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Comparative efficiency of chemical compounds for *in vitro* and *in vivo* activity against *Clavibacter michiganensis* subsp. *michiganensis*, the causal agent of tomato bacterial canker

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ABSTRACT

Bacterial canker of tomato caused by *Clavibacter michiganensis* subsp. *michiganensis* produces considerable economic losses in many countries because effective control measures are lacking. The extent to which bactericides control this disease effectively is low and has not yet been well documented for Southern European conditions. In this study the bactericidal effect of several products on this pathogen was assessed *in vitro* and *in vivo* in tomato plants under greenhouse conditions. Seven antibacterial substances (bronopol, copper sulphate, kasugamycin, oxolinic acid, oxytetracycline, streptomycin and 8-hydroxy-quinoline), three commercial formulates (Antibak RZ, an oligoelements mixture containing copper plus zinc; Orthopol, a potassium soap; and Param, a resistance inductor) and combinations thereof were tested. *In vitro* assessment showed that minimal inhibitory concentration (MIC) of antibacterial substances was between 4–8 $\mu g\,ml^{-1}$, except for copper sulphate with a MIC value of 150 $\mu g\,ml^{-1}$ and kasugamycin, which was not active at 500 $\mu g\,ml^{-1}$. MIC values of commercial formulates ranged between 5 and 40 $\mu l\,ml^{-1}$. Furthermore, combinations of 8-hydroxy-quinoline+copper sulphate, 8-hydroxy-quinoline+Antibak RZ, streptomycin+Antibak RZ and streptomycin+Orthopol showed a synergistic effect at sub-inhibitory concentrations. Treatments containing copper sulphate greatly reduced disease symptoms on plants sprayed with the bacteria, whereas streptomycin was less effective. In two independent trials, the percentage of leaves showing symptoms was significantly lower (2.4% and 11.9%) after treatment with copper sulphate combined with 8-hydroxy-quinoline at half-dose, than in inoculated controls (75.1% and 59.6%). These results were better than copper sulphate alone. However, plants inoculated by pricking rapidly developed systemic infection, which no product managed to control significantly, although several treatments did reduce symptoms. We conclude that copper sulphate combined with 8-hydroxy-quinoline may be useful in controlling external symptoms of this disease in greenhouses, and is environmentally friendly, reducing the amount of copper applied to crops.

1. Introduction

Bacterial canker of tomato produced by *Clavibacter michiganensis* subsp. *michiganensis* (Smith) Davis et al. (1984) is considered the most important bacterial disease affecting tomato crops (OEPP/EPPO, 2005), and is a quarantine organism in the European Union (EU) (Anonymous, 2000). Seedlings infected with this seed-borne pathogen can become stunted plants that eventually succumb to the disease and soon die (Hausbeck et al., 2000). Systemic infection produces characteristic symptoms, like wilting of leaflets and cankers on stems and petioles. When the infection occurs after epiphytic spread of the pathogen, marginal necrosis of leaves and small white blister-like spots are common, and lesions, named bird's-eye spots, occasionally appear on fruits (Carlton et al., 1998). After this, the bacteria can penetrate the vascular tissues and plants develop systemic symptoms.

The typical start-and-stop pattern demonstrated by this pathogen hinders its control, which has primarily been based on

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the use of pathogen-free seeds and seedlings (Strider, 1969; Thyr et al., 1973; Gleason et al., 1993). However, this measure does not always give satisfactory results, and the crop becomes infected with the bacteria. Control measures must then be adopted to reduce losses and prevent secondary infection in order to decrease epiphytic pathogen populations (Moffet and Wood, 1984; Gleason et al., 1993; Carlton et al., 1994; Hausbeck et al., 2000; Medina-Mora et al., 2001; Werner et al., 2002). Plant activators for resistance induction (Soylu et al., 2003) and biocontrol employing different bacteria (El-Abyad et al., 1993; Boudyach et al., 2001; Umesha, 2006) have also been proposed to improve control strategies, but at present, they are still far from providing successful control in the field. Similarly, progress in developing genetic resistance to bacterial canker has been modest, and few commercial cultivars have shown significant field tolerance to the disease (Gleason et al., 1993).

Studies focusing on *C. michiganensis* subsp. *michiganensis* chemical control are scarce and afford variable results. With respect to standard bactericides, secondary spread of the pathogen in the field can only be reduced by treating seedlings with streptomycin and copper compounds (Hausbeck et al., 2000; Werner et al., 2002). However, little information is available about their efficacy on greenhouse-cultivated tomatoes, like those grown in the Canary Islands and Southern Europe. Alternative substances like aqueous extract of propolis (Bianchini and Bedendo, 1998), resinous exudates (Modak et al., 2004) and essential oils (Daferera et al., 2003; Kizil et al., 2005) have shown *in vitro* efficacy, but their effects *in vivo* have never been evaluated. Given the absence of information, the aim of this study was to (i) screen *in vitro* a range of antimicrobial agents and their synergistic effect against *C. michiganensis* subsp. *michiganensis*; and (ii) determine *in vivo* to what extent they effectively control the pathogen under greenhouse conditions.

2. Materials and methods

2.1. Bacterial cultures and growth conditions

Five strains of *C. michiganensis* subsp. *michiganensis* were used: 185-2.1, C-8.2 and 118-1.1 (isolated in the Laboratorio de Sanidad Vegetal del Gobierno de Canarias; The Canary Islands) and IVIA 613 and IVIA 873 (from the Instituto Valenciano de Investigaciones Agrarias collection; Valencia, Spain). Strains were routinely cultured in yeast–peptone–glucose agar (YPGA: yeast extract, 5 g; bactopeptone, 5 g; glucose, 10 g; agar, 15 g; in 1 l of distilled water) and incubated at 25 °C. Yeast–peptone–glucose broth (YPGB: yeast extract, 5 g; bactopeptone, 5 g; glucose, 10 g; in 1 l of distilled water) was used for liquid cultures.

2.2. In vitro assays

The activity of seven antimicrobial substances and three commercial formulates was evaluated against four strains of *C. michiganensis* subsp. *michiganensis* (185-2.1, C-8.2, IVIA 613 and IVIA 873). The antimicrobial substances used were bronopol, copper sulphate, oxolinic acid, oxytetracycline, streptomycin and 8-hydroxy-quinoline (Sigma-Aldrich, Spain), kasugamycin (Lainco, Spain), and the commercial formulates: Antibak RZ containing copper (1%) and zinc (1%) (Stoller Iberica S.L., Spain), Orthopol (potassium soap with 50% potassium oleate, PBCF S.L., Spain) and Param (menadione sodium bisulphite, Impla S.A, Spain).

The minimal inhibitory concentrations (MIC) and minimal bactericide concentrations (MBC) were determined by the broth macrodilution method (Peterson and Shanholtzer, 1992) in 2 ml of YPGB. Antimicrobial substances were dissolved in sterile water, except for oxolinic acid for which DMSO was used, and added to YPGB at concentrations of 1, 2, 4, 6, 8, 10, 12, 15 and 20 $\mu g\,ml^{-1}$. Additional concentrations of 50, 80, 100, 150, 250 and 400 $\mu g\,ml^{-1}$ were prepared for copper sulphate and up to 500 $\mu g\,ml^{-1}$ for kasugamycin. Commercial formulates were assayed at 1, 3, 5, 8, 10, 12, 15, 20, 40, 60 and 100 $\mu l\,ml^{-1}$. Sterile water or DMSO was used as controls. The starting bacterial inoculum was $1–5 \times 10^6$ cfu ml^{-1}, and bacterial populations were monitored at 0, 24, 48, 72 and 96 h by cfu counts on YPGA plates. The MIC and MBC were defined respectively as the lowest concentrations of compound at which growth was inhibited, or reduced $\geqslant$99.9%, after 96 h of incubation in a rotatory shaker at 25 °C.

Furthermore, all substances were assayed at sub-inhibitory concentrations ($\frac{1}{2}$ and $\frac{1}{4} \times$ MIC) separately and combined in pairs to search for possible synergistic effects. All these assays were done in quadruplicate.

2.3. Greenhouse assays

2.3.1. Plant material and inoculations

Experiments were carried out with tomato plants cv. Roma, susceptible to bacterial canker. Plants were grown under greenhouse conditions (with a minimum temperature of 15 °C and a maximum of 28 °C), in multi-pots (7 × 7 cm per pot) containing peat as substrate. Plants were fertilized twice a week with 2.5 ml l^{-1} of Actigil AA 8-8-6 (Bayer CropScience, Spain) solution and were inoculated with a virulent strain of *C. michiganensis* subsp. *michiganensis* (118-1.1) on reaching the four to six fully expanded-leaf stage (7–8 weeks old) (Meier, 2001). Cells were obtained from a 48 h culture at 25 °C in YPGB, after centrifugation at 2000 *g* for 5 min at 4 °C. The pellet was rinsed twice in sterile distilled water and adjusted to $OD_{550nm} = 0.06$ (10^7 cfu ml^{-1}) for inoculations. Two inoculation systems were used to evaluate chemical treatment effectiveness: (i) foliar-spray inoculation with the bacterial suspension (18–20 ml per 12 plants) after manual injury by applying finger pressure to plant stems to break the trichomes at three points of height (top, middle and base of the stem), and (ii) pricking inoculation placing a drop (10 μl) of bacterial suspension in the node of the first true leaf with the stem, and immediately pricking the stems with an insulin needle. After inoculation, plants were covered with polyethylene bags for 5 d.

2.3.2. Chemical treatments

Antimicrobial substances were applied at the following concentrations: bordeaux mixture (10 g of copper sulphate plus 0.5 g of hydrated lime per litre of water); Beltanol-L (50% 8-hydroxy-quinoline, Probelte, Spain), 1 ml l^{-1}; Kasumin (8% kasugamycin, Lainco, Spain), 500 mg l^{-1}; streptomycin, 100 mg l^{-1}; Antibak RZ, 21 ml l^{-1}; Param, 1.5 ml l^{-1}; and Orthopol, 5 ml l^{-1}. Additionally, combinations of Beltanol-L+bordeaux mixture, Beltanol-L+Antibak RZ, streptomycin+Antibak RZ and streptomycin+Orthopol were applied at half dosages each. Chemicals were sprayed on 12 tomato plants per treatment until runoff (approximately 300 ml), using a hand-held spray. Two applications were made per treatment, the first, 5 d before pathogen inoculation and the second, 6 d after. After being sprayed, plants were allowed to dry until no liquid droplets were visible. Control plants were sprayed with the same volume of sterile tap water. All the experiments were run twice from March to April of 2005 and 2006.

2.3.3. Disease assessment

Symptoms caused by *C. michiganensis* subsp. *michiganensis* in tomato plants were evaluated 4 weeks after inoculation. Data concerning height and fresh/dry weight were obtained for all plants. The percentage of leaflets displaying symptoms was recorded for spray-inoculated plants. Furthermore, the presence of blister-like spots on the stems in the injured zone, previously described by Gleason et al. (1993), was rated on a scale from 0 to 3, where 0 = no lesions, 1 = <5 small and dispersed blister, 2 = >5 dispersed blisters, and 3 = blisters occupying all injured areas. Samples of vascular tissues next to blisters were taken for pathogen isolation. In plants inoculated by pricking, the percentage of wilting leaves was calculated to assess disease severity. Additionally, stems from each plant were cut longitudinally for visual inspection and the length of vascular tissues affected was measured. Samples from vascular tissues at 3 cm from the inoculation sites were taken for pathogen isolation.

For pathogen isolation, small pieces from the samples were soaked in 1 ml of sterile water and streaked on YPGA plates, which were then incubated for 5 d at 25 °C. To confirm the identity of putative *C. michiganensis* subsp. *michiganensis* colonies, randomly selected ones were tested by nitrocellulose membrane ELISA (De León et al., 2006) and by PCR using primers developed by Dreier et al. (1995).

2.4. Statistical analysis

Analysis of variance (ANOVA) was performed using the Systat Statistical Software Package version 10 (SPSS Inc.), and means were separated by Fisher's least significant difference test at $p<0.05$. Prior to analysis, homogeneity of variance was verified by Bartlett's test (Little and Hills, 1975). Data for percentage of wilt and incidence of margin necrosis on leaves were subjected to angular transformation ($Y=\text{arcsine}\ [\%]^{1/2}$) to stabilise variance. For similar reasons, treatments whose values for all data were zero (disease symptoms on uninoculated controls) were excluded from ANOVA. In addition, the relationship between the symptom intensity in spray-inoculated plants and pathogen isolation from internal tissues was tested by regression analysis.

3. Results

3.1. Effect of antimicrobial compounds on C. michiganensis subsp. michiganensis growth

The MIC and MBC values of 10 chemical compounds tested are listed in Table 1. All antimicrobial compounds inhibited bacterial growth after 96 h of incubation, except kasugamycin, which was not active at the highest assayed concentration (500 $\mu g\,ml^{-1}$). Similar effects were observed for each compound among the different strains under study. Oxytetracycline (MIC 4 $\mu g\,ml^{-1}$), streptomycin, 8-hydroxy-quinoline, oxolinic acid (MIC 6 $\mu g\,ml^{-1}$) and bronopol (MIC 8 $\mu g\,ml^{-1}$) exhibited low MIC values, whereas copper sulphate was active at a higher concentration (MIC 150 $\mu g\,ml^{-1}$). The MICs of commercial formulates were 5 $\mu l\,ml^{-1}$ for Antibak RZ and Param and 40 $\mu l\,ml^{-1}$ for Orthopol.

In addition, MBC values were below 2 × MIC for streptomycin, 8-hydroxy-quinoline and Orthopol. Addition of these compounds at MIC values produced a >4-$\log_{10}$ reduction in the initial inoculum of bacterial cultures after 24 h of incubation, but after 48 h incubation, a slight regrowth of the cultures was observed for streptomycin and 8-hydroxy-quinoline. The other compounds showed a bacteriostatic effect on cell cultures with <2−$\log_{10}$ cfu ml^{-1} reduction after 96 h (data not shown).

Table 1
Minimal inhibitory and bactericide concentrations (MIC and MBC) of chemical compounds against four strains of *C. michiganensis* subsp. *michiganensis*

Chemical compounds	Strains of *C. michiganensis* subsp. *michiganensis*			
	185. 2. 1 MIC/MBC[a]	C 8.2 MIC/MBC	IVIA-613 MIC/MBC	IVIA-873 MIC/MBC
Bronopol	8/>20	8/>20	8/>20	8/>20
Copper sulphate	150/>400	150/>400	150/>400	150/>400
Kasugamycin	>500/>500	>500/>500	>500/>500	500/>500
Oxolinic acid	6/>20	6/>20	6/>20	6/>20
Oxytetracycline	4/>20	4/>20	4/>20	4/>20
Streptomycin	6/8	6/10	6/8	6/10
8-Hydroxy-quinoline	6/10	6/12	6/12	6/12
Antibak RZ	5/15	5/12	5/15	5/15
Orthopol	40/60	40/60	40/60	40/60
Param	5/40	5/40	5/40	5/40

[a] MIC and MBC are expressed in $\mu g\,ml^{-1}$ except for commercial compounds Antibak RZ, Orthopol and Param, which are in $\mu l\,ml^{-1}$.

When the joint action of the tested compounds was evaluated in pairs at sub-inhibitory concentrations ($\frac{1}{2}$ and $\frac{1}{4}\times$ MIC), the following combinations improved the antibacterial effect compared to each compound separately: 8-hydroxy-quinoline+copper sulphate; 8-hydroxy-quinoline+Antibak RZ; streptomycin+Antibak RZ and streptomycin+Orthopol. For those combinations, subinhibitory concentrations of each component drastically reduced cfu counts (>99.9%) from the initial inoculum (Table 2).

3.2. Bactericidal effects on tomato plants inoculated by spraying

The ability of seven out of the 10 compounds evaluated *in vitro* to reduce symptoms produced by *C. michiganensis* subsp. *michiganensis* was evaluated in plants sprayed with the pathogen (Table 3). Early symptoms, such as yellow leaf margins and blister-like spots, appeared in inoculated plants 1 week after inoculation. Moreover, among inoculated controls and plants treated with Orthopol, Param and 8-hydroxy-quinoline, some displayed sideways leaf wilt, revealing an obstruction of vascular tissues due to systemic infection.

Copper sulphate, streptomycin, 8-hydroxy-quinoline+copper sulphate and 8-hydroxy-quinoline+Antibak RZ significantly reduced ($p<0.05$) the percentage of affected leaves in both assays, whereas kasugamycin, Antibak RZ, Param, streptomycin+Antibak RZ and streptomycin+Orthopol did so only in one. The highest reduction in percentage of leaf symptoms was achieved by applying 8-hydroxy-quinoline+copper sulphate (97.6% and 88.1%) and by copper sulphate (92.7% and 81.9%) in both trials (Table 3). These compounds also significantly reduced ($p<0.05$) the presence of blister-like spots on stems in both trials, compared with inoculated controls. For streptomycin, Antibak RZ, 8-hydroxy-quinoline+Antibak RZ, and streptomycin+Orthopol, this reduction was significant in only one of the two trials. Likewise, treatments with 8-hydroxy-quinoline, 8-hydroxy-quinoline+copper sulphate, 8-hydroxy-quinoline+Antibak RZ and streptomycin+Orthopol resulted in higher fresh weight in plants in one of the two trials (data not shown).

Clavibacter michiganensis subsp. *michiganensis* was easily isolated in samples obtained from symptomatic leaves and blister-like spots. Furthermore, the pathogen was isolated from internal tissues in more than 70% of inoculated plants except for those treated with copper sulphate (50%) and the combination 8-hydroxy-quinoline+copper sulphate (16.6% and 25%) in two independent experiments (Table 3). Significant lineal correlation was observed between pathogen isolation and development of blister-like spots on stems ($r^2=0.79$; $p<0.0001$) or leaf symptoms ($r^2=0.73$; $p<0.0001$) (Fig. 1).

Table 2
Effect of individual chemical compounds and combinations at sub-inhibitory concentrations ($\frac{1}{2}$ and $\frac{1}{4}\times$ MIC) that showed *in vitro* synergistic effect on *C. michiganensis* subsp. *michiganensis*, after 96 h of incubation

Initial inoculum (cfu ml^{-1})[a]	Combination of chemical compounds	Recovered cells (cfu ml^{-1})[a]
$1.17\pm0.09\times10^6$	$\frac{1}{2}$ 8-Hydroxy-quinoline (3 μg ml^{-1})	$6.32\pm2.01\times10^9$
	$\frac{1}{2}$ Copper sulphate (75 μg ml^{-1})	$6.88\pm1.62\times10^9$
	$\frac{1}{2}$ 8-Hydroxy-quinoline+ $\frac{1}{2}$ copper sulphate	$1.03\pm0.93\times10^2$
	$\frac{1}{2}$ 8-Hydroxy-quinoline+ $\frac{1}{4}$ copper sulphate	0
	$\frac{1}{4}$ 8-Hydroxy-quinoline+ $\frac{1}{2}$ copper sulphate	$1.63\pm1.19\times10^3$
	$\frac{1}{4}$ 8-Hydroxy-quinoline+ $\frac{1}{4}$ copper sulphate	$8.75\pm5.95\times10$
$1.17\pm0.09\times10^6$	$\frac{1}{2}$ 8-Hydroxy-quinoline (3 μg ml^{-1})	$5.48\pm2.02\times10^9$
	$\frac{1}{2}$ Antibak RZ (2.5 μl ml^{-1})	$8.88\pm5.81\times10^9$
	$\frac{1}{2}$ 8-Hydroxy-quinoline+ $\frac{1}{2}$ Antibak RZ	$3.75\pm3.60\times10$
	$\frac{1}{2}$ 8-Hydroxy-quinoline+ $\frac{1}{4}$ Antibak RZ	$2.25\pm2.60\times10$
	$\frac{1}{4}$ 8-Hydroxy-quinoline+ $\frac{1}{2}$ Antibak RZ	$1.08\pm0.47\times10^2$
	$\frac{1}{4}$ 8-Hydroxy-quinoline+ $\frac{1}{4}$ Antibak RZ	$1.25\pm1.44\times10$
$1.16\pm0.07\times10^6$	$\frac{1}{2}$ Streptomycin (3 μg ml^{-1})	$6.76\pm1.53\times10^9$
	$\frac{1}{2}$ Antibak RZ (2.5 μl ml^{-1})	$4.34\pm1.93\times10^9$
	$\frac{1}{2}$ Streptomycin+ $\frac{1}{2}$ Antibak RZ	$9.73\pm7.51\times10^2$
	$\frac{1}{2}$ Streptomycin+ $\frac{1}{4}$ Antibak RZ	$1.30\pm0.71\times10^4$
	$\frac{1}{4}$ Streptomycin+ $\frac{1}{2}$ Antibak RZ	$5.23\pm1.98\times10^3$
	$\frac{1}{4}$ Streptomycin+ $\frac{1}{4}$ Antibak RZ	$6.48\pm4.36\times10^5$
$1.16\pm0.07\times10^6$	$\frac{1}{2}$ Streptomycin (3 μg ml^{-1})	$7.74\pm2.32\times10^9$
	$\frac{1}{2}$ Orthopol (20 μl ml^{-1})	$5.27\pm1.66\times10^9$
	$\frac{1}{2}$ Streptomycin+ $\frac{1}{2}$ Orthopol	0
	$\frac{1}{2}$ Streptomycin+ $\frac{1}{4}$ Orthopol	5.00 ± 5.77
	$\frac{1}{4}$ Streptomycin+ $\frac{1}{2}$ Orthopol	6.25 ± 4.33
	$\frac{1}{4}$ Streptomycin+ $\frac{1}{4}$ Orthopol	1.38 ± 9.82

[a] Values presented are means (± SE) for four repetitions.

Table 3
Effect of chemical treatments on tomato leaf symptoms, presence of stem blisters and isolation of *C. michiganensis* subsp. *michiganensis* from internal tissues after spray inoculation of *C. michiganensis* subsp. *michiganensis* (two greenhouse trials)

Treatments[a]	Symptomatic leaves (%)[b,c]		Blisters in stem (SI)[b,d]		Pathogen isolation[e]	
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
Uninoculated control[f]	0.0	0.0	0.0	0.0	0/12	0/12
Inoculated control	75.1a	59.6ab	2.58ab	2.42a	12/12	12/12
8-Hydroxy-quinoline	70.7ab	55.9a–c	2.58ab	2.58a	12/12	9/12
Copper sulphate	7.3h	18.1e	0.42f	0.08c	6/12	6/12
Kasugamycin	51.1de	60.1a	2.17b–d	2.50a	12/12	11/12
Streptomycin	14.3g	47.1d	1.92de	2.33a	10/12	10/12
Antibak RZ	54.7cd	54.5a–d	2.08cd	1.92ab	12/12	9/12
Orthopol	71.3ab	58.0a–c	2.66a	2.50a	12/12	11/12
Param	63.6bc	59.6ab	2.50a–c	2.08ab	12/12	11/12
8-Hydroxy-quinoline+copper sulphate	2.4i	11.9f	0.41f	0.08c	2/12	3/12
8-Hydroxy-quinoline+Antibak RZ	66.8b	51.0cd	1.58e	2.33a	11/12	11/12
Streptomycin+Antibak RZ	43.2d	52.2b–d	2.42a–c	2.25a	12/12	11/12
Streptomycin+Orthopol	29.2f	55.6a–c	2.42a–c	1.5b	9/12	9/12

[a] Treatments were applied twice, 5 d before and 6 d after inoculation with the pathogen at the doses indicated in the text.
[b] Values are the mean of 12 plants per treatment. Numbers followed by the same letter are not significantly different according to Fisher's least significant difference (LSD) test ($p<0.05$).
[c] Data from percentage of symptomatic leaves were transformed to angular ($Y=\text{arcsine}\,[\%]^{1/2}$) for analysis of variance.
[d] Blisters on stems were evaluated following a disease severity index (SI) as described in Section 2.
[e] Positive isolation of the pathogen from samples obtained from the internal tissues of 12 tomato plants.
[f] Values equal to zero obtained from the uninoculated control were excluded from the analyses of variance.

3.3. Bactericidal effects on tomato plants inoculated by pricking

The compounds previously tested in plants inoculated by spraying were also assessed in tomato plants inoculated by pricking, as were the combinations of compounds at sub-inhibitory concentrations that provided satisfactory results in laboratory conditions (Table 4). After 2 weeks, inoculated tomato plants developed canker at the inoculation site and leaflet

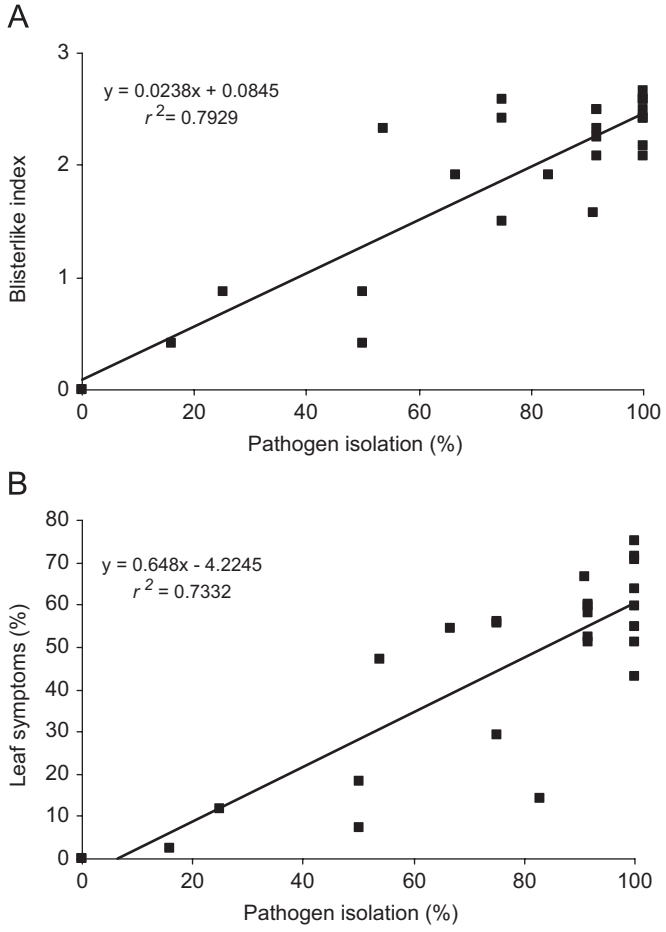

Fig. 1. Relationship between pathogen isolation from internal tissues and (A) development of blister-like spots on stems expressed as disease index on a scale of 0–3, or (B) leaf symptoms for plants spray-inoculated with *C. michiganensis* subsp. *michiganensis*, then treated with different compounds.

wilt, sometimes laterally in the first stages of the disease. The analyzed parameters showed that application of the assayed compounds or their combinations did not provide significant reduction of foliar wilt or vascular symptoms compared with untreated plants (Table 4), although in many treatments symptoms were reduced by varying degrees. Copper sulphate, Param, Orthopol, Antibak RZ and the combination of streptomycin+Antibak RZ significantly reduced ($p<0.05$) the length of vascular tissue affected in one of the two trials. Likewise, the height of plants treated with streptomycin+Antibak RZ and fresh weight of plants treated with streptomycin and Orthopol were significantly higher than untreated plants in one out of two independent experiments (data not shown). The pathogen was isolated from internal tissue in all inoculated plants.

4. Discussion

The use of antibacterial compounds is one of the first choices after outbreaks of bacterial plant diseases. However, such substances commercialized as generic bactericides have often not been evaluated specifically against the causal agent of the disease.

The results of this study, obtained *in vitro*, showed that the antibiotics oxolinic acid, oxytetracycline and streptomycin exerted the strongest effect against the four tested strains of *C. michiganensis* subsp. *michiganensis*, whereas kasugamycin was not effective at a concentration of 500 $\mu g\,ml^{-1}$. Similar results were reported for kasugamycin by Theodoro and Maringoni (2000), finding that a concentration of 1000 $\mu g\,ml^{-1}$ was necessary to inhibit *in vitro* growth of this bacterium. Other compounds like 8-hydroxy-quinoline, bronopol, copper sulphate, Antibak RZ and commercial potassium soap (Orthopol) also exerted *in vitro* antibacterial activity. Potassium oleate is a widely used insecticide in pest control (Parry et al., 1989; Trdan et al., 2006), although *in vitro* inhibition of Gram-positive bacteria has also been reported (Hinton and Ingram, 2003). A commercial formulation of menadione sodium bisulphite (Param), described as an effective resistance activator in banana (Borges et al., 2004) and with antifungal activity against *Fusarium oxysporum* f.sp. *lycopersici*

Table 4
Effect of chemical treatments on tomato leaf wilting and vascular symptoms in plants inoculated by pricking with *C. michiganensis* subsp. *michiganensis* (two greenhouse trials)

Treatments[a]	Wilting leaves (%)[b,c]		Vascular symptoms (cm)[b,d]	
	Trial 1	Trial 2	Trial 1	Trial 2
Uninoculated control[e]	0.0	0.0	0.0	0.0
Inoculated control	73.5a–c	64.2a–d	22.0a–c	25.6a–c
8-Hydroxy-quinoline	74.9a–c	70.9a–d	18.8a–d	27.5a–c
Copper sulphate	72.9a–c	81.4ab	13.2ef	29.5ab
Kasugamycin	82.4ab	56.5b–d	22.7ab	21.8c–e
Streptomycin	57.2c	47.5d	22.7ab	26.4a–c
Antibak RZ	62.2bc	64.1a–d	17.5c–e	19.4de
Orthopol	60.9a–c	50.8cd	20.9a–c	17.5e
Param	72.7a–c	56.1b–d	10.0f	24.2b–d
8-Hydroxy-quinoline+copper sulphate	83.3a	87.1a	23.2a	28.2ab
8-Hydroxy-quinoline+Antibak RZ	69.9a–c	59.4b–d	18.3a–d	24.6a–d
Streptomycin+Antibak RZ	61.8bc	88.8a	15.7de	30.3a
Streptomycin+Orthopol	71.5a–c	73.6a–c	18.1b–e	27.8ab

[a] Treatments were applied twice, 5 d before and 6 d after inoculation with the pathogen at the doses indicated in the text.
[b] Values are the mean of 12 plants per treatment. Numbers followed by the same letter are not significantly different according to Fisher's least significant difference (LSD) test ($p<0.05$).
[c] Data from percentage of wilting leaves were transformed to angular ($Y=\text{arcsine}\,[\%]^{1/2}$) for analysis of variance.
[d] Length of visible vascular symptoms.
[e] Values equal to zero obtained from the uninoculated control were excluded for the analyses of variance.

(Borges et al., 2000), also showed *in vitro* antibacterial activity in our assays.

A selection of antimicrobial compounds was made based on the *in vitro* results for *in vivo* assays in greenhouse-grown tomato plants inoculated by spraying and pricking. For both inoculation methods, typical reproducible symptoms were visible 1 week after inoculation. *In vivo* assays revealed that pricking inoculation with *C. michiganensis* subsp. *michiganensis* produced symptoms of systemic infection, while spraying did so only in some plants. Spray inoculation produced superficial infection in the early stages, whereas the vascular tissues of plants inoculated by pricking were invaded very rapidly, hindering treatment efficacy, even of the antibiotics streptomycin and kasugamycin. By contrast, streptomycin did reduce leaf symptoms produced by spray inoculation in two independent trials, and the blister-like spots on the stems in one.

Differences in disease control according to the inoculation method have been previously observed (Theodoro and Maringoni, 2000). Hausbeck et al. (2000) reported that streptomycin applied to seedlings inoculated by misting increased their survival after transplant and prevented severe disease symptoms from developing in the field. In our study, streptomycin was used as a positive control as its application is forbidden for agricultural practices in the EU. Kasugamycin, an antibiotic registered in several EU countries and recommended for the treatment of bacterial diseases, was not effective as already reported (Theodoro and Maringoni, 2000).

Our study revealed that copper sulphate combined with 8-hydroxy-quinoline and copper sulphate alone were the most effective treatments in reducing symptoms in plants inoculated with *C. michiganensis* subsp. *michiganensis* by spraying. Products containing copper have been reported to significantly reduce foliar blight and/or fruit spotting produced by this pathogen (Gleason et al., 1993). Furthermore, copper treatments were more active when mixed with mancozeb, suggesting a synergistic effect because mancozeb alone did not reduce populations or spread (Hausbeck et al., 2000). Such enhanced activity has also been reported on *Pseudomonas syringae* pv. tomato when copper is combined with carbamate fungicides. Copper ions bind to many organic substances in media and on plant surfaces and are not toxic to bacteria in this form. Copper-chelating carbamates may prevent the formation of these complexes, increasing toxic-copper availability (Cooksey, 1990). Similarly, 8-hydroxy-quinoline is known to form chelate complexes with divalent metal ions (Albert and Gledhill, 1947). Antibacterial activity of 8-hydroxy-quinoline plus copper sulphate was markedly enhanced on Gram-positive bacteria like *Staphylococcus aureus* (Rohde et al., 1976).

Data from this study evidence the synergistic effects of 8-hydroxy-quinoline+copper sulphate against *C. michiganensis* subsp. *michiganensis*. Combinations of both compounds at half concentration provided a significantly higher reduction of bacterial symptoms than copper sulphate alone or 8-hydroxy-quinoline alone, which did not significantly reduce disease symptoms. Application of intensive copper treatments to commercial crops over many years can lead to copper accumulation in soils, with subsequent negative effects on plants and the environment (Ninot et al., 2002). Furthermore, copper tolerance of plant-pathogenic bacteria seems to have increased since the 1980s (Andersen et al., 1991; Scheck et al., 1996; Sholberg et al., 2001). Consequently, copper applications on commercial crops should be reduced (Ninot et al., 2002). Our results show that copper sulphate at reduced dosages in combination with 8-hydroxy-quinoline or alone may be useful as a protective compound to prevent the pathogen spreading, or even as a post-infection treatment to reduce the risk of new external infections in greenhouse-cultivated tomatoes.

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