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Relationships between copper content in orange leaves, bacterial biofilm formation and citrus canker disease control after different copper treatments

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A B S T R A C T

Citrus canker is caused by *Xanthomonas citri* subsp. *citri* (*X. citri*), a severe and wide-spread bacterial pathogen. Repeated copper sprays are part of integrated management systems applied in countries where canker is endemic. The objective of this research was to evaluate the effect of a low copper compound, copper sulphate pentahydrate, as soil applications and sprays, on canker intensity, in comparison with traditional application of copper oxychloride. Two independent trials were conducted in 2010 and 2011 in a canker endemic sweet orange orchard. Products were monthly applied during springs. Copper content in young leaves and fruits after each application was assessed through an atomic absorption spectrometer. In addition, the ability of treatments to interfere with bacterial biofilm formation was evaluated under controlled conditions. Soil application of copper sulphate pentahydrate did not reduce citrus canker incidence. In 2011, the pulverization of copper sulphate pentahydrate and copper oxychloride reduced the incidence of the disease compared with untreated trees. Notably, copper content in leaves sprayed with the first compound was significantly lower than in leaves treated with copper oxychloride. However, both treatments showed the same ability to avoid biofilm formation, the first step in establishment of canker disease. Consequently, the pulverization of copper sulphate pentahydrate, integrated with the monitoring of the phenological tree stages and the weather conditions, could constitute a useful tool for the management of citrus canker disease, of high importance to avoid the adverse environmental effects, the risk of phytotoxicity, and the development of copper resistant bacterial populations.

1. Introduction

Citrus canker is caused by *Xanthomonas citri* subsp. *citri* (*X. citri*) (Schaad et al., 2006), which is the most adverse and extensive bacterial disease which affects citrus crops. Typical symptoms of the disease are necrotic canker lesions on fruit, stems and leaves. Defoliation, twig dieback and early fruit drop are the main damages of the disease, decreasing fruit quality and yield (Gottwald et al., 2002; Graham et al., 2004). Moreover, the most significant impact of the disease results in the restrictions of interstate and international fruit trade issued from canker-affected areas (Gottwald et al., 2002). In Argentina, citrus canker became an endemic disease after the failure of the eradication programs (Canteros, 2004).

X. citri is exuded and dispersed when free water is present on canker lesions. Rainfall and wind throughout the period of shoot and fruit growth are the main ecological factors that aggravate canker infection (Bock et al., 2005; Graham et al., 2004). After dispersion, *X. citri* grows epiphytically over the leaf surface forming a biofilm structure to obtain more favorable environment conditions for growth and survival before entering the tissues (Favaro

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et al., 2014; Rigano et al., 2007). Biofilm formation has been correlated with the virulence of *X. citri* and canker development (Malamud et al., 2011; Rigano et al., 2007; Yaryura et al., 2015). Moreover, the interference with biofilm formation has been linked to canker resistance in citrus plants (Favaro et al., 2014). Recently, it has been shown that copper sulphate is able to partially inhibit *X. citri* biofilm formation (Redondo et al., 2015). Likewise, the combination of this compound with small molecules such as D-leucine and 3-indolylacetonitrile avoid *X. citri* biofilm formation and reduce the symptoms of citrus canker (Li and Wang, 2014).

Chemical control and cultural practices are part of integrated management systems applied in countries where canker is endemic. Integrated control measures include planting pathogen-free certified trees, replacement of susceptible citrus species with resistant material, reduction of bacterial dispersion by surrounding orchards with windbreaks, insecticide treatments to reduce leaf-miner damage, and copper sprays (Behlau et al., 2010a, 2010b; Canteros, 2004; Leite and Mohan, 1990). Copper bactericides are strictly preventive, and fixed forms of copper such as copper oxychloride are the formulations most widely used (Behlau et al., 2010a, 2010b; Graham et al., 2010; Leite and Mohan, 1990; McGuire, 1988; Timmer, 1988). Pulverizations with copper are frequent during the growing season, when the susceptibility of tree organs is higher and meteorological conditions predispose pathogen infection (Behlau et al., 2010a). Bacterial ingress occurs when leaves reach 50 to 100% of its expansion (Gottwald et al., 2002), whereas the susceptibility of fruits is higher as they grow from 2 to 6 cm in diameter for a period of 90–120 days, depending on citrus species (Graham et al., 1992; Stall et al., 1981). An essential aspect when applying copper to control citrus canker, is the fact that the formation of new susceptible tissue may not be uniform between the trees, leading to unequal protection (Behlau et al., 2010a). In this context, the utilization of a systemic compound which could be applied to soil and act after bacterial penetration could be useful. Preliminary results using soil drenches of copper sulphate pentahydrate to control the Huanglongbing vascular pathogen *Candidatus* Liberibacter asiaticus, suggest that copper moves to recently expanded leaves and may act as a systemic bactericide in citrus plants (Graham, 2014). However, the behavior of this formulation for citrus canker control when is applied as a soil drench is still unknown.

The repeated utilization of copper bactericides has several disadvantages including induction of copper-resistance in *X. citri* populations (Behlau et al., 2013; Canteros, 2004) and copper increment in soils with its possible phytotoxic and negative ecological effects (Alva et al., 1995; Fan et al., 2011; Zhou et al., 2011). The use of bactericides with lower copper concentration but with the same efficacy is desirable in order to avoid these disadvantages.

The objective of this research was to evaluate the effect of a low copper compound as soil applications and sprays on citrus canker intensity, in comparison with traditional application of copper oxychloride. Meteorological and phenological data were associated with copper content in plant organs in order to understand the obtained results. In addition, the effect of foliar-applied copper treatments on bacterial biofilm formation was evaluated under controlled conditions.

2. Materials and methods

2.1. Trial description

Two independent, single-season trials were conducted in a commercial grove located in citrus canker endemic areas of Esperanza, Santa Fe, Argentina (31°26′ S; 60°56′ W; 40 m above sea level) during two consecutive years (2010–2011). According to Köppen classification (1936), the climate of this region is humid subtropical without a dry season. Mean temperatures of the region are 11 °C in winter and 25 °C in summer. Annual precipitations are of 938 mm distributed mainly throughout spring and early summer (between September and December), and autumn. Storms and average wind speed increase from August to December (García et al., 2002).

Ten year-old ‘Lanelate’ sweet orange (*Citrus sinensis* L. Osbeck) trees, grafted onto *Poncirus trifoliata* (L.) Raf. rootstocks were employed for the trial. Trees were planted on a 5 × 3 m spacing in a silt–loamy soil with complementary drip irrigation. Crop fertilization and pest control were made according to normal commercial practices in the region, and a natural windbreak barrier surrounds the grove (Favaro et al., 2014).

2.2. Copper treatments

During 2010 and 2011 springs, different treatments of copper-based bactericides were applied monthly from September to November. Products utilized were copper oxychloride (CO) (50% metallic copper), at the rate of 2 g L^{-1}, and copper sulphate pentahydrate (CSP) (21% metallic copper) at the rate of 2.5 mL L^{-1}. Products were mixed with water and spray-applied with a hand-gun, 2 L of solution per tree, and 1370 kPa of air pressure. A nonionic wetting agent (nonylphenyl polyethyleneglycol ether 20% w/w) was added to copper compounds at 0.05%. CSP was also applied at the same rate as a soil drench at 4 L per tree. To facilitate infiltration, soil was irrigated for ten minutes before drench treatment. Untreated control trees were sprayed with water. The dates of applications of copper compounds are listed in Table 1. The experimental design was a randomized complete block design with fifteen replications per treatment.

2.3. Field assessment of citrus canker disease

At the beginning of the growing season four branches in each tree were randomly selected in the four quadrants of the middle portion of the tree canopy (Favaro et al., 2014). Phenological stages of the branches were registered according to the Biologische Bundesanstalt, Bundessortenamt and Chemical Industry (BBCH) scale for citrus (Agustí et al. (1997). Canker incidence was measured throughout the growing season as the percentage (%) of leaves with canker lesions in each branch (Leite et al., 1987). The evaluation of disease severity was performed in the five most diseased leaves of each shoot, employing diagrammatic scales for small, medium and big cankers (Belasque et al., 2005).

The number of fruits per tree was registered before harvest and 30 fruits per tree were randomly selected and weighed to estimate fruit yield. Disease incidence was calculated on each plant as the proportion of symptomatic fruits. The evaluation of canker severity was performed in the ten most infected fruits of each tree, estimating the percentage of the fruit with symptoms.

Monthly meteorological data were obtained from the automatic meteorological Station situated in the Experimental Field of the Facultad de Ciencias Agrarias, Universidad Nacional del Litoral (31°26′ S; 60°56′ W; 40 m above sea level), and compared with the mean data for the last 30 years (Fig. 1).

2.4. Copper content in leaves and fruits

In order to quantify the effect of the treatments on the plant copper levels throughout the growing season, samples consisting in leaves of the new vegetative growth and small fruits were randomly collected from trees, approximately 20 days after each

Table 1
Dates of applications of copper treatments and phenological stages registered during years 2010 and 2011 in ‘Lanelate’ oranges trees.

2010			
Dates of applications	8/09	7/10	9/11
Phenological Stages[a]	BBCH11 first leaves visible BBCH55 flowers visible, still close, green bud	BBCH19 first leaves fully expanded BBCH60 first flowers open	BBCH91 shoot growth complete, foliage fully green BBCH71 fruit set, fruits with a diameter of 1.5–3 cm
2011			
Dates of applications	18/09	14/10	11/11
Phenological Stages[a]	BBCH15 more leaves visible, not yet at fully size BBCH56 flowers petals elongating, white bud	BBCH19 first leaves fully expanded BBCH69 petal fall	BBCH91 shoot growth complete, foliage fully green BBCH71 fruit set, fruits with a diameter of 1.5–3 cm

[a] Phenological stages registered in new shoots according to the BBCH scale for citrus (Agustí et al., 1997).

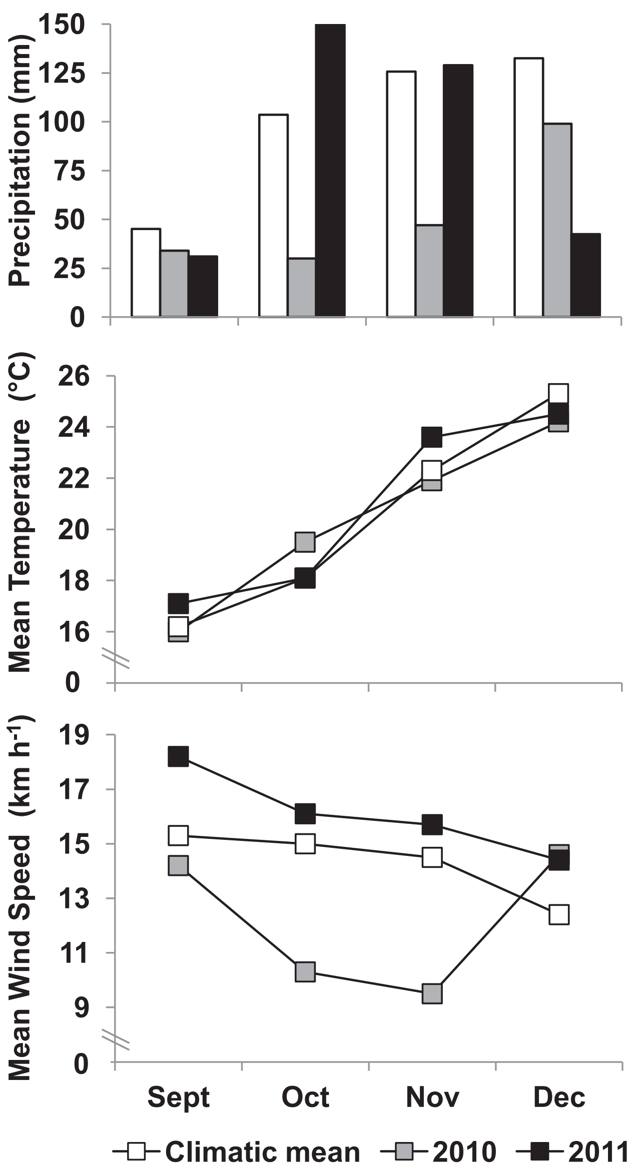

Fig. 1. Comparison between climatic mean and meteorological variables registered in years 2010 and 2011, in Esperanza, Argentina (31°26′ S; 60°56′ W; 40m over sea level). Meteorological data were obtained from the automatic Station situated in the Experimental Field of the Facultad de Ciencias Agrarias, Universidad Nacional del Litoral.

treatment application (Table 1). The samples were washed 3 times with tap water and 3 times with distillated water to eliminate soil particles, and subsequently dried for 48 h in an oven at 60 °C (Poschenrieder et al., 2001). Thereafter, 500 mg dry weight of each sample were grounded and digested following USEPA Method 200.3 (McDaniel, 1991), which uses repeated additions of nitric acid (HNO_3) and hydrogen peroxide (H_2O_2) to extract copper from the samples. Total copper in the digested solution was determined using an atomic absorption spectrometer (Perkin-Elmer, AAnalyst 200). Three replicates were evaluated for each treatment and date of collection.

2.5. Bacterial epiphytic growth and biofilm formation

Young flushes of one-year old ‘Lanelate’ plants grown under controlled greenhouse conditions were chosen for analysis of biofilm formation. CO and CSP were prepared as previously described and spray-applied to leaves at the BBCH15 phenological stage (Agustí et al., 1997) corresponding to a 50–80% of expansion. Leaves of three plants were sprayed with 10 mM $MgCl_2$ as control. A week later, leaves of the different treatments were pulverized with a suspension of 10^7 colony-forming units (CFU) mL^{-1} of *green fluorescent protein*-tagged *X. citri* cells (*X. citri*-GFP) prepared in 10 mM $MgCl_2$ solution (Favaro et al., 2014). Treated plants were kept under controlled conditions, with temperatures between 25 and 28 °C, relative humidity above 95%, a 14 h photoperiod and a light intensity of 150–200 μE m^{-2} s^{-1}. At 7 days post inoculation (dpi), leaves samples of approximately 1 cm^2 were cut and mounted on the adaxial surface under glass coverslips. Biofilm formation was monitored with an inverted confocal laser scanning microscope (CLSM, C1 Eclipse TE-2000-E2, Nikon Instruments Inc.) as previously described (Favaro et al., 2014; Rigano et al., 2007). Simulated three-dimensional images and sections were made using Nikon EZ-C1 3.9 Free Viewer software. Experiments involved a minimum of two shoots per plant and three plants for each treatment (Favaro et al., 2014).

2.6. Statistical analysis

Data of copper contents and yield parameters were subjected to a one-way analysis of variance (ANOVA) and the treatment means were separated using Tukey's test, $P < 0.05$ through InfoStat Software v2011 (Di Rienzo et al., 2011). In the particular case of incidence and severity, percentages were converted to proportion values and subjected to the arcsine-square root transformation before ANOVA and Tukey's test analysis.

3. Results

3.1. Influence of copper treatments on citrus canker intensity

The progress of citrus canker disease was different in the two years assessed. This behavior can be explained by variations in the phenology of the new growth and the environmental conditions (Table 1; Fig. 1). Phenological stages were similar between treatments during both years (Table 1). The two first applications were done between the phenological stages BBCH11 (first leaves were visible), and BBCH19 (first leaves fully expanded). Regarding reproductive stages, treatments correspond with BBCH55 (green bud) and BBCH69 (petals falling). Thus, these two applications protect the spring flush during a critical susceptibility period (Table 1). At the time of the third application, shoot growth was complete and the foliage was fully green (BBCH91) but the treatment was necessary to protect fruits during fruit set and expansion (BBCH71), a period of maximum fruit susceptibility (Table 1).

Average wind velocity and rainfalls from September to November during the first year were notably lower in comparison with the 30 years mean. Precipitations during the year 2010 were reduced 48% in comparison with the mean (Fig. 1). These meteorological conditions delayed the formation of canker symptoms in spring flushes to mid-November, after the third application. At this time, regardless of treatment applied, low levels of canker incidence, similar to those of untreated trees, were observed (Table 2). At the end of November canker incidence increased in all treatments. Incidence and severity were significantly higher in trees drenched with CSP compared with untreated trees and plants sprayed with CO or CSP (Table 2). Cankers were predominantly small in leaves of plants sprayed with CSP and CO, and in untreated trees, whereas they were predominantly medium size in plants drenched with CSP. None of the treatments reduced the canker intensity in fruits, although the soil application of CSP increased the incidence and severity (+208 and 109%, respectively) (Table 2).

In the following year, average wind velocity and temperature were above the 30-years mean throughout the growing season (Fig. 1). Total precipitations were slightly lower than mean but they were concentrated at the beginning of the growing season (October), allowing an anticipation of citrus canker outbreak (Fig. 1, Table 2). Little canker symptoms were evident in spring flushes since mid-October, after the second application. At this time, plants drenched with CSP showed significantly higher values of canker incidence than untreated plants and trees sprayed with CO, whereas plants sprayed with CSP showed significantly lower levels (Table 2). At the end of October, significant differences in incidence were found between all the treatments. Leaves of plants drenched with CSP showed the highest values of incidence, whereas leaves sprayed with CSP showed the lowest values (Table 2). Cankers were predominantly small in leaves of untreated plants and in spray treatments, whereas they were predominantly medium size in plants drenched with CSP (Table 2). At the end of November, after the third application, the incidence in trees sprayed with CSP and CO remained low and significant differences were found respect to untreated trees (−83 and −70%, respectively). Instead, the foliage of trees drenched with CSP achieved high levels of citrus canker incidence, similar to those found in untreated plants (Table 2). Cankers were medium size in untreated, CSP and CO sprayed leaves, but they were big in trees subjected to soil application of CSP (Table 2). Differences in canker severity between treatments were less notorious than incidence during the growing season (Table 2).

In fruits, the pulverization of CO and CSP significantly reduced the incidence of citrus canker compared with untreated plants (−67 and −65%, respectively), whereas soil drenches of CSP significantly increased this parameter (+208%; Table 2). Fruits of plants sprayed or drenched with CSP showed a significant lower (−58%) and higher (+109%) disease severity than untreated trees, respectively (Table 2).

3.2. Influence of the copper treatments in fruit size and yield

During both years of study, application of copper compounds did not significantly alter the mean weight of fruits compared with untreated trees. However, the number of fruits per tree was significantly lower in plants drenched with CSP comparing with untreated plants (−25 and −51%, in years 2010 and 2011,

Table 2
Effect of different copper treatments on incidence and severity of citrus canker in leaves and fruits of 'Lanelate' oranges trees, during years 2010 and 2011.

Measurement Date	Leaves						Fruits	
	15/11/10			31/11/10			10/03/11	
Treatment	Inc[a]	Sev[b]	Si[c]	Inc[a]	Sev[b]	Si[c]	Inc[a]	Sev[b]
Untreated trees	0.9 a	1.7 b	S	2.1 b	2.6 b	S	4.1 b	4.4 b
CO sprays	0.9 a	1.4 ab	S	1.5 b	2.6 b	S	4.8 b	5.0 b
CSP sprays	0.8 a	1.3 ab	S	1.4 b	1.8 b	S	4.7 b	4.8 b
CSP drenches	0.4 a	1.2 a	S	3.6 a	4.9 a	M	30.4 a	8.0 a
ANOVA *F*, *P* values	*F* = 2.25 *P* = 0.08	*F* = 4.64 *P* = 0.004		*F* = 14.7 *P* = 0.00	*F* = 7.8 *P* = 0.000		*F* = 787.5 *P* = 0.000	*F* = 41.85 *P* = 0.000

Measurement Date	Leaves									Fruits	
	17/10/11			31/10/11			25/11/11			05/03/12	
Treatment	Inc[a]	Sev[b]	Si[c]	Inc[a]	Sev[b]	Si[c]	Inc[a]	Sev[b]	Si[c]	Inc[a]	Sev[b]
Untreated trees	1.9 b	0.8 ab	S	4.4 b	1.39 ab	S	16.8 a	2.8 ab	M	25.8 b	20.2 b
CO sprays	2.3 b	0.3 b	S	2.9 c	0.8 b	S	4.9 b	1.3 ab	M	8.3 c	13.4 bc
CSP sprays	0.4 c	1.0 ab	S	1.0 d	0.8 b	S	2.9 c	0.9 b	M	8.9 c	8.3 c
CSP drenches	5.5 a	1.2 a	S	11.4 a	2.1 a	M	16.9 a	3.7 a	B	79.6 a	42.2 a
ANOVA *F*,*P* values	*F* = 90.38 *P* = 0.000	*F* = 4.61 *P* = 0.007		*F* = 154.7 *P* = 0.000	*F* = 2.95 *P* = 0.039		*F* = 140.34 *P* = 0.000	*F* = 4.19 *P* = 0.007	*F* = 37.77 *P* = 0.000	*F* = 53.44 *P* = 0.000	

Percentages of incidence and severity were converted to proportion values and subjected to the arcsine-square root transformation before ANOVA.
Means followed by different letters in each column indicate significant differences according to Tukey's test, $P < 0.05$.
[a] Inc: canker incidence measured as percentage (%) of leaves or fruits with canker lesions over total examined.
[b] Sev: evaluation of canker severity in the five most infected leaves of each shoot or in the ten most infected fruits of each tree.
[c] Si: canker sizes according to Belasque et al. (2005): S: small, M: medium, B: big.

respectively). As a result, yield was notably reduced by soil application of CSP (Fig. 2).

3.3. Copper content in plant organs

The evolution of copper content in plant organs throughout the growing season was explored during the second year of study. After the first copper application, just the CO spray treatment generated significantly higher copper levels in the leaves of the new vegetative growth (+174%; Fig. 3). After the second application, copper content in the foliage of trees sprayed with CSP and CO was

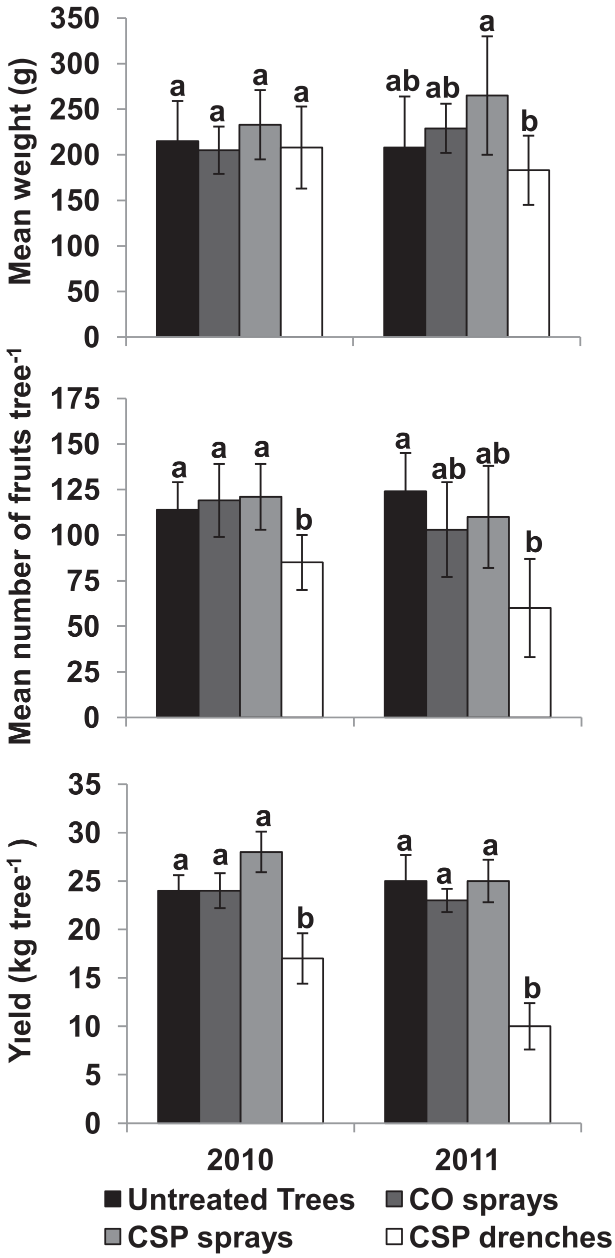

Fig. 2. Effect of different copper treatments on yield parameters of 'Lanelate' oranges trees during years 2010 and 2011. The number of fruits per tree was registered before harvest and 30 fruits per tree were randomly selected and weighed to estimate fruit yield. Columns followed by different letters in the same year differ statistically according to Tukey's test, *P*<0.05. CO: copper oxychloride; CSP: copper sulphate pentahydrate.

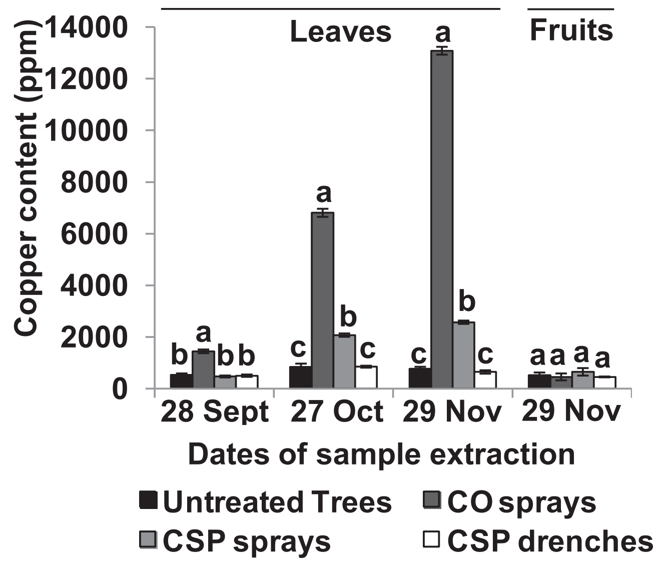

Fig. 3. Copper content in leaves and fruits of 'Lanelate' oranges trees after different treatments. During year 2011 leaves of the new vegetative growth and small fruits were collected after each treatment application and digested to extract copper. The content of copper in the digested solution was determined using an atomic absorption spectrometer. Three replicates were evaluated for each treatment and date of collection. Columns followed by different letters in the same date differ statistically according to Tukey's test, *P*<0.05. CO: copper oxychloride; CSP: copper sulphate pentahydrate.

significant higher than that of untreated plants (+146 and +708%, respectively) and CSP drenched trees (Fig. 3). The same trend was observed after the third application. Notably, copper contents after the second and third application, were approximately 3 and 5-fold lower in spring flushes sprayed with CSP than in those sprayed with CO. On the other hand, copper content in fruits was similar among all treatments 20 days after the third application (Fig. 3).

3.4. Copper influence on biofilm formation

In order to determine if copper application interferes with biofilm formation on the leaf surface, 'Lanelate' leaves were treated with CSP and CO and sprayed with *X. citri*-GFP suspensions a week later. CLSM was employed to visualize the bacterial aggregates formed during canker development at 7 dpi, the time at which biofilms structures could easily be distinguished (Rigano et al., 2007).

Colonization of *X. citri*-GFP was notorious in control leaves sprayed with $MgCl_2$ at 7 dpi, particularly surrounding the cells of the stomata, the main entrance pathway for bacteria. In the ZX axis projected images it was possible to distinguish the formation of three-dimensional bacterial aggregates (Fig. 4). On the contrary, *X. citri*-GFP cells could not be observed when bacteria were sprayed one week after copper application (Fig. 4). Notably, it was not possible to found differences among CSP and CO treatments at 7 dpi. Thus, both copper formulations interfered in the same way with bacterial survival and biofilm formation.

4. Discussion

The efficacy of copper sprays to decrease citrus canker has been previously demonstrated and depends on many factors such as the copper formulation, frequency, rate and application method; initial inoculum levels; host susceptibility; tree phenological stage at the time of the applications; meteorological conditions; and the

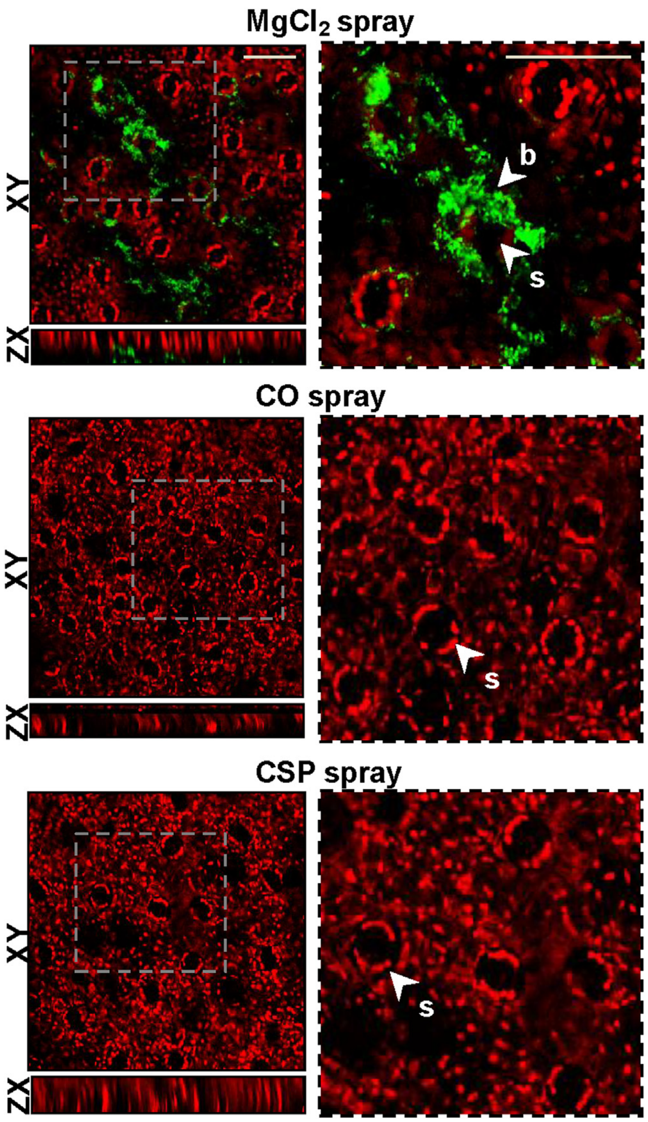

Fig. 4. Copper application interferes with biofilm formation of *Xanthomonas citri* subsp. *citri* (*X. citri*) in 'Lanelate' leaves. Epiphytic growth and biofilm formation of the green fluorescent protein (GFP)-tagged *X. citri* strain (*X. citri*-GFP) monitored with confocal laser scanning microscope (CLSM). Orange leaves were sprayed with bacterial suspensions (10^7 CFU mL^{-1}) one week after the application of copper compounds. Sections from the photographs in the left are shown enlarged at right. Red indicates chlorophyll autofluorescence and green GFP-tagged bacteria. XY and XZ are the XY and XZ axis projected images, respectively. Scale bars, 50 μm. s: stomata; b: bacteria. The experiment involved a minimum of two shoots per plant and three plants for each treatment. CO: copper oxychloride; CSP: copper sulphate pentahydrate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

utilization of another management strategies (Behlau et al., 2010a; Graham et al., 2010; Leite et al., 1987; McGuire, 1988; Stein et al., 2007; Timmer, 1988). Likewise, the capacity of *X. citri* to infect *Citrus* spp. is related to host susceptibility and phenology, and the environmental conditions (Bock et al., 2005; Favaro et al., 2014; Graham et al., 2004). In this study, taking account the weather conditions and phenology of the trees during 2011, monthly sprays of CSP and CO were enough to maintain low incidence and severity of citrus canker in spring flushes. In year 2010, unfavorable meteorological conditions for pathogen infection and dispersion delayed the formation of canker symptoms in all treatments. Conversely, disease incidence was higher in 2011 when the weather conditions allowed the progress of the disease. Hence, differences among sprayed (CSP and CO) and untreated trees were significant in the second year (Table 2).

In year 2011, copper sprays increased the level of this element in leaves from the spring flushes and consequently reduced the incidence of diseased leaves and fruits compared with untreated trees (Table 2; Fig. 3). Notably, copper content in leaves sprayed with CSP was significantly lower than those of leaves treated with CO (Fig. 3). This preservation of control efficacy after the decrease of the copper quantity implicates that greater amounts of copper might be being applied excessively. Thus, as shown in the present study, the amount of metallic copper applied could be reduced notably without compromising control disease. Similarly, Scapin et al. (2015) showed that is possible to diminish markedly the quantity of water and copper applied keeping the efficacy of the treatments, applying the tree-row-volume methodology for management of *X. citri* on *Citrus sinensis* plants. The reduction in the copper quantity applied to the trees is of high importance to avoid the accumulation of this element in soils, with a possible negative ecological impact, and the risk of phytotoxicity and stippling of the fruit (Alva et al., 1995; Graham et al., 2010; McGuire, 1988; Stein et al., 2007). Moreover, the use of low-copper compounds would be prudent in order to avoid the development of copper resistant *X. citri* populations, which already exists in Argentina (Behlau et al., 2013; Meneguim et al., 2007).

In addition to the mentioned advantages, sprays of CSP or CO showed the same capacity to impede bacterial biofilm formation under controlled conditions (Fig. 4). Proper biofilm formation is a prerequisite to reach maximal virulence, is essential for *X. citri* epiphytic survival and canker development (Malamud et al., 2011; Rigano et al., 2007; Yaryura et al., 2015). Bacterial attachment to a surface, microcolonies conformation, and finally biofilm maturation are the three steps of biofilm development (Stanley and Lazazzera, 2004). When biofilm is mature, it functions as a protection strategy that enables bacterial aggregates to overcome abrupt environmental changes (Rigano et al., 2007). In this regard, in a recent study, Redondo et al. (2015) showed that once biofilms are established, copper sulphate is not able to completely disrupt them. The results obtained in our work demonstrated that when copper is applied before to epiphytic colonization of *X. citri*, acts as an environmental agent which interferes with the initial stages of biofilm formation, avoiding inoculums increment in young tissues and establishment of canker disease. Similarly, Redondo et al. (2015) showed a partial inhibition in biofilm formation with $CuSO_4$ in lemon leaves. However, some important differences should be noted between both studies. Plant host (orange versus lemon) and the inoculation procedure were different. In the cited work, *X. citri* was cultured in liquid media amended with $CuSO_4$ at 0.0125% and then sprayed on leaves of lemon, whereas in our study, plants were first treated with the bactericide and then sprayed with bacteria after a week, simulating the conditions of a protectant treatment in the field. Thus, the obtained results show that the role of copper bactericides is strictly preventive (Behlau et al., 2010a; Scapin et al., 2015).

Soil application of CSP did not reduce citrus canker incidence over each of the two seasons (Table 2). Moreover, copper levels in spring flush were similar to those found in untreated trees (Fig. 3). Taken together, these results suggest the inability of CSP to be translocated to new vegetative growth in oranges when is applied to the soil. In *Citrus* sp., copper accumulated in roots in proportion to the amount added to soil (Graham et al., 1986). It has been demonstrated that copper toxicity in roots has an impact on root function and mineral nutrition, interfering absorption of nutrients and weakening plant health (Graham et al., 1986). Copper increment in soils has been reported to diminish the quantity and variability of microorganisms (Zhou et al., 2011). Thus, the reduced yield and the effect of higher incidence and severity of the disease on fruit drop during the growing season of trees drenched with CSP, could be a consequence of the increment of soil and roots copper content affecting fruit set. Recently, Graham (2014) found that a chelate formulation of copper sulphate pentahydrate moved to recently expanded leaves when is applied as soil drenches with the objective of control Huanglongbing, but the treatment was ineffective and also cause phytotoxicity. The discrepancy in results of copper mobility between this study and our work could be due to the different CSP formulation and concentration employed, and to the use of different host age and cultivar between both studies.

The frequency of the treatments in this work was not enough to cover the whole fruit susceptibility period to *X. citri*, because at the time of the last copper application, fruits only had reached 1.5–3 cm diameter (Table 1), explaining why the incidence and severity of the disease in fruits were higher than in leaves, even in the more effective treatments. Copper content in fruits was similar among treatments after the third application (Fig. 3). This is probably because fruits were small at the time of the application, and fruit surface area increased rapidly before sample extraction, diluting differences in copper content among treatments. Moreover, although CSP and CO pulverizations reduced the intensity of citrus canker in fruits, this reduction did not produce differences in yield respect to untreated trees (Fig. 2). Similarly, Behlau et al. (2010a), found that monthly copper sprays were effective for decreasing citrus canker incidence on orange organs. However, a greater number of applications was necessary to significantly decrease yield loss. Nevertheless, copper applications are necessary in order to reduce disease intensity and inoculums quantity on the plant during the favorable period for canker formation and bacterial dispersion, and to reduce bacterial survival for the following growing season (Behlau et al., 2010b).

This work demonstrated that soil CSP application produces a negative effect on canker control and citrus tree yield. However, the pulverization of this low copper compound, integrated with the monitoring of the phenological tree stages and the weather conditions, could constitute a useful tool for the management of citrus canker disease, preventing, like traditional CO, *X. citri* biofilm formation and avoiding inoculum build-up on new leaf flush.

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