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Chemical control of bacteria *Xanthomonas hortorum* pv. *gardneri* and *Xanthomonas euvesicatoria* pv. *perforans in vitro*

Monteiro FP, Ogoshi C and Mallmann G

Researchers at the EPAGRI – Agricultural Research and Rural Extension Enterprise of Santa Catarina, Abílio Franco, 1500, Bom Sucesso, PO Box 591, Zip code 89.501-032, Caçador, Santa Catarina, Brazil

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Abstract

Tomato bacterial leaf spot is a worldwide disease that causes high losses in processed and fresh tomatoes. This disease is caused by four species of bacteria (*Xanthomonas hortorum* pv. *gardneri*, *Xanthomonas euvesicatoria* pv. *perforans*, *Xanthomonas euvesicatoria* pv. *euvesicatoria* and *Xanthomonas euvesicatoria* pv. *vesicatoria*). *Xanthomonas hortorum* pv. *gardneri* and *Xanthomonas euvesicatoria* pv. *perforans* are the phytopathogenic bacteria most frequently found in tomato crops. The objectives of this work were to identify potential bactericides and to study the mancozeb and copper oxychloride in the management of bacterial spot caused by *Xanthomonas hortorum* pv. *gardneri* and *Xanthomonas euvesicatoria* pv. *perforans*. Forty-four active ingredients were tested at 1% of the commercial product (1000g c.p./100L H_2O) as first screening. Posteriorly, those that inhibited bacterial multiplication were tested at their recommended doses. When used at a dose of 1000g c.p./100L H_2O, the products that inhibited the multiplication of *X. hortorum* pv. *gardneri* and *X. euvesicatoria* pv. *perforans* were benzalkonium chloride, acetic acid, cuprous oxide, mancozeb, copper hydroxide, mancozeb + famoxadone, copper oxychloride, metiram + pyraclostrobin, Bordeaux mixture and Viçosa mixture. At the doses recommended in the package insert, the products that inhibited the multiplication of *X. hortorum* pv. *gardneri* and *X. euvesicatoria* pv. *perforans* were benzalkonium chloride, acetic acid, mancozeb, mancozeb + famoxadone and metiram + pyraclostrobin. Copper oxychloride at the recommended dose of 0.2% (200g c.p./100L H_2O – 168g a.i./100L H_2O) did not inhibit the multiplication of *X. hortorum* pv. *gardneri*. It has been estimated that 0.004% is resistant to the recommended dose of copper oxychloride. At doses higher than 0.4% (400g c.p./100L H_2O – 336g a.i./100L H_2O) there was no bacterial growth. However, once the bacteria grow in a culture medium containing 0.2% copper oxychloride of commercial product (200g c.p./100L H_2O – 168g a.i./100L H_2O), it can be multiplied in a culture media even at a dose of 1000g c.p./100L H_2O (840g a.i./100L H_2O), which would be equivalent to a dose of 10kg c.p./ha. Mancozeb inhibited bacterial growth from the dose of 0.1% (100g c.p./100L H_2O – 80g a.i./100L H_2O). However, when *X. hortorum* pv. *gardneri* resistant to copper oxychloride 1% (1000g c.p./100L H_2O – 840g a.i./100L H_2O) is striated in a culture medium containing mancozeb, the bacteria multiply up to a dose of 0.5% mancozeb (500g c.p./100L H_2O – 400g a.i./100L H_2O). The *in vitro* results indicate that doses equal to or less than 0.2% copper oxychloride (200g c.p./100L H_2O – 168g a.i./100L H_2O) select colonies resistant to high doses of copper oxychloride and also decrease the efficiency of mancozeb.

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Corresponding Author: Fernando P. Monteiro – e-mail – fernandomonteiro@epagri.sc.gov.br

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Introduction

The top nine tomato producers in 2020 in descending order were China (64,865,807t), India (20,573,000t), Türkiye (13,204,015t), United States of America (12,227,402t), Egypt (6,731,220t), Italy (6,247,910t), Iran (5,787,094t), Spain (4,137,342t) and Mexico (4,137,342t) (FAOSTAT 2022). Brazil occupied the tenth position in the ranking of the biggest producers in 2020 with 3,753,595 tonnes of tomato fruits. Santa Catarina state occupied the seventh position in Brazil. The top five state producers are São Paulo (26.2%), Goias (26.1%), Minas Gerais (14.2%), Paraná (5.7%) and Bahia (5.4%) (IBGE 2021). The tomato bacterial spot may affect production potential by causing losses estimated as higher as 52% (Quezado-Soares et al. 1998).

The tomato bacterial spot has four etiological agents worldwide, belonging to the genera *Xanthomonas*: *X. hortorum* pv. *gardneri*, *X. euvesicatoria* pv. *perforans*, *X. euvesicatoria* pv. *euvesicatoria* and *X. euvesicatoria* pv. *vesicatoria* (Jones et al. 2004). In Brazil, despite the time that has elapsed since the Jones et al. (2004) published their study about the reclassification of the xanthomonds associated with bacterial spot disease of tomato, the product package insert still uses old names such *Xanthomonas vesicatoria* or *Xanthomonas campestris* pv. *vesicatoria*. Furthermore, the existence of the complex of bacteria that cause the disease is disregarded. In fact, the concomitant occurrence of more than one species in the same field was reported (Costa et al. 2012). In the region of Alto Vale do Rio do Peixe (Santa Catarina, Brazil), from a total of 44 strains, 80%, 11% and 9% were identified as *X. hortorum* pv. *gardneri*, *X. euvesicatoria* pv. *perforans* and *X. euvesicatoria* pv. *vesicatoria*, respectively (Costa et al. 2012). The prevalence of *X. hortorum* pv. *gardneri* in fresh market tomato crop of some South region of Brazil was also demonstrated by Pereira et al. (2011).

Tomato cultivars resistant to bacterial spot do not exist, then the control is mainly carried out using registered cupric bactericides for tomato crops. Tomato growers have recurrent complaints about the inefficiency of these products when using the recommended doses since 1960s (Marco & Stall 1983). The emergence of resistant strains to active ingredients may explain the inefficiency observed in the field (Quezado-Duval et al. 2003). Later was found that the addition of ethylene-bis-dithiocarbamates to Cu bactericides provided better disease control and improved Cu solubility (Marco & Stall 1983, Conover & Gerhold 1981). However, Cu/ethylene-bis-dithiocarbamates become ineffective to control the bacterial spot of tomato when the environmental conditions are optimal and copper-tolerant strains are present (Jones & Jones 1985, Obradovic et al. 2004a).

This work aimed to identify potential bactericides and to study the relationship between mancozeb and copper oxychloride in the management of bacterial spot caused by *Xanthomonas hortorum* pv. *gardneri* and *Xanthomonas euvesicatoria* pv. *perforans*.

Materials & Methods

Identification of the bacterial strains

The bacteria *Xanthomonas hortorum* pv. *gardneri* (ON890384 strain EPAGRI-Xhg6), *Xanthomonas euvesicatoria* pv. *perforans* (ON890387 strain EPAGRI-Xep20), *Bacillus velezensis* (ON890388 strain EPAGRI-Bvseptoria1) and *Pseudomonas rhizosphaerae* (ON890389 strain EPAGRI-Pr1) were identified by sequencing the 16S ribosomal RNA gene. In addition, Koenraadt et al. (2007) proposed primers were used to confirm the identity of *X. hortorum* pv. *gardneri* and *X. euvesicatoria* pv. *perforans*. PCR was performed in thermocycler TC-9639 (Loccus) with the following program: 94ºC for 1 min followed by 30 cycles of 94ºC for 1 min, 55ºC for 1 min, 72ºC for 2 min, and a final extension at 72ºC for 10 min. PCR products were analyzed by agarose gel (0.7%) electrophoresis in 0.5x TBE buffer conducted at 90V for 1h and scanned using the imaging system L-PIX EX (Loccus).

Effect of fungicides/bactericides at 1% of commercial product as first screening of potential efficient control

To know the potential effect of fungicides/bactericides registered for tomato plants 44 products were tested at 1% of commercial product (1000g c.p./100L H_2O). This was the first screening of potential promising control agent and represent doses much higher than recommended in the package insert. For this reason, this dose should not be used in field applications. Petri dishes poured with YDC-medium base 31 (glucose 20 g/L, yeast extract 10 g/L, $CaCO_3$ 20 g/L and agar 20 g/L) plus fungicides/bactericides at 1% of commercial product was used to support the bacterial growth of *X. hortorum* pv. *gardneri* EPAGRI-Xhg6 and *X. euvesicatoria* pv. *perforans* EPAGRI-Xep20. The following fungicides/bactericides were used in this study: benzalkonium chloride 10% (the percentage means the amount of active ingredient present in the commercial product used), acetic acid (with nickel 0.05% and molybdenum 0.05%), cuprous oxide 56% (metallic copper 50%), mancozeb 80%, copper hydroxide 69.1% (metallic copper 45%), mancozeb 62.5% + famoxadone 6,25%, copper oxychloride 84% (metallic copper 50%), metiram 55% + pyraclostrobin 5%, propineb 70%, metiram 70%, mancozeb 44% + copper oxychloride 30%, mancozeb 64% + metalaxyl-M 4%, cymoxanil 30% + famoxadone 22.5%, mancozeb 64% + cymoxanil 8%, fluazinam 50%, tebuconazole 21.3%, chlorothalonil 75%, methyl-thiophanate 70%, metconazole 9%, difenoconazole 25%, azoxystrobin 50%, pyraclostrobin 25%, isopropyl bentiavalicarb 3.75% + chlorothalonil 37.5%, pyrimethanil 30%, iprodione 50%, casugamicin 2%, procymidone 50%, mandipropamide 25%, propamocarb hydrochloride 72.20%, propamocarb hydrochloride 62.50% + fluopicolide 6.25%, metalaxyl-M 4% + chlorothalonil 40%, captan 48%, dimethomorph 50%, trifloxystrobin 10% + tebuconazole 20%, boscalid 50%, azoxystrobin 20% + difenoconazole 12.5%, isopropyl bentiavalicarb 10% + fluazinam 25%, fluxapyroxad 16.7% + pyraclostrobin 33.3%, methyl-thiophanate 37.50% + fluazinam 37.50%, *Melaleuca alternifolia* extract 22.25%, tetraconazole 10%, lime sulfur (S 50%, Ca 5%), Bordeaux mixture (Cu 20%, S 10% and Ca 3%) and Viçosa mixture (K_2O 8%, B 3.5%, Mg 0.8%, Cu 9%, S 8% and Zn 3%). The analysis was qualitative using the positive signal (+) for the treatment that allows the bacterial growth and the negative signal (-) for those that inhibit the bacterial growth.

Effect of fungicides/bactericides in their recommended doses according to the package insert

After the screening of 44 potential products, 17 were selected to be tested in their recommend doses. The experiment was performed as described above. The dose of active ingredient (g/mL a.i./100L H_2O) of each commercial product was described at the Table 2.

Effect of copper oxychloride on the *X. hortorum* pv. *gardneri*, *X. euvesicatoria* pv. *perforans*, *Bacillus velezensis* and *Pseudomonas rhizosphaerae*

The copper oxychloride was tested in different concentration (0.02%, 0.05%, 0.10%, 0.20%, 0.39%, 0.78%, 1.56%, 3.13%, 6.25%, 12.50% and 25% of commercial product containing 80% copper oxychloride) added to YDC medium on Petri plates, where *X. hortorum* pv. *gardneri* strain EPAGRI-Xhg6, *B. velezensis* strain EPAGRI-Bvseptoria1, *X. euvesicatoria* pv. *perforans* strain EPAGRI-Xep20 and *P. rhizosphaerae* strain EPAGRI-Pr1 were striated to grow. 0.1% means 100g c.p./100L H2O or 84g a.i./100L H2O. The analysis was qualitative using the positive signal (+) for the treatment, which allow the bacterial growth and the negative signal (-) for those which inhibit the bacterial growth.

***X. hortorum* pv. *gardneri* population naturally resistant to copper oxychloride at 0.2% (recommended dose of commercial product)**

To estimate the population of *X. hortorum* pv. *gardneri* is naturally resistant to copper oxychloride at 0.2% c.p.(168g a.i/100L H_2O), Petri dishes poured with YDC medium amended with the copper oxychloride was used to perform this experiment. The check was the YDC medium without the bactericide. The suspension was adjuted to OD600 1.000 and a dilution until 10^8 was

performed. 100µL of each dilution was added per Petri plates and the suspension was spread on the plate with Drigalski handle. The total and the resistant population were estimated.

X. hortorum pv. _gardneri_ adapted to high doses of copper oxychloride

Initially *X. hortorum* pv. *gardneri* was spread onto Petri dishes poured with YDC medium amended with copper oxychloride at crescent concentrations (0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9% and 1.0% of commercial product containing 84% copper oxychloride and 50% metallic copper). 0.1% means 100g c.p./100L H2O or 84g a.i./100L H2O. One week later, the bacteria which grown at 0.2% of copper oxychloride was reisolated and striated in Petri dishes poured with YDC medium amended with copper oxychloride at the concentration of 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9% and 1.0% to demonstrated how easy the *X. hortorum* pv. *gardneri* can adapt or become resistant.

Effect of mancozeb in different concentrations on _X. hortorum_ pv. _gardneri_

The *X. hortorum* pv. *gardneri* was spread onto Petri dishes poured with YDC medium amended with mancozeb at crescent concentrations (0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9% and 1.0% of commercial product containing 80% mancozeb). 0.1% means 100g c.p./100L H_2O or 80g a.i./100L H_2O. The analysis was qualitative using the positive signal (+) for the treatment that allows the bacterial growth and the negative signal (-) for those that inhibit the bacterial growth. One week later, the bacteria which grown at YDC medium with 1% of commercial product containing 84% copper oxychloride was reisolated in previous experiment and striated in Petri dishes poured with YDC medium amended with mancozeb at the concentration of 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9% and 1.0% of commercial product to study the resistance to mancozeb when a *X. hortorum* pv. *gardneri* resistant to 1% commercial product containing 84% copper oxychloride was present to verify the possibility of cross resistance between mancozeb and copper oxychloride.

Effect of mancozeb 0.5% (500g c.p./100L H_2O – 400g a.i./100L H_2O) in combinantion with copper oxychloride at 0.5% (500g c.p./100L H_2O – 420g a.i./100L H_2O) and benzalconium chloride 0.25% (250mL c.p./100L H_2O – 25mL a.i./100L H_2O)

Petri dishes with YDC culture medium with the addition of mancozeb 0.5% c.p. + copper oxychloride 0.5% c.p. and mancozeb 0.5% c.p. + copper oxychloride 0.5% + benzalconium chloride 0.25% c.p. were used as a substrate for the growth of the bacterium *X. hortorum* pv. *gardneri*, because it was considered efficient treatment on previous experiments. A strain of the bacterium *X. hortorum* pv. *gardneri* EPAGRI-Xhg6 susceptible to copper oxychloride and a strain of the same bacteria resistant to 1% copper oxychloride (c.p.) were spread on those Petri dishes with Drigalski handle. Analyzes were qualitative using the positive signal (+) for the treatment which allow the bacterial growth and the negative signal (-) for those which inhibit the bacterial growth.

Experimental design and replicates

All experiments were performed in a completely randomized design with 3 replicates. Analyzes were qualitative, considering the presence or absence of CFU (colony forming unit).

Results

Identification of the bacterial strains

X. hortorum pv. *gardneri* EPAGRI-Xhg6 (ON890384) matched with *X. hortorum* pv. *gardneri* strain BF14 (MZ754163) and *X. hortorum* pv. *gardneri* strain JS749-3 (CP018728) with 100% of query cover and identity, while *X. euvesicatoria* pv. *perforans* EPAGRI-Xep20 (ON890387) matched with *X. perforans* 91-118 (CP019725) and *X. perforans* strain MGH1 (OM212464) with 100% of query cover and identity (Fig. 1).

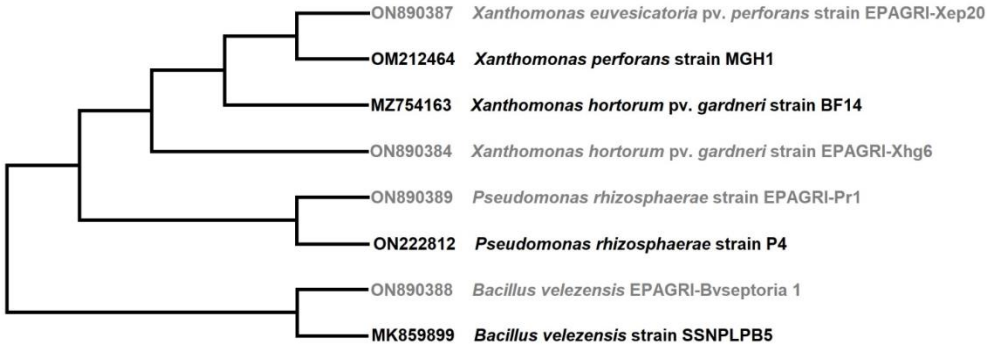

Fig. 1 – Evolutionary analysis inferred by using the maximum likelihood method and Tamura-Nei model of 16S ribosomal RNA gene.

The isolates *X. hortorum* pv. *gardneri* EPAGRI-Xhg6 and *X. euvesicatoria* pv. *perforans* EPAGRI-Xep20 were additionally identified via specific primers proposed by Koenraadt et al. (2007) (Fig. 2).

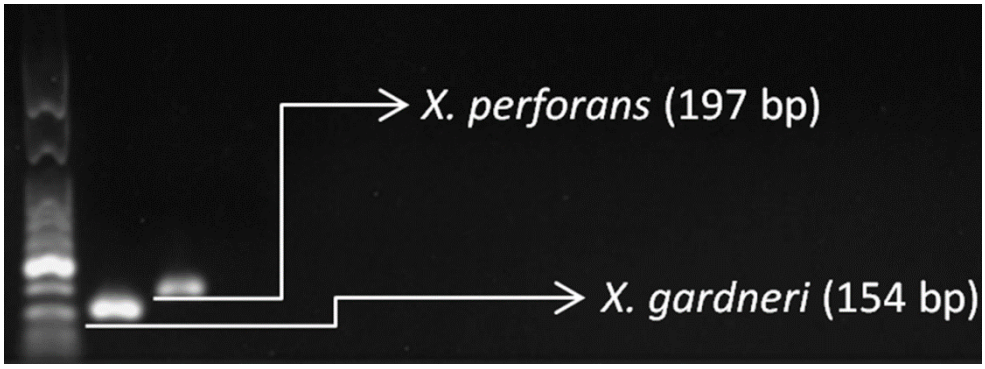

Fig. 2 – Agarose gel with *X. hortorum* pv. *gardneri* EPAGRI-Xhg6 and *X. euvesicatoria* pv. *perforans* EPAGRI-Xep20 PCR products. *The strongest DNA band in the 50 bp ladder has 250 bp.

Effect of fungicides/bactericides at 1 % of commercial product as first screening of potential efficient control

Among 44 active ingredients at 1 % of the commercial products, only ten active ingredients inhibited the growth of both *X. hortorum* pv. *gardneri* EPAGRI-Xhg6 and *X. euvesicatoria* pv. *perforans* EPAGRI-Xep20 (Table 1). Sixteen active ingredients inhibited *X. euvesicatoria* pv. *perforans* EPAGRI-Xep20, but six of them allow the growth of *X. hortorum* pv. *gardneri* EPAGRI-Xhg6 (Table 1).

Effect of fungicides/bactericides in their recommended doses according to the package insert

After the first screening, 17 active ingredients were selected to be applied in their recommended dose, and among them only five could control both *X. hortorum* pv. *gardneri* EPAGRI-Xhg6 and *X. euvesicatoria* pv. *perforans* EPAGRI-Xep20 (Table 2). Mancozeb + cymoxanil inhibited only *X. euvesicatoria* pv. *perforans* EPAGRI-Xep20 (Table 2).

Effect of copper oxychloride on the *X. hortorum* pv. *gardneri*, *X. euvesicatoria* pv. *perforans*, *Bacillus velezensis* and *Pseudomonas rhizosphaerae*

The copper oxychloride was not sucessful in the control of *X. hortorum* pv. *gardneri* EPAGRI-Xhg6 and *X. euvesicatoria* pv. *perforans* EPAGRI-Xep20. It was selected because it has been used as a standard treatment by tomato growers to control the tomato bacterial spot in the field. From the dose 0.78% (780g c.p./100L) the multiplications of all bacteria was inhibited

(Table 3, Fig. 3). At the label recommended dose 0.2% (200g c.p./100L H_2O – 168g a.i./100L H_2O), only *X. gardneri* was able to growth (Table 3, Fig. 3). *B. velezensis* was inhibited at the dose of 0.1% (100g c.p./100L H_2O – 84g a.i./100L H_2O) (Table 3, Fig. 3)). Even using twice the indicated dose of copper oxychloride, *X. hortorum* pv. *gardneri* EPAGRI-Xhg6 *gardneri* was not completely inhibited (Fig. 4).

Table 1 Effect of fungicides/bactericides at the dose of 1% of commercial product (1000g c.p./100L) against *X. hortorum* pv. *gardneri* EPAGRI-Xhg6 and *X. euvesicatoria* pv. *perforans* EPAGRI-Xep20.

Fungicides / Bactericides	***X. gardneri***	***X. perforans***	**Fungicides / Bactericides**	***X. gardneri***	***X. perforans***
Benzalkonium chloride	-	-	Pyraclostrobin	+	+
Acetic acid	-	-	Bentiavalicarb + Chlorothalonil	+	+
Cuprous oxide	-	-	Pyrimethanil	+	+
Mancozeb	-	-	Iprodione	+	+
Copper hydroxide	-	-	Casugamicin	+	+
Mancozeb + Famoxadone	-	-	Procymidone	+	+
Copper oxychloride	-	-	Mandipropamide	+	+
Metiram + Pyraclostrobin	-	-	Propamocarb hydrochloride	+	+
Bordeaux mixture	-	-	Azoxystrobin	+	+
Viçosa mixture	-	-	Lime sulfur	+	+
Propineb	+	-	Propamocarb hydrochloride + Fluopicolide	+	+
Metiram	+	-	Metalaxyl-M + Chlorothalonil	+	+
Mancozeb + Copper oxychloride	+	-	Captan	+	+
Mancozeb + Metalaxyl-M	+	-	Dimethomorph	+	+
Cymoxanil + Famoxadone	+	-	Trifloxystrobin + Tebuconazole	+	+
Mancozeb + Cymoxanil	+	-	Boscalid	+	+
Fluazinam	+	+	Azoxystrobin + Difenoconazole	+	+
Tebuconazole	+	+	Bentiavalicarb + Fluazinam	+	+
Chlorothalonil	+	+	Fluxapyroxad + Pyraclostrobin	+	+
Methyl-thiophanate	+	+	Methyl-thiophanate + Fluazinam	+	+
Metconazole	+	+	*Melaleuca alternifolia* extract	+	+
Difenoconazole	+	+	Tetraconazole	+	+

+ When an observable colony was seen after seven days of incubation (25º C);
- No growth was observed after seven days of incubation (25º C).

Table 2 Effect of fungicides/bactericides in their package insert doses against *X.* hortorum pv. *gardneri* EPAGRI-Xhg6 and *X. euvesicatoria* pv. *perforans* EPAGRI-Xep20.

Fungicides / Bactericides	**Dose (g/mL a.i./100L H_2O)**	**Growth**	
		gardneri	***perforans***
Benzalkonium chloride	25	-	-
Acetic acid	N.A.[1]	-	-
Mancozeb	400	-	-
Mancozeb + Famoxadone	100 + 10	-	-
Metiram + Pyraclostrobin	220 + 20	-	-
Mancozeb + Cymoxanil	192 + 24	+	-
Bordeaux mixture 0.3%[2]	300	+	+
Viçosa mixture 0.3%[3]	300	+	+
Lime sulfur 0.3%[4]	300	+	+
Copper hydroxide[5]	172.75 (112.5)	+	+
Copper oxychloride	168 (100)	+	+
Propineb	210	+	+
Metiram	210	+	+
Mancozeb + Copper oxychloride	88 + 60	+	+
Mancozeb + Metalaxyl-M	192 + 12	+	+
Cymoxanil + Famoxadone	24 + 18	+	+
Cuprous oxide	134.4 (120)	+	+

[1] The percentage of active ingredient is not available at the product package insert. The comercial dosage is 200 mL/100L H_2O;
[2] Formulated with copper 20%; sulfur 10% and calcium 3%;
[3] Formulated as mixed mineral fertilizer (K_2O 8%, Mg 0.8%, S 8%, B 3.5%, Cu 9% and Zn 3%);
[4] Formulated with calcium sulfate (S 50% and Ca 5%).[5] The number in parentheses is the equivalent in metallic copper.

Table 3 Effect of copper oxychloride on *X. hortorum* pv. *gardneri* EPAGRI-Xhg6, *B. velezensis* EPAGRI-Bvseptoria1, *X. euvesicatoria* pv. *perforans* Epagri-Xep20 and *P. rhizosphaerae* EPAGRI-Pr1 on Petri plates with YDC medium after seven days of incubation.

Doses (%) c.p.	**g a.i./100L[1]**	***Xanthomonas hortorum* pv. *gardneri***	***Bacillus velezensis***	***Xanthomonas euvesicatoria* pv. *perforans***	***Pseudomonas rhizosphaerae***
25	21,000	-[2]	-	-	-
12.50	10,500	-	-	-	-
6.25	5,250	-	-	-	-
3.13	2,629.2	-	-	-	-
1.56	1,310.4	-	-	-	-
0.78	655.2	-	-	-	-
0.39	327.6	+[3]	-	-	-
0.20	**168**	+[4]	-	-	-
0.10	84	+	-	+	+
0.05	42	+	+	+	+
0.02	16.8	+	+	+	+
Control	-	+	+	+	+

[1] Dose of active ingredient in grams per 100 L of water considering a commercial product with 84% copper oxychloride;
[2]When an observable colony was seen after seven days of incubation (25º C);
[3]No growth was observed after seven days of incubation (25º C);
[4]The gray highlighted line means the label dose recommended in field pulverization.

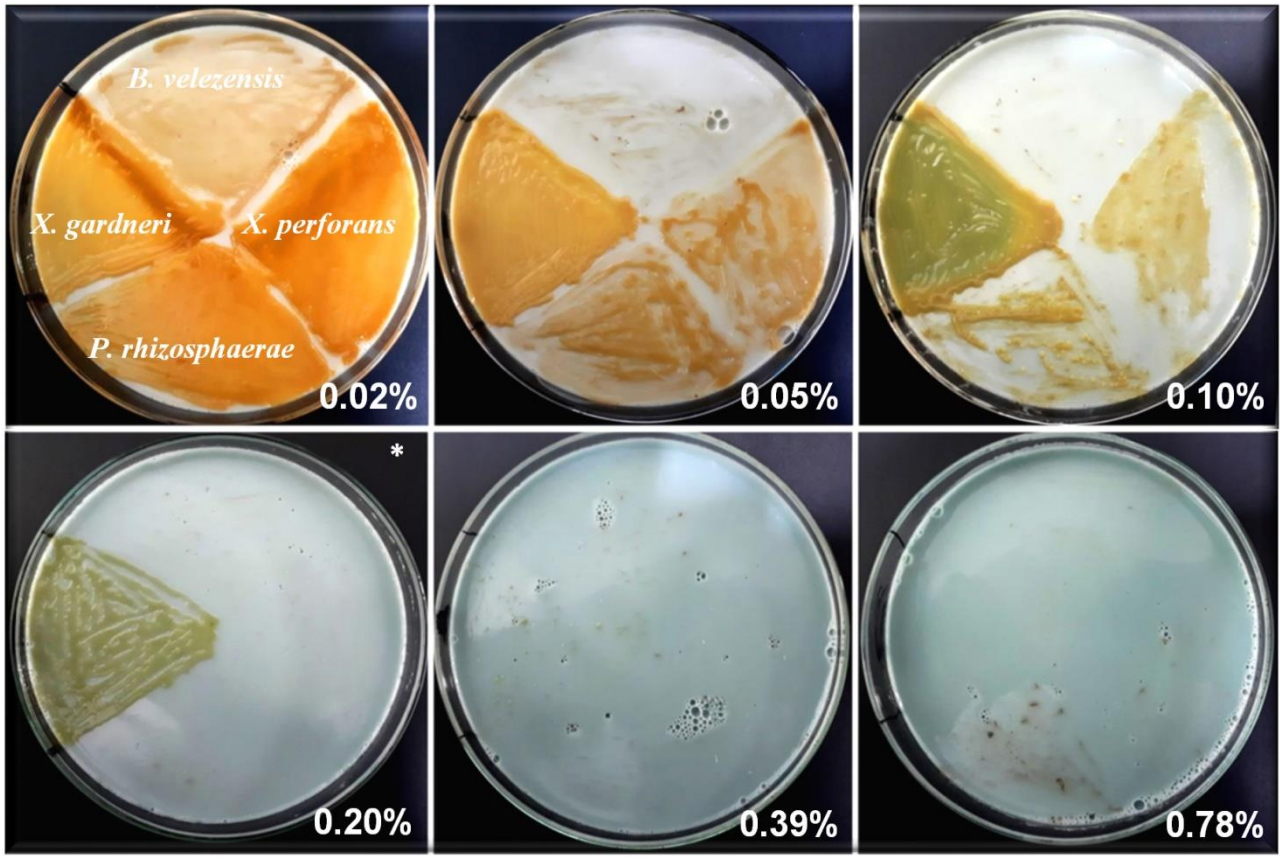

Fig. 3 – Effect of copper oxychloride in percentage of the commercial product on *X. hortorum* pv. *gardneri* EPAGRI-Xhg6, *B. velezensis* EPAGRI-Bvseptoria1, *X. euvesicatoria* pv. *perforans* Epagri-Xep20 and *P. rhizosphaerae* EPAGRI-Pr1 on Petri plates with YDC medium after seven days of incubation.

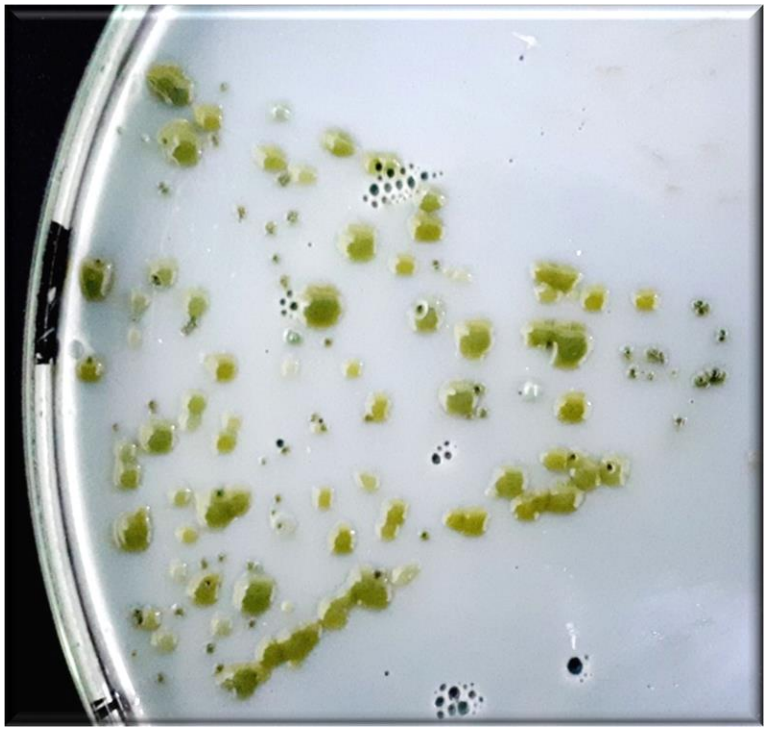

Fig. 4 – *X. hortorum* pv. *gardneri* growing in a Petri dish with YDC plus copper oxychloride at 0.39% after 17 days of incubation at 25ºC.

***Xanthomonas hortorum* pv. *gardneri* population naturally resistant to copper oxychloride at 0.2% (recommended dose of commercial product)**

An estimate was performed for *X. hortorum* pv. *gardneri* EPAGRI-Xhg6 population using the recommended dose of copper oxychloride (200g c.p./100L H_2O – 168g a.i./100L H_2O) to study the adaptation or a possible resistance to this bactericide, and was found that 0.004% of CFU (colony-forming-unit) was naturally adapted to dose of 0.2% (200g c.p./100L H_2O – 168g a.i./100L H_2O) (Table 4).

Table 4 Percentage of *X. hortorum* pv. *gardneri* EPAGRI-Xhg6 naturally adapted to the label recommended dose of copper oxychloride at 0.2%.

CFU/mL at 1.000 O.D. A600 nm	CFU/mL adapted to copper oxychloride at 0.2% c.p.	% naturally adapted to recommend dose (0.2% c.p.)
$3.1x10^9 \pm 1.4x10^8$	$1.1x10^5 \pm 6.8x10^3$	0.004%

***X. hortorum* pv. *gardneri* adaptated to high doses of copper oxychloride**

At doses equal to or greater than 0.4% copper oxychloride (400g c.p./100L H_2O – 336g a.i./100L H_2O), *X. hortorum* pv. *gardneri* EPAGRI-Xhg6 was inhibited (Fig. 5A). However, when the CFUs were grown in culture medium containing 0.2% copper oxychloride (200g c.p./100L H_2O – 168g a.i./100L H_2O), these adapted bacteria were able to grow in YDC containing 1% copper oxychloride (1kg c.p./100L H_2O – 840g a.i./100L H_2O) (Fig. 5B).

Effect of mancozeb in different concentration on *X. hortorum* pv. gardneri

Mancozeb was able to inhibit the multiplication of *X. hortorum* pv. *gardneri* EPAGRI-Xhg6 from a dose of 0.1% (100g c.p./100L H_2O – 80g a.i./100L H_2O) (Figure 6A). But when an *X. hortorum* pv. *gardneri* EPAGRI-Xhg6 adapted to a dose of 1% of copper oxychloride was striated, bacterial multiplication was observed in Petri dishes containing mancozeb up to a dose of 0.5% (500g c.p./100L H_2O – 400g a.i./100L H_2O), with no CFU being observed at higher doses (Fig. 6B).

Effect of mancozeb 0.5% (500g c.p./100L H_2O – 400g a.i./100L H_2O) in combinantion with copper oxychloride at 0.5% (500g c.p./100L H_2O – 420g a.i./100L H_2O) and benzalconium chloride 0.25% (250mL c.p./100L H_2O – 25mL a.i./100L H_2O)

When the susceptible bacteria were striated on the medium containing the mixture (0.5% c.p. mancozeb + 0.5% c.p. copper oxychloride) and on the medium containing the mixture plus 0.25% c.p. benzalkonium chloride, the bacteria did not multiply (Fig. 7A). However, when bacteria adapted to copper oxychloride 1% c.p. (840g a.i./100L H_2O) were striated in these culture medium, bacterial multiplication occurs (Fig. 7B).

Discussion

On tomato crop the use of copper worldwide vary from 0.5-6 kg $ha^{-1}year^{-1}$ and the applications may vary between 4 and 10 according to outside weather conditions and infection intensity (Katsoulas et al. 2020). The selection of copper-resistant strains is the major reason for disease control failures (Behlau et al. 2012). As demonstrated herein, 0.004% of the population develops the copper resistance easily, if the dose of 0.2% copper oxychloride (200g c.p./100L H_2O – 168g a.i./100L H_2O) is applied.

The bacterial resistance was observed in 1983 in *Xanthomonas campestris* pv. *vesicatoria* (Marco & Stall 1983). In bacteria the copper resistance is regulated by several genes (Cooksey 1990) generally located in mobile elements like plasmids and transposons (Bondarczuk & Piotrowska-Seget 2013). Yin et al. (2017) reported the main mechanisms regulating copper resistance in bacteria. Copper ions act non-specifically (multisite) at the cell membrane level, leading to the denaturation of structural and enzymatic proteins and altering membrane

semipermeability (La Torre et al. 2018). Herein, as bacterial isolates resistant to copper oxychloride multiplied in Petri dishes containing mancozeb, it is believed that copper and mancozeb may share some sites of action in controlling the bacteria leading to a cross-resistance.

X. euvesicatoria pv. *perforans* seems to be more susceptible to the treatments used, since the bacteria were inhibited when in contact with some fungicides/bactericides incapable of inhibiting *X. hortorum* pv. *gardneri*. This indicates that the control of *X. hortorum* pv. *gardneri* is a bigger challenge.

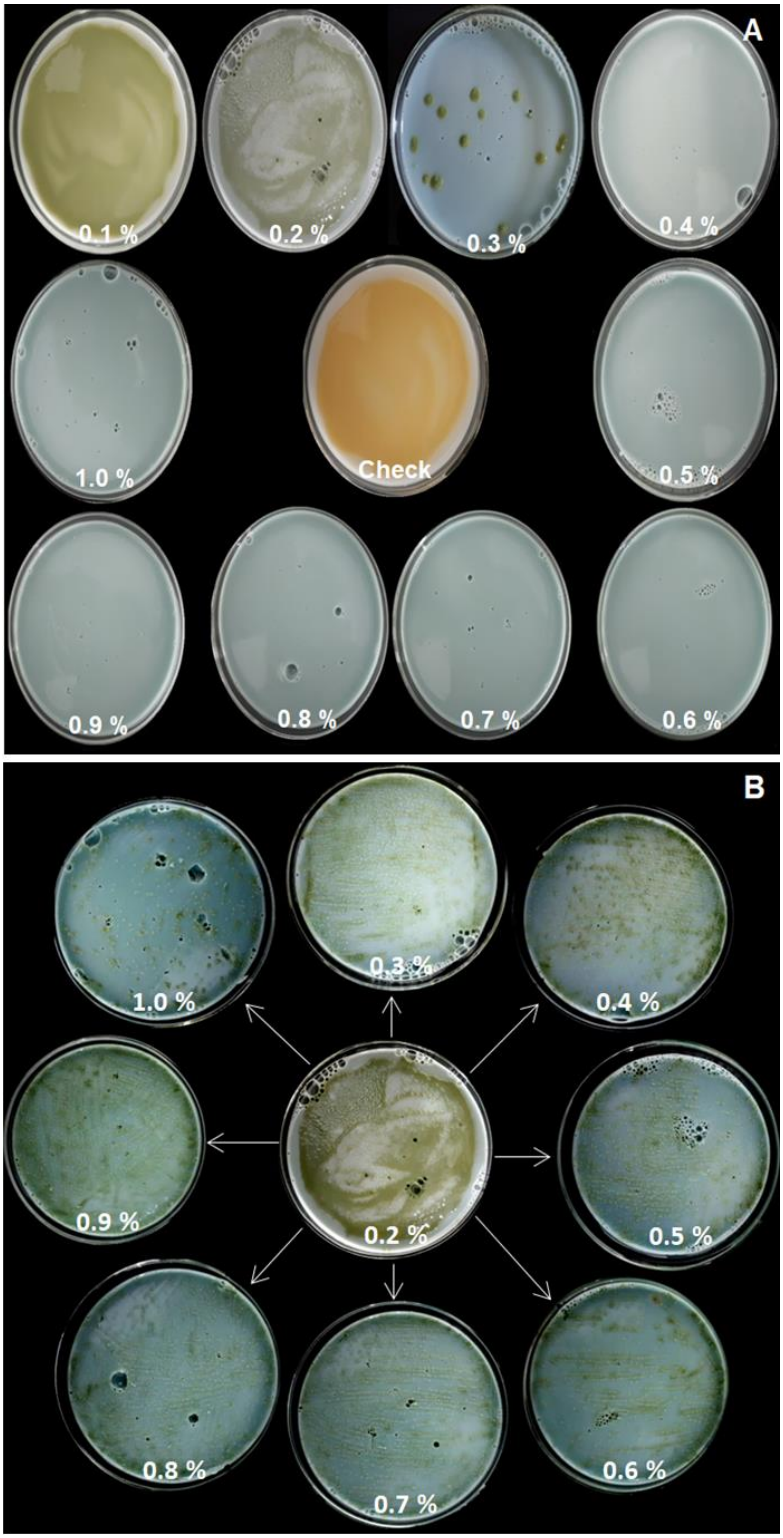

Fig. 5 – Adaptability/Resistance of *X. hortorum* pv. *gardneri* to copper oxychloride in a range of doses. A *X. hortorum* pv. *gardneri* growing in Petri dishes with YDC medium amended with 0.1,

0.2 and 0.3% commercial product (c.p.) containing 84% copper oxychloride. B *X. hortorum* pv. *gardneri* isolated from the YDC Petri dish with 0.2% commercial product containing 84% copper oxychloride growing in Petri dishes with YDC medium amended with copper oxychloride in a range of concentration (0.3-1% c.p.). *0.1% means 100g c.p./100L H_2O or 84g a.i./100L H_2O.

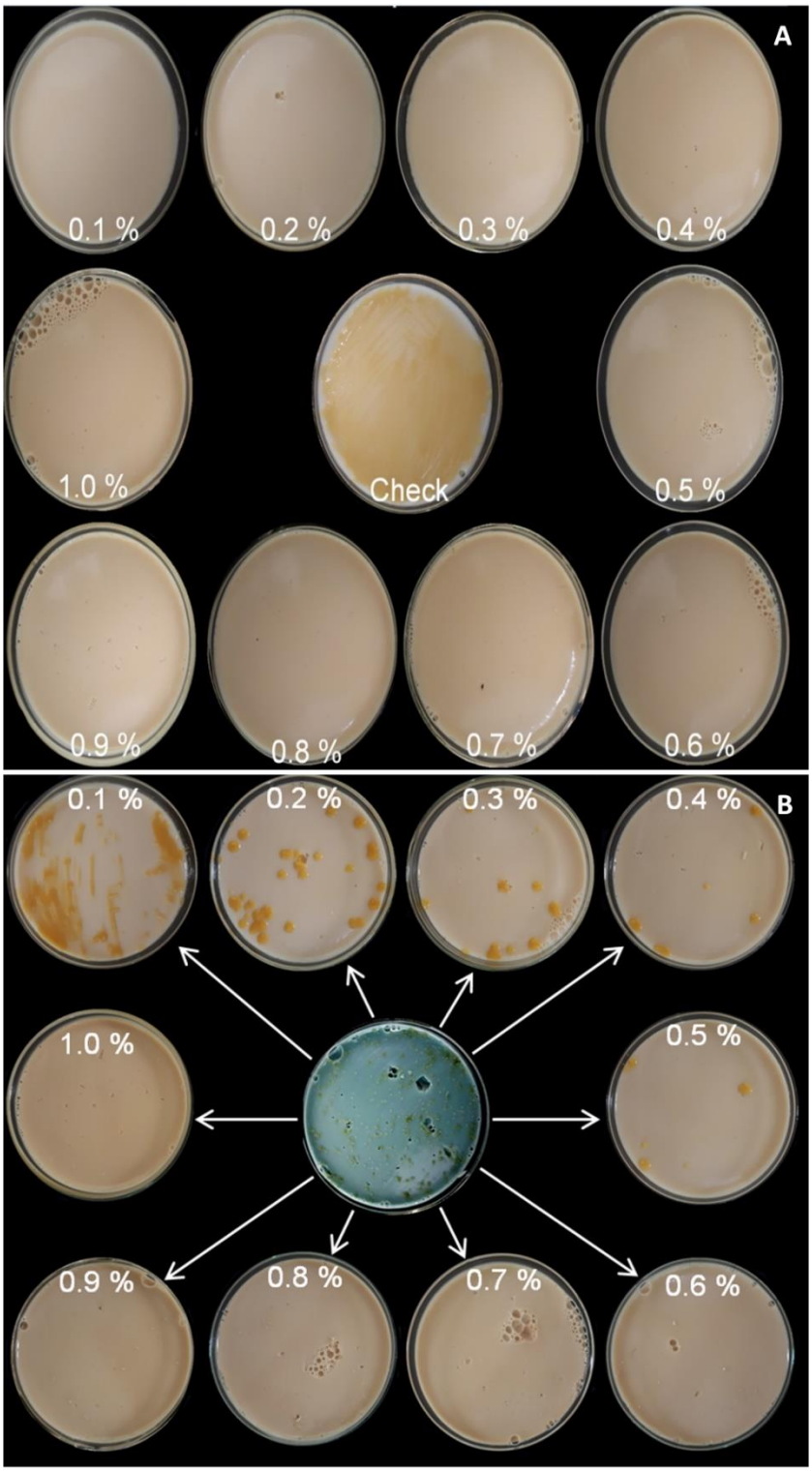

Fig. 6 – Adaptability/Resistance of *X. hortorum* pv. *gardneri* to mancozeb in a range of doses using a copper oxychloride-adapted-strain and a susceptible strain. A Susceptible *X. hortorum* pv. *gardneri* striated in Petri dishes with YDC medium amended with mancozeb at 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0% concentration of commercial product (80% mancozeb). B *X. hortorum* pv. *gardneri* isolated from the YDC Petri dish with 1% of copper oxychloride striated in

Petri dishes with YDC medium amended with mancozeb in a range of concentration (0.1-1%). *0.1% means 100g c.p./100L H_2O or 80g a.i./100L H_2O.

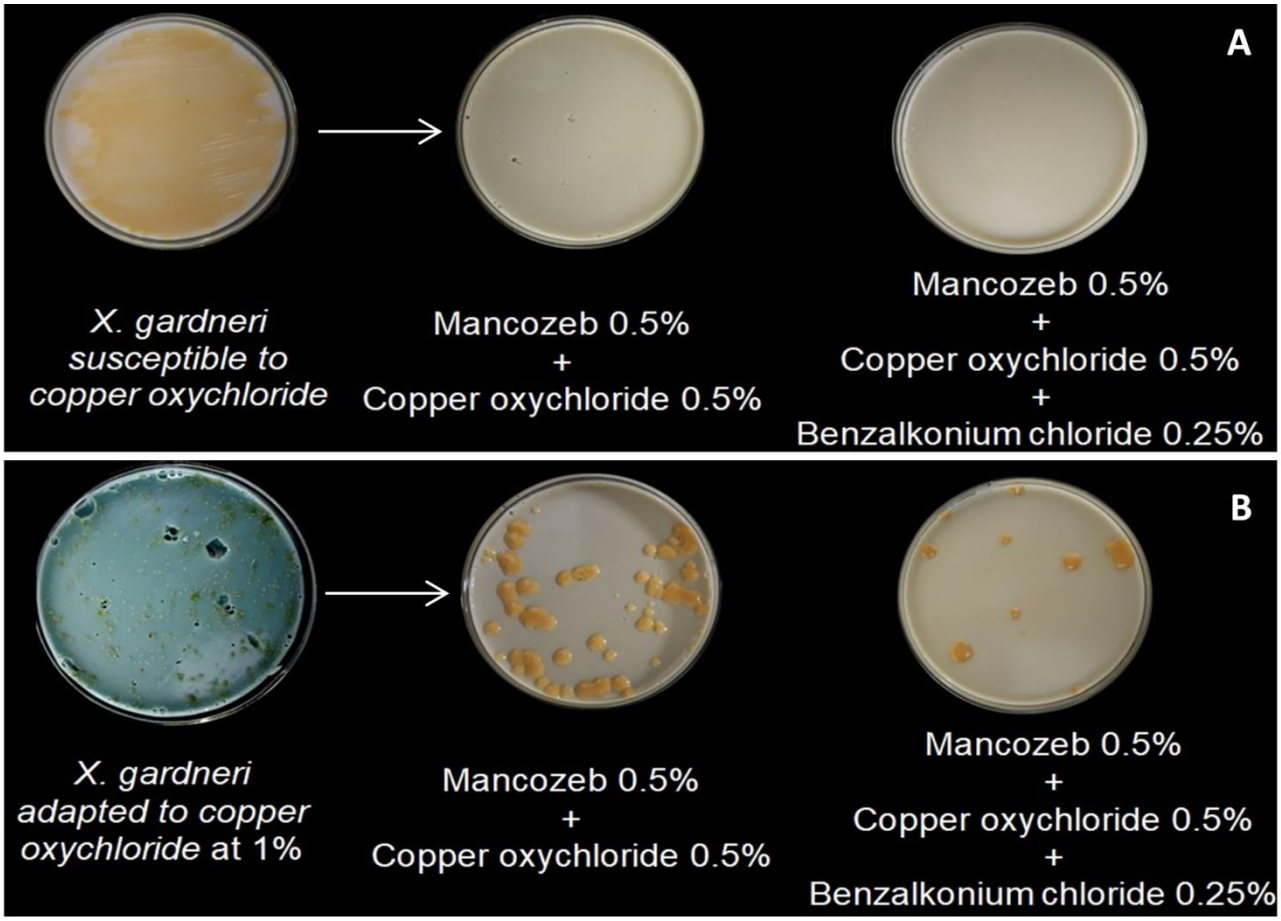

Fig. 7 – Differences between *X. hortorum* pv. *gardneri* EPAGRI-Xhg6 susceptible and adapted to copper oxychloride at 1% (commercial product) to mancozeb 0.5% c.p. + copper oxychloride 0.5% c.p. and to mancozeb 0.5% c.p. + copper oxychloride 0.5% c.p. + benzalkonium chloride 0.25% c.p. A *X. hortorum* pv. *gardneri* susceptible to copper oxychloride did not grown on the YDC with mancozeb, copper oxychloride and benzalkonium chloride. B -*X. hortorum* pv. *gardneri* adapted to copper oxychloride at 1% (840g a.i./100L H_2O) grown on the YDC with mancozeb, copper oxychloride and benzalkonium chloride.

All 28 bactericides registered for tomato bacterial spots in Brazil target the *Xanthomonas vesicatoria* or *Xanthomonas campestris* pv. *vesicatoria* instead *X. hortorum* pv. *gardneri* or *X. euvesicatoria* pv. *perforans*. This can be a problem because the frequency of the target bacteria reprensent only 9% of the occurencies in tomato crop, while *X. hortorum* pv. *gardneri* and *X. euvesicatoria* pv. *perforans* are responsible for 91% of the occurencies of the tomato bacterial spots in the Alto Vale do Rio do Peixe, Santa Catarina region (Costa et al. 2012). The *X. euvesicatoria* pv. *perforans* are the most recurrent bacteria in tomato crops causing bacterial spot worldwide (Horvath et al. 2012).

Some studies suggest the joint application of *Bacillus* in a tank mixture with copper-based bactericides (Abbasi & Weselowski 2015). As demonstrated in this work, at a dose of 42g a.i./100L H_2O, four times less than the recommended dose of copper oxychloride, the growth of *B. velezensis* was inhibited. As the bacterium can produce fungicidal/bactericidal metabolites during its multiplication to make the commercial product and dead bacteria may induce resistance in plants, it is possible that there will be some increase in disease control when used together in the spray tank. However, the biological control agent would be failing to act via competition for space and

nutrient, antibiosis *in loco* using the resources of the environment in which it is inserted for the production of antimicrobial substances or even predation since the bacterium would die when in contact with copper in the recommended doses for the control of diseases in tomato.

It is known that fungal communities are more resistant to copper than bacterial communities (Rajapaksha et al. 2004). The dose considered inefficient in inhibiting the multiplication of *X. hortorum* pv. *gardneri* may still be efficient to inhibit the native microbiota present in the leaves, as demonstrated here for *B. velezensis* in Petri plates, aggravating the problem with tomato bacterial leaf spot, as it prevents the natural competition for space and leaf nutrients promoted by native populations.

Despite the inefficiency of the package insert dose shown here, the application of the commercial product should only be recommended in its package insert doses to comply with current legislation and also to ensure food safety until the manufacturers of the product carry out a review of the package insert.

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