Research Article

Bacillus subtilis QST 713, copper hydroxide, and their tank mixes for control of bacterial citrus canker in Saudi Arabia

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Abstract

Citrus bacterial canker (CBC) is a devastating disease that affects production of almost all commercial citrus cultivars in subtropical citrus growing regions worldwide. In this study, the effect of monthly foliar sprays of wettable powder formulation Serenade® MAX of *Bacillus subtilis* QST 713, alone or as tank mixes with copper hydroxide, on CBC disease development was evaluated under greenhouse and open air nursery conditions. The Serenade® MAX as a tank mix with copper hydroxide significantly reduced the disease severity and incidence, followed by the copper hydroxide treatment, compared to the control. The disease incidence on leaves of inoculated trees treated with a combination of copper with Serenade® MAX was never higher than 19%, whereas the disease incidence reached 43% for non-sprayed trees. It was possible to reduce the number of copper sprays down to 6 sprays per season when it was mixed with the bio-fungicide Serenade® MAX to effectively control CBC, compared with 8 sprays of copper hydroxide alone per season. Based on our results, the application of Serenade® MAX may have not only the potential for CBC management in conjunction with copper hydroxide or other disease control measures, but may also reduce the frequency or rate of copper sprays in citrus groves.

Keywords: Citrus bacterial canker, bio-fungicide, disease control, foliar sprays

Introduction

Citrus bacterial canker (CBC), caused by *Xanthomonas citri* subsp. *citri* (*Xcc*), is a challenging and widespread disease of citrus, causing extensive losses in fruit quantity and quality (Gottwald et al. 2002). There are no highly effective strategies for controlling CBC disease development. Copper-based bactericides have been used widely for control of CBC and other foliar bacterial diseases (Dumestre et al. 1999). Copper applications provide satisfactory levels of citrus canker control where the disease is endemic by reducing inoculum buildup on new leaf flushes and protecting expanding fruit surfaces from infection (McGuire 1988; Timmer 1988; Gottwald and Timmer 1995; Graham and Leite 2004; Behlau et al. 2008). The average number of copper sprays needed may range from 2 to 7 sprays per season (Leite and Mohan 1990) and depends on many factors such as environmental conditions, the susceptibility of the citrus cultivar, and adoption of other control measures (Stall and Seymour 1983; Gottwald et al. 2002). Behlau et al. (2010) reported that control of CBC increased as spray interval decreased; they found 7 days interval applications of copper oxychloride reduced foliar canker incidence on leaves and fruits compared to 14 to 28 days intervals. The problems with the continuous application of copper compounds are the accumulation and contamination of soils with potentially phytotoxic compounds, in addition to the development of copper resistant *Xcc* strains, which have been reported in Argentina (Canteros et al. 2008). To manage the copper-resistant strains, manzeb or mancozeb mixed with copper-based bactericides or with new chemistries were reported (Roberts et al. 2008; Fayette et al. 2012). In recent years, alternative tools to reduce CBC disease were investigated with some degree of success, including the use of plant resistance activators (Graham and Leite 2004), bacteriophage (Balogh et al. 2008), and plant extracts of neem (*Azadirachta indica*), ginger (*Zingiber officinale* Roscoe), and curcuma rhizomes (*Curcuma longa* L.) (Vechet et al. 2009). These biological approaches could also be used in integrated disease management programs. Programs using multiple products with diverse modes of action have shown promise for management of bacterial diseases (Obradovic et al. 2004; Gent and Schwartz 2005; Ji et al. 2006). *Bacillus subtilis* QST 713, a bio-fungicide and bactericide available in a wettable powder formulation (Serenade® MAX) (Bayer, CropScience), has been used on various crops to control foliar bacterial and fungal diseases (Roberts et al. 2008; Gilardi et al. 2009; Abbasi and Weselowski 2014; Paçe et al. 2016). There is little scientific data on the efficacy of Serenade® MAX, a commercial bio-fungicide, either alone or in tank mixes with copper hydroxide to control CBC disease. Because Serenade® MAX is a novel control measure against CBC in Saudi Arabia, greenhouse and nursery experiments were conducted to establish its efficacy in this country.

Materials and Methods

Bacterial strain and inoculum preparation

The *Xcc* strain JQ890095 used in this study was isolated from Mexican lime (*Citrus aurantifolia*) leaves grown in a commercial citrus orchard in the Jazan region, Saudi Arabia (Al-Saleh et al. 2014). *Xcc* inoculum was prepared in nutrient broth (Merck Millipore, Germany) and grown at 28 °C for 24 h to log phase and the bacterial suspension was centrifuged at 10,000 x g for 20 min, washed and re-suspended in phosphate buffer saline (PBS; 40 mM Na_2HPO_4, 25 mM KH_2PO_4), and adjusted to 10^5 cfu mL^{-1}. The optical density of the suspension was measured using a JENWAY 6175 spectrophotometer (Bibby Scientific limited, Staffordshire, UK) at A_{600nm}.

Greenhouse experiments

Nine-month-old seedlings of Mexican lime were used in this study. Seedlings were fertilized twice a month with 20-10-20 (NPK) and supplemented with Essential Minor Elements (NAFCO Co., Saudi Arabia). Four weeks prior to the treatments, seedlings were cut back to approximately 40 cm, and only 1 shoot per plant was allowed to grow to approximately 20 to 30 cm in order to obtain 4 to 5 immature leaves (75% expanded) suitable for treatment and inoculation. Approximately 3 weeks later, the emerging foliage was inoculated with *Xcc* suspension (10^5 cfu/ml) from 9:00 to 12:00 AM when stomata were fully open. The plants were irrigated and covered with polyethylene bags for about 48 hours to promote maximum humidity, followed by inoculation. Serenade® MAX and copper hydroxide fungicide (CuOH, Kocide 2000, 35% metallic Cu) were sprayed alone or as tank mixes (Table 1). Treatment suspensions were made fresh prior to each spray and applied directly onto seedlings. Monthly applications of copper hydroxide and Serenade® MAX at the rate of 1.5 g/L^{-1} and 10^7 cells ml^{-1} respectively were applied from September to April in each greenhouse experiment. In each experiment, a total of 8 sprays were applied (Table 1). Sprays were applied with a backpack sprayer (Gloria Co. Germany) calibrated to deliver approximately 2 liters of spray material per tree. Control plants were sprayed with water. Copper and Serenade® MAX applications started 7 days before inoculations and continued until the last rating was taken. Plants were arranged in a completely randomized design and the experiment was repeated twice with 3 replications and 8 plants in each replication. Tests to confirm efficacy of Serenade® MAX against *Xcc* were conducted in a quarantine greenhouse at the Department of Plant Protection, King Saud University. The day/night temperatures were 27/23 ± 2 °C.

Table 1

Spray materials applied to Mexican lime plants in the greenhouse or an open air nursery at King Saud University nursery, Riyadh, Saudi Arabia.

Treatment number[a]	Product name	Active ingredient	Product rate (g/L)
1	Untreated control	Water	-
2	Kocide 2000[b]	Copper hydroxide	1.5 g/L^{-1}
3	Serenade® MAX QST 713	*B. subtilis*[d]	10^7 cells ml^{-1}
4	Kocide + Serenade® MAX QST 713[c]	Copper hydroxide + *B. subtilis*	1.5 g/L^{-1} + 10^7 cells ml^{-1}

[a]Freshly made suspensions were applied onto foliage monthly and 8 sprays in each experiment at greenhouse; [b]Applied as wettable powder; [c]Treatment suspensions were applied in tank mixes; [d] Applied as dry flowable formulation.

CBC nursery experiments

Based on the results of the greenhouse experiments, Serenade® MAX was evaluated in spray programs with and without copper hydroxide in 2 experiments carried out during 2013/2014 and 2014/2015 in an open air nursery at King Saud University, Riyadh, Saudi Arabia. The experimental design for the nursery experiments was a randomized block with 5 blocks of 5 Mexican lime per treatment (5 trees in a row, 25 trees per treatment) for 4 treatments. Average monthly temperatures and rainfall totals for October to September of each year were recorded (Fig. 1). Treatments and applications are described in Table 1.

Disease assessment and data analysis

Disease incidence (percentage of diseased leaves) was evaluated monthly from October through April in each greenhouse/open air nursery experiment (Leite et al. 1987). Citrus canker severity was estimated using the Belasque et al. (2005) scale. Data were subjected to ANOVA using SAS PROC GLM version 9.1. Means were analyzed using Fisher's Least Significant Difference ($P<0.05$). The equation proposed by Shaner and Finney (1977) was used to calculate the area under disease progress curve (AUDPC). In the repeated greenhouse experiments, disease incidence and severity of different treatments were not significantly different between the 2 experiments; therefore, data were pooled and analyzed.

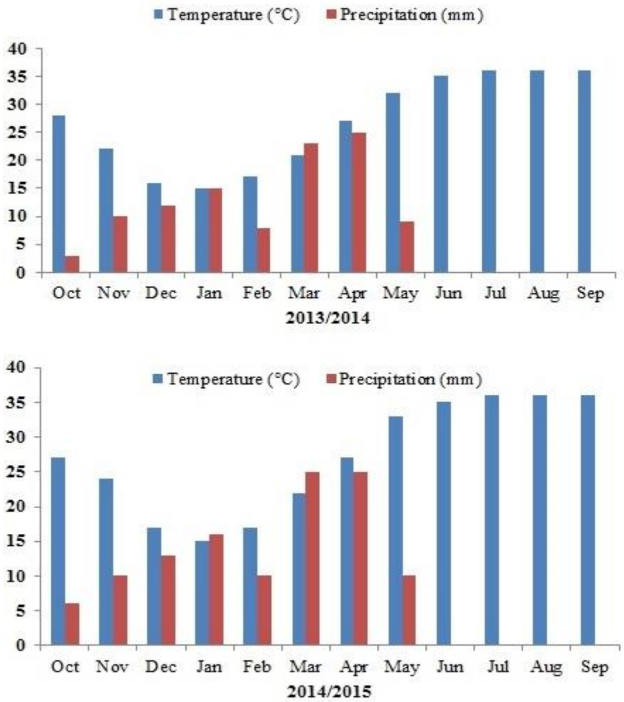

Fig1. Monthly average temperature (°C) and cumulative precipitation (mm) from October to September during 2013/2014 and 2014/2015 growing seasons, at an open air nursery at King Saud University, Riyadh, Saudi Arabia.

Results

Greenhouse experiments

Generally, from our experiments, the disease incidence and severity were significantly reduced for all treated plants with different control levels (P = 0.0001) compared to the untreated inoculated control plants (Table 2, Fig. 2). The lowest values of disease incidence and severity were observed in plants treated with a combination of copper and Serenade® MAX (8.72 and 1.4, respectively) followed by the copper treatment alone (10.3 and 1.8, respectively) (Table 2). However, Serenade® MAX application significantly reduced citrus canker incidence and severity, but was less effective than copper hydroxide alone or their combination in both experiments (Table 2). Peaks of citrus canker incidence on leaves showed similar magnitude in both experiments (Fig. 2). The citrus canker incidence on leaves reached the highest percentage in March at 46% in the untreated plants in the 2 experiments (Fig. 2). The efficacy of copper combined with Serenade® MAX in the first and second experiments at 6 sprays (8%) in significantly reducing canker incidence on foliage was equal to that obtained with the copper treatment alone (13% and 14%, respectively) at 8 sprays.

Nursery experiments

The average temperature and rainfall did not differ between the 2 seasons for the same period. Temperatures were relatively warmer (25 to 36 °C) during the rainy season (November to March) while, in the following 2014/2015 season, a longer rainy season associated with warmer temperature increased disease progress. Based on greenhouse experiments, Serenade® MAX or copper alone were applied in the 2 seasons at 8 sprays while Serenade® MAX in combination with copper was applied at 6 sprays (Table 3). Overall, no treatment tested was able to completely control the development of the CBC disease, where all plants showed leaf canker symptoms 2 weeks after spray-inoculation. However, there was significant control of disease incidence by all treatments compared to the untreated inoculated plants in 2 successive seasons (Table 3).

Table 2
Effects of Serenade® MAX, copper hydroxide, and their tank mixes on Xcc-inoculated citrus plants under greenhouse conditions.

Treatments	Mean disease incidence on leaves[x]	% Disease control	Mean disease severity on diseased leaves[z]
Copper	10.30c[y]	85.55	1.80c
Serenade® MAX	15.20b	78.68	2.40b
Copper + Serenade® MAX	8.72bc	87.79	1.40c
untreated control	71.30a	0	4.80a

[x] Disease incidence: percentage of plants exhibiting CBC symptoms; [y] Means within the same row followed by the same letter are not significantly different (LSD at P = 0.05); [z] Disease severity: percentage of diseased plant surface area.

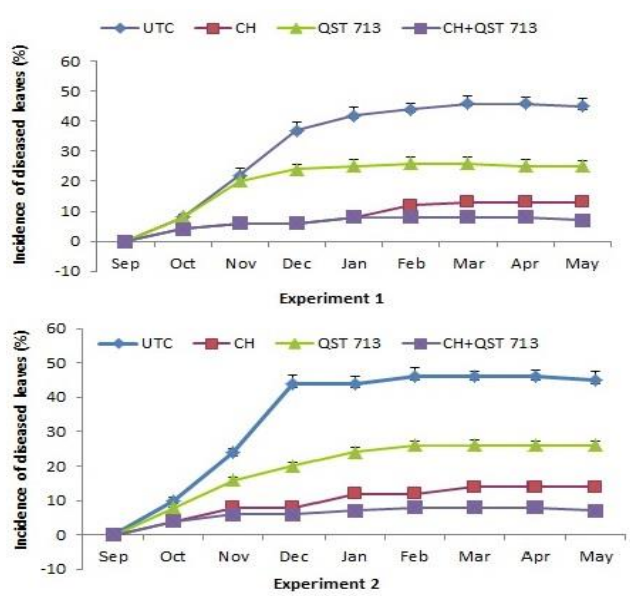

Fig.2. Citrus canker incidence evaluated monthly during the 2 greenhouse experiments on plant leaves treated with Serenade MAX, copper hydroxide, and their tank mixes. UTC = untreated control, CH = copper hydroxide, and QST 713 = Serenade® MAX.

Table 3
The mean canker incidence on leaves, the area under the disease progress curve (AUDPC) for canker incidence on leaves, and the percentage of disease control using different spray treatments under open air nursery conditions during 2013/2014 and 2014/2015 seasons.

Treatments	No. of applications	Season 2013/2014			Season 2014/2015		
		Incidence on leaves	AUDPC	% Disease control	Incidence on leaves	AUDPC	% Disease control
UTC	-	37.36a	20.30a	0	40.16a	21.60a	0
Copper	8	4.72c	2.70c	86.69	5.40cd	4.130c	80.87
QST 713	8	18.80b	6.40b	68.47	23.70b	6.20b	71.29
Copper + QST 713	6	5.88c	2.43c	88.02	7.70c	3.70bc	82.87

Means followed by the same letter are not significantly different (LSD at P=0.05)
UTC= untreated control, Copper= copper hydroxide, and QST 713= Serenade® MAX.

In the first season of 2013/2014, the disease incidence of the treated plants ranged from 4.72% to 18.8% compared to the 37.36% on the untreated control plants (Table 3). The environmental conditions in October of the first season where the recorded temperature and precipitation were 33 °C and 0 mm may explain the lower rate of disease incidence on the untreated control plants. Serenade® MAX was less effective when used alone than the copper treatment (Table 3). The same trend was also observed in the second season of 2014/2015, where copper hydroxide mixed with Serenade® MAX significantly reduced canker incidence by 86.5% compared to the untreated control. The disease incidence on untreated trees was up to 9 times higher than observed on the trees treated with a combination of copper with Serenade® MAX (Table 3). Also, the disease incidence on the leaves was directly related to AUDPC values.

The AUDPC values clearly demonstrated that all treatments were effective against *Xcc* compared with the untreated control. The AUDPC values for treatments of either copper alone or its mixture with Serenade® MAX followed the same trend as the 2013/2014 season and peaked at 4.13 and 3.7 respectively (Table 3). The percentage of disease control for these application regimes ranged from 68.47% to 88.02% compared to the untreated control. Collectively, 6 sprays of a combination of copper and Serenade® MAX treatment were not significantly different from copper alone at 8 sprays in controlling CBC disease (Table 3). At the end of the experiments the plants were destroyed due to the appearance of CBC disease, thus prohibiting further experiments.

Discussion

One of the most popular CBC chemical control strategies in citrus production regions, including Saudi Arabia, is the application of copper bactericides. However, the long-term use and multiple applications of these bactericides can develop copper resistant strains of *Xcc*, which have already occurred in Argentina (Canteros et al. 2008). Moreover, the intensive use of copper-based bactericides can lead to environmental pollution and copper accumulation in soils (Alva et al. 1995). To reduce the copper impact on the environment as well as the selection pressure on *Xcc* populations, this study was undertaken to evaluate the efficacy of Serenade® MAX, a commercial bio-fungicide, alone and in tank mixes with copper hydroxide for CBC management.

The most consistently effective treatment for reducing CBC disease development was achieved through the integration of copper with Serenade® MAX. Under nursery conditions, the disease incidence on leaves of *Xcc*-inoculated trees treated with a combination of copper with Serenade® MAX was less than 19%. In contrast, the disease incidence was as high as 43% for non-sprayed *Xcc*-inoculated trees. The application of copper bactericides in combination with bio-fungicide may increase production costs but sometimes is more effective and environmentally sounds than bactericide-dependent programs. Similar results were also achieved in controlling early blight of tomato using foliar sprays of Serenade® MAX integrated with copper (Abbasi and Weselowski 2014), without altering the number of chemical sprays. Abbasi and Weselowski (2014) found that weekly sprays of Serenade® MAX in tank mixes with copper hydroxide consistently reduced foliar disease severity under highly conducive disease conditions.

In this study, it was possible to reduce the number of copper sprays to 6 sprays when copper hydroxide was mixed with the bio-fungicide Serenade® MAX to effectively control CBC compared with 8 sprays of copper hydroxide alone. The use of copper sprays to control CBC in susceptible to highly susceptible cultivars requires the integration of different control measures to reduce disease development on citrus fruits, taking into consideration that the copper should not be used as a standalone measure (Leite and Mohan 1990). The application of Serenade® MAX alone did not significantly reduce disease incidence of CBC on Mexican lime leaves as compared to copper alone but it was more effective than untreated trees inoculated with *Xcc*.

The mode of action of Serenade® MAX or its effect on the CBC pathogen was not investigated in this study. However, in other studies, Serenade® MAX has been shown to be significantly or partially effective against *Pseudomonas syringae* pv. *syringae* and *Xanthomonas* on tomato plants under greenhouse conditions (Roberts et al. 2008; Gilardi et al. 2009) and *Erwinia amylovora* on apple trees (Sundin et al. 2009). When *B. subtilis* strain QST 713 was mixed with chemical fungicides, Alternaria leaf blight of ginseng was more effectively controlled (Li et al. 2008). Chen et al. (2009) reported that Serenade® MAX produces various antibiotics and these findings can be correlated with the ability of *B. subtilis* in controlling phytopathogenic bacteria. In addition, it has been reported that Serenade® MAX produces the polyketide compounds bacilysin, macrolactin, and difficidin; and the lipopeptides Surfactin and Iturin A that may have significant roles in controlling phytopathogens (Chen et al. 2009; Kinsella et al. 2009). The other possible mode of action of Serenade® MAX is its ability to induce the plant defense resistance (Lahlali et al. 2013).

As demonstrated in the present study, the application of copper hydroxide alone was effective in reducing CBC disease development in the 2 successive experiments (2013/2014 and 2014/2015), but the same results were obtained with 6 sprays of a mixture of Serenade® MAX with copper. The extensive use of copper-based bactericides (e.g. 26 sprays per season) may provide better protection than the combination of copper with Serenade® MAX for reducing disease incidence on leaves and fruits of sweet orange (Behlau et al. 2010). However, the ability of bacteria to grow and survive in an environment with high concentrations of heavy metals leads them to develop resistance against these heavy metals (Teixeira et al. 2008; Rensing and Grass 2003). Therefore, the integration of copper with Serenade® MAX could be employed to decrease the chance of developing copper resistant bacterial strains by reducing the frequency of copper application for highly susceptible young trees. Citrus canker incidence on leaves during the 2 growing seasons peaked in February, March, and April as a result of rainfall levels and moderate temperatures conducive to disease development. The application of 6 sprays of a combination of copper with Serenade® MAX in an integrated control program may act as a safeguard when the weather is conducive to CBC.

In summary, copper hydroxide in tank mixes with Serenade® MAX sprayed monthly was sufficient for controlling citrus canker in Mexican lime groves, when young trees frequently produce new leaf flushes during the early years of CBC establishment. In addition, reduction in copper use would be beneficial to reduce the risk of fruit stippling and to lower copper accumulation in the soil. Further studies are needed to evaluate the effectiveness of this combination on large-scale applications to control CBC in commercial citrus orchards in the southwestern region of Saudi Arabia, where citrus canker is considered an endemic citrus disease.

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