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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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ABSTRACT

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 leafminer 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