. De forma geral, observou-se uma evolução no crescimento da tolerância ao cobre ao longo dos anos, com as estirpes brasileiras apresentando maior tolerância que a estirpe-tipo, coletada em 1972 na Índia. As diferenças observadas em sensibilidade ao cobre entre as estirpes de *X. campestris* pv. *viticola* podem levar, com o uso contínuo de compostos cúpricos na região, à seleção e dominância das estirpes mais tolerantes na população bacteriana.
Palavras-chave: *Vitis vinifera,* tolerância ao cobre, cancro bacteriano da videira.

In Brazil, grape (*Vitis vinifera* L.) production has significantly increased in the irrigated areas of the São Francisco river valley, in the states of Pernambuco and Bahia. This region is responsible for 32% of the total table grape production in the country, corresponding to approximately 98% of Brazilian annual grape export (Valexport, 2005). One of the diseases that affect grapevines in that region is bacterial canker, caused by *Xanthomonas campestris* pv. *viticola* (Nayudu) Dye. Disease management relies on several cultural practices aiming at reducing inoculum and pathogen dissemination, use of more resistant varieties and protective chemical control with copper sprays, especially after pruning (Malavolta Jr. et al*.,* 1999; MAPA, 2006). Although there are no registered products for bacterial canker control in Brazil, the spraying of copper compounds is recommended as a protection method to minimize disease damage and spread (Malavolta Jr. et al., 1999; Lima & Moreira, 2002).

Copper compounds have been widely used for management of bacterial diseases of vegetable and fruit crops. These fungicides are capable of inhibiting or delaying bacterial multiplication (Marco & Stall, 1983; Romeiro, 1995). However, the efficacy of copper sprays in control of plant bacterial diseases has been variable and is often associated with the occurrence of copper tolerant strains (Marco & Stall, 1983; Cooksey, 1990). Resistance to copper was not detected in plant pathogenic bacteria until the 1980s, probably because the presence of copper-resistant strains in the field did not always lead to failure of control with copper sprays (Cooksey, 1990). Since then, several studies have focused on characterizing strain sensitivity and the genetic mechanisms of copper resistance in plant-

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pathogenic bacteria (Cooksey, 1990; Silver & Phung, 1996; Voloudakis et al., 2005; Teixeira et al., 2008).

Copper resistance has been detected and characterized in *Xanthomonas* and *Pseudomonas*. Resistance is widespread in *X. campestris* pv. *vesicatoria* (Doidge 1920) Dye, causal agent of bacterial spot of pepper and tomato, in several geographical regions, but it was first reported in Florida (Marco & Stall,1983), where copper was not effective for disease control. In Brazil, studies have also shown low efficacy of copper compounds to control bacterial spot of sweet pepper (Carmo et al., 2001; Aguiar et al., 2003). Copper-resistant strains have also been described in *Pseudomonas syringae* pv. *tomato* (Okabe 1933) Young et al. causing tomato bacterial speck, since copper hydroxide sprays did not reduce disease severity (Cooksey, 1990).

In India, several copper based-compounds and antibiotics were screened for grapevine bacterial canker control, but none of these compounds showed a significant reduction in disease severity (Chand et al., 1991). During a four-year trial conducted in a nursery, grape plants with variable levels of infection were monitored with applications of several copper compounds and antibiotics. At the end of the experiment, the pathogen had acquired resistance to both copper and antibiotics. Tolerant strains were viable after exposure to 600-1800 µg/mL Cu^{2+} (Chand et al., 1994).

In Brazil, several attempts have been made to determine effectiveness of bactericides for bacterial canker control (Nascimento & Silva, 1999; Lima & Mashima, 2000). A mixture of gentamycin sulfate and copper oxychloride was the most effective for inhibiting pathogen growth when compared to other compounds; however, the use of copper sulfate or copper solution to treat propagative plant material with low levels of infection was ineffective (Silva et al., 2000). Tolerance to copper sulfate in five Brazilian strains of *X. campestris* pv. *viticola* varied from 50 to 300 µg/mL Cu^{2+}. The most sensitive strain was unable to grow after 1-hour exposure to concentrations higher than 50 µg/mL Cu^{2+} (Araújo 2001; Araújo et al., 2003).

Considering the possibility of reduced sensitivity to copper compounds leading to the prevalence of tolerant strains in the Brazilian populations of this pathogen, the objective of this work was to determine sensitivity to copper of *X. campestris* pv. *viticola* strains collected in different locations and over a period of years, from 1998 to 2006. This information could contribute to better assess the effectiveness of the control practices based on copper spraying currently adopted in the producing areas.

A total of 21 strains were selected for *in vitro* studies of copper sensitivity (Table 1). Eighteen strains were obtained from diseased plants collected in Pernambuco

TABLE 1 - Origin and sensitivity to copper of *Xanthomonas campestris* pv.*viticola* strains used in this study

Strain	Host cultivar	Location and year	$CuSO_4$	$Cu_2(Cl(OH)_3)$
NCPPB 2475	Anab-e-Shahi	India, 1972	10[a]	10[a]
IBSBF 1369	Red Globe	Petrolina – PE, 1998	40	60
IBSBF 1385	Itália	Teresina – PI, 1998	40	30
UnB 1183	Red Globe	Area 1, Petrolina – PE, 1998	40	20
UnB 1190	Red Globe	Area 2, Petrolina – PE, 1998	40	30
UnB 1204	Red Globe	Area 3, Juazeiro – BA, 1999	30	30
UnB 1205	Itália	Area 4, Sobradinho– BA, 2000	20	30
UnB 1216	Red Globe	Area 5, Petrolina – PE, 2000	30	40
UnB 1212	Itália	Area 2, Petrolina – PE, 2001	40	30
UnB 1222	Perlette	Area 5, Petrolina – PE, 2001	30	30
UnB 1292	Red Globe	Area 6, Juazeiro – BA, 2003	50	50
UnB 1293	Superior x IAC 766	Area 1, Petrolina – PE, 2003	20	30
UnB 1294	Thompson x Paulsen	Area 7, Petrolina - PE, 2003	40	30
UnB 1295	Festival	Area 5, Petrolina – PE, 2004	30	20
UnB 1298	Itália	Area 5, Petrolina – PE, 2004	50	30
UnB 1299	Thompson	Area 8, Petrolina – PE, 2004	60	40
UnB 1301	Thompson	Petrolina – PE, 2004	50	40
UnB 1310	Festival	Area 9, Petrolina – PE, 2005	40	50
UnB 1314	Red Globe	Area 7, Petrolina – PE, 2005	40	60
UnB 1316	Red Globe	Area 10, Juazeiro– BA, 2005	40	60
UnB 1318	BRS – Morena	Petrolina – PE, 2006	50	40

[a]Minimum inhibitory concentration (µg/mL Cu^{2+}) on MMCC (medium minimal complexing copper)

and Bahia and were identified through biochemical and molecular tests according to Lima et al. (1999) and Trindade et al. (2007), respectively. Two strains were obtained from Instituto Biológico (IBSBF 1385 and 1369) and the type strain (NCPPB 2475) from India was obtained from the National Collection of Plant Pathogenic Bacteria, Central Science Laboratory (Sand Hutton, York, UK). The strains were selected to represent different locations and years of isolation, from 1998 to 2006.

Two copper sensitivity assays were performed. In the first one the MMCC (medium minimal complexing copper) medium was used due to its low ability to complex copper ions (Pohronezny et al., 1992). As sources of copper ions, solutions of copper sulfate ($CuSO_4$) and copper oxychloride ($Cu_2Cl(OH)_3$) were used. The stock solutions were prepared at a concentration of 2×10^4 µg/mL and aseptically added to the medium for final concentrations of: 0, 10, 20, 30, 40, 50 and 60 µg/mL Cu^{2+}. Bacterial cultures were recovered from stock cultures maintained in 30% glycerol at -80 ºC and transferred to 523 medium (Kado & Heskett, 1970). After 72 h, bacterial cell suspensions were prepared in sterile distilled water and adjusted to 2.5×10^8 cfu/mL, using a digital spectrophotometer UV-1203 (Shimazu Corporation) at 550 nm wave length and 0.575 absorbance. The suspensions were diluted to 10^{-5} (1:100,000), and 50 µL were streaked on the copper–containing medium (MMCC) at the desired concentrations. The plates were kept at 28 ºC and the experiment was performed with three replicates for each strain/concentration combination. Plates without added copper were included as controls. After 72 h, the number of colonies on each plate was counted and expressed as cfu/mL considering the average of three replicates.

To determine levels of copper sensitivity, a second experiment was conducted according to the method described by Marco & Stall (1983), which was previously used to evaluate copper tolerance of five strains of *X. campestris* pv. *viticola* (Araújo, 2001). Copper sulfate was used to prepare copper solutions at the following final concentrations: 0, 50, 100, 150, 200, 250, 300, 350 and 400 µg/mL Cu^{2+}. The bacterial suspensions were prepared as described above; 50 µL of each suspension was added to 1 mL of copper solutions at each different concentration. After 1 h exposure, 100 µL-aliquots were transferred to nutrient-agar medium (NA), with no added copper. Strain sensitivity was evaluated after 72 h of incubation at 28ºC, based on the presence or absence of bacterial growth.

Sensitivity of *X. campestris* pv. *viticola* strains to copper was determined through colony counts on MMCC amended with increasing concentrations of copper. For all treatments with both products (copper sulfate and oxychloride), there was a decrease in the number of bacterial colonies with the increase in copper concentration, compared to the control. The MIC value was defined as the lowest concentration in which bacterial growth was totally inhibited. The results showed variation among *X. campestris* pv. *viticola* strains in their response to copper (Table 1) and a general tendency of increasing tolerance over the years, from 1998 to 2006 (Figure 1).

The MIC value in the treatments with copper sulfate varied from 10 to 60 µg/mL (Table 1) and the type strain NCPPB 2475, from India, was the only highly sensitive strain, with no growth at the lowest concentration tested. Brazilian strains were inhibited at concentrations between 20 and 60 µg/mL Cu^{2+}. One strain, UnB 1299, had the highest MIC value, and was able to grow at 50 µg/mL Cu^{2+}, but completely inhibited at 60 µg/mL Cu^{2+}. The MIC value in the treatments with copper oxychloride also varied from 10 to 60 µg/mL (Table 1) and the type strain NCPPB

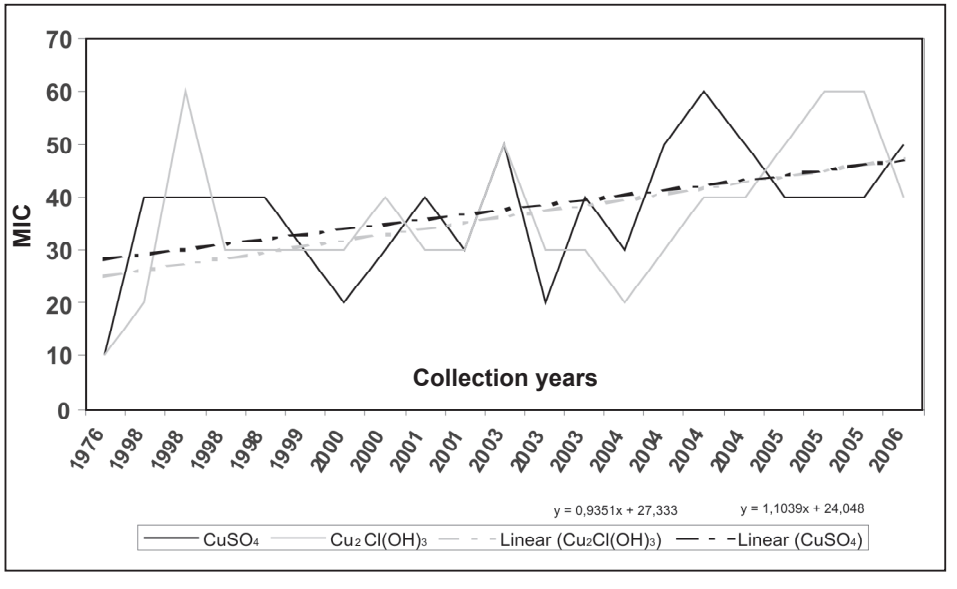

FIGURE 1 - Evolution of copper tolerance in *Xanthomonas campestris* pv. *viticola* strains, collected from 1998 to 2006, expressed as the minimal inhibitory concentration (MIC) of copper (µg/mL) on MMCC (medium minimal complexing copper).

2475 was, again, the only highly sensitive strain, with no growth at the lowest copper concentration tested (10 µg/mL). Almost half of the strains were inhibited at 30 µg/mL, but three strains IBSBF 1369, UnB 1314 and 1316 showed MIC values of 60 µg/mL.

Regarding their geographical distribution (Table 1) strains collected in the same area differed in sensitivity to copper in some cases. In Pernambuco, area 2, tolerance was uniform among the two strains collected in 1998 and 2001; in areas 1, 5 and 7, however, tolerance varied among collection years and between the two copper compounds tested. Generally, higher MIC values were observed in strains collected after 2003. For instance, strains with MIC of 50 µg/mL of copper sulfate or higher were collected in 2003, 2004 and 2006 (Table 1).

Seven strains, representing a range in copper sensitivity, were tested using the method described by Marco & Stall (1983) which employs nutrient-agar medium and the bacterial suspension exposed directly to copper solutions. Copper tolerance varied from 0 to 350 µg/mL (Table 2). Sensitivity of the type strain NCPPB 2475 was confirmed, but strains IBSBF 1385, UnB 1298 and UnB 1301 were also sensitive, being completely inhibited in the lowest concentration tested (50 µg/mL). Strain UnB 1292 showed tolerance up to 200 µg/mL, UnB 1299 up to 250 µg/mL, and the most tolerant strain, UnB 1318, collected in 2006, was able to grow after being exposed to 350 µg/mL Cu^{2+}.

The results of the present study showed that Brazilian strains of *X. campestris* pv. *viticola* differ in their sensitivity to copper sulfate and copper oxychloride, and that more tolerant strains occur naturally in vineyards in Pernambuco and Bahia states. Araújo (2001) examined five strains from Petrolina, PE, but in the present study, a larger number of strains from Pernambuco were included, along with strains collected in Bahia, Piauí and the type strain, from India. The occurrence of more tolerant strains of *X. campestris* pv. *viticola* may be explained by their introduction associated with propagative material from India, which occurred probably before 1998 when the disease was first detected in Brazil. In 1994, Chand et al. had already detected tolerant strains in India. The continuous use of copper compounds as one of the measures adopted to prevent disease dissemination may have led to the selection of these more tolerant strains (Araújo, 2001).

The variability in tolerance reported here among and within states and areas suggests that tolerant strains already established in the vineyards were then disseminated through propagative plant material to different areas, contributing to the dissemination and maintenance of these populations with the continuous use of copper compounds. Another factor that could be also associated with this process is horizontal gene transfer through conjugation (Cooksey, 1990). It is known that copper-resistant genes are plasmid-borne but can also be present in the bacterial chromosome (Basim et al., 2005).

The exclusive use of copper compounds may not be effective as a control measure for *X. campestris* pv. *viticola* (Araújo, 2001). The gradual increase in tolerance over the years reported here has also been observed in other important plant pathogenic bacteria. Decreased copper-sensitivity of strains of *X. campestris* pv. *vesicatoria* has been reported in Florida, USA, for many years (Marco & Stall, 1983) and also in Brazil (Carmo et al., 2001; Aguiar et al., 2003; Quezado-Duval et al., 2003). The same has been reported for *P. syringae* pv. *tomato* in California, USA (Cooksey, 1990) and in Brazil (Silva & Lopes, 1995). In several cases, cupric and cuprorganic compounds were proven ineffective to control these pathogens, when applied alone. Higher efficiency has been reported when copper is combined with a copper-chelating carbamate fungicide, such as mancozeb. Moreover, in many cases, a reduction in the efficacy to control bacterial diseases has led to an increase in the dosage and frequency of copper applications (Carzola et al., 2002).

In vitro tests are usually performed to determine tolerance to bactericides; however, the results are greatly

TABLE 2 - Copper tolerance of *Xanthomonas campestris* pv. *viticola* strains, defined as the presence of bacterial colonies on NA medium after exposure of bacterial cells to copper solutions in various concentrations

Strain	Collection year	Origin	Tolerance (µg Cu^{2+}/mL)[a,b]
NCPPB 2475	1972	India	0
IBSBF 1385	1998	Teresina – PI	0
UnB 1292	2003	Juazeiro – BA	200
UnB 1298	2004	Petrolina – PE	0
UnB 1299	2004	Petrolina – PE	250
UnB 1301	2004	Petrolina – PE	0
UnB 1318	2006	Petrolina – PE	350

[a]Copper sulfate
[b]Maximum concentration that supported bacterial growth

dependent on the method and the culture medium used. Copper ions bind to many organic substrates in media and on plant surfaces (Cooksey, 1990); thus, several media are known to chelate copper ions, and make them unavailable to inhibit bacterial growth. Tolerance in *P. syringae* pv. *tomato* strains from processing tomato was assessed using four different culture media: King's B, NA, 523 and MMCC. Differences in resistance ranged from 250 to 1800 ppm and depended greatly on the culture medium used (Silva & Lopes 1995). The same was observed by Rezende (2006), when comparing growth of *Erwinia psidii* on 523 and MMCC amended with copper.

In the present study, the levels of tolerance, expressed as MIC values, for *X. campestris* pv. *viticola* ranged from 10 to 60 µg/mL Cu^{2+}, when copper ions were added to the medium. Using the same method, *E. psidii* strains showed growth of up to 30 µg/mL Cu^{2+} for $CuSO_4$ and up to 50 µg/mL for copper oxychloride. The second method used for *X. campestris* pv. *viticola* employs free copper solutions in direct contact with bacterial cells. This may activate resistance genes present in some strains, and through different mechanisms of reduced uptake or detoxification, allow their growth on higher concentrations (200-350 µg/mL) of the metal. The structure of the copper-tolerant populations of *X. campestris* pv. *viticola* could change over time, so a more extensive sampling of strains should be considered in future studies. It should also be of interest to investigate the genetic mechanisms of tolerance in this pathogen.

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