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Laboratory Bioassay and Field Evaluation of Copper Gluconate as a New Potential Bactericide Against *Xanthomonas campestris* pv. *citri* (Hasse) Dye

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Abstract

Laboratory bioassays and field trials were carried out to study the toxicity and effectiveness of copper gluconate (CG) against *Xanthomonas campestris* pv. *citri* (Hasse) in comparison with copper hydroxide (CH) and DT (copper succinate + copper glutarate + copper adipate). The results of laboratory experiment indicated that CG recorded significant control against the bacteria at the concentration of 40 g/L or more. In particular, CG had a similar result to those of CH at the concentration of 100 g/L. Meanwhile, field experiment confirmed that CG had a similar efficacy compared with those of CH, DT, and Mancozeb. It was suggested that CG (1: 1000 dilutions) could be used as effective bactericide for managing citrus canker.

Keywords: copper gluconate, citrus canker, *Xanthomonas campestris*, bioassay, toxicity

1. Introduction

Citrus canker, caused by *Xanthomonas campestris pv. citri* (Hasse) which is associated with various citrus bacterial diseases, was one of the most disastrous plant diseases in most citrus production area worldwide (Verniere et al., 1993). In China, citrus canker is also one of important citrus disease in many citrus orchards. It caused defoliation, shoot die-back, and fruit drops, resulting fruit yield loss.

Several strategies were used to control citrus canker in citrus production. Basically, these strategies were initially exclusion and/or eradication of the pathogen (Leite, 1990; Gimenes-Fernandes et al., 2000). At present, the integrated control measures, such as resistant cultivars and sprays with copper bactericides, have been developed around citrus production regions (Leite et al., 1987; Leite & Mohan, 1990; Leite, 2000). Copper-based bactericides have become a common control measure for citrus canker worldwide (Leite & Mohan, 1990; Das, 2003). However, some researchers found that exclusive use of copper was only effective against citrus canker infested in the moderately resistant cultivars. Some highly susceptible cultivars may require the integration of management measures to reduce the number of lesions on the fruit to manageable levels (Kuhara, 1978; Leite & Mohan, 1990). In past years, several copper products with bactericidal activity had been evaluated against the bacterial pathogen. Among the most effective were copper oxychloride (Pereira et al., 1981; Medina-Urrata & Stapleton, 1985; Leite et al., 1987; Leite, 1990), copper sulphate (Medina-Urrata & Stapleton, 1985; McGuire, 1988), copper hydroxide (Leite et al., 1987; Leite, 1990), copper oxide (Pereira et al., 1981), and ammonia-copper carbonate (McGuire, 1988; Timmer, 1988; Gottwald & Timmer, 1995). In China, Copper hydroxide was the most common and effective bactericide to manage the disease. Other copper bactericide, such as DT (copper succinate + copper glutarate+ copper adipate, Guangdong Academy), Copper ammonium (Chen, 1998), Cupric Acetate (Copper Acetate) (Chen, 2008), were also used to inhibit the citrus canker. However, these over use of traditional copper bactericides had resulted in metal accumulates in soil and caused phytotoxicity or

Cu^{2+} pollution (Fu et al., 2009). Therefore, the development of new copper formulations with other acid radicals or improvements of current copper formulations were essential.

Copper gluconate (CG), a potential new copper bactericide, has not widely used because of its higher costs. However, copper gluconate with inorganic acid radical gluconic was favorable to Cu^{2+} absorption and diffusion, and was more safety to environment and plants in comparison with the traditional copper bactericides. It may become a new copper bactericide in plants disease management while the bactericide cost decreased in the future. The goal of the study was to determine the efficacy of copper gluconate against citrus canker in the laboratory bioassay and field trials. Specific objective were: 1) Laboratory comparison of efficiency of four copper bactericides at different concentrations. 2) Field trial to evaluate the disease progress on citrus plants before and after usage of bactericides and spray methods.

2. Materials and Methods

2.1 Preparation of Three Copper Products

In this study, 95% copper gluconate (CG) (CINO-CHEM Science and Tech. Co. Ltd. Beijing) designed as a potential copper bactericide for evaluation of their use in plant protection. Copper hydroxide 77% (DuPont, Shanghai, China); 30% DT (copper succinate + copper glutarate+ copper adipate) (Guangdong Academy, China), which have widely been used in China to control the plant disease as common bactericides; Distilled water was used as blank control**.** Three copper bactericides were dissolved in distilled water before use.

2.2 Isolation of Bacterial Species

Bacterial species, *Xanthomonas campestris* pv. *citri* **(**Hasse) Dye**,** were isolated from infested citrus plants based on a modified procedure described by (Chao et al., 2005), and maintained the colony in the incubator (27°C, RH=75 ± 5% L: D=14:10).

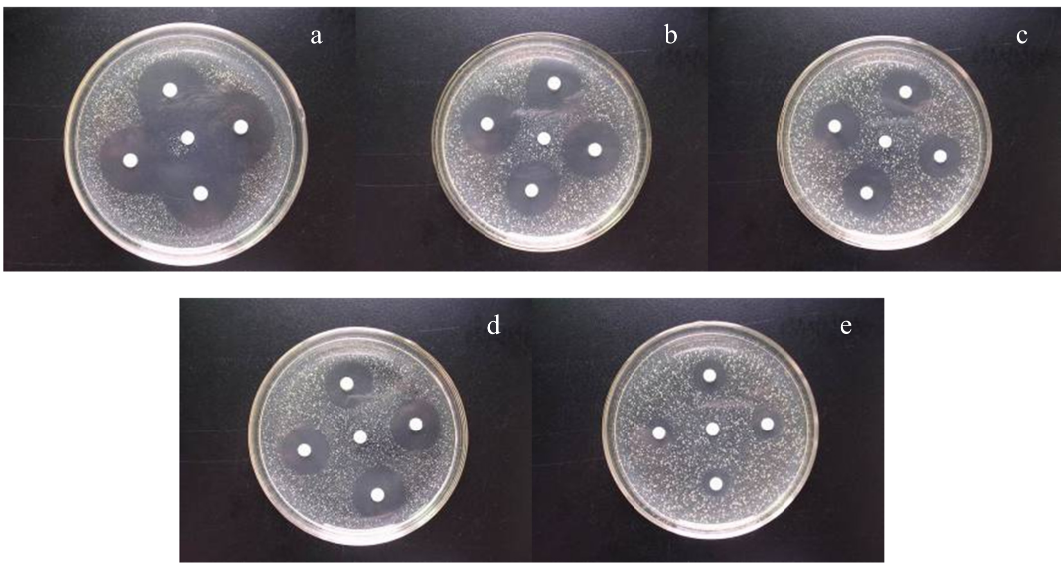

Figure 1. The inhibiting effect of copper gluconate on *X. campestris* (a: 100; b: 80; c: 60; d: 40; e: 20 g/L)

2.3 Laboratory Bioassay of Copper Products Against X. campestris

A modified paper disc agar diffusion method was employed to perform bioassay activity of copper-based bactericides against *X. campestris***.** The utilized protocol was as following: 1) Pure cultivation: the bacteria were picked from colonies on slant agar that had infected into nutrient broth containing beef extract and were cultured in incubator shaker at 27°C for 20 h. 2) Concentration gradient of copper based bactericides: Each copper bactericide was diluted in water to form five concentration series: 100, 80, 60, 40, and 20g/l in sterile Erlenmeyer flask. 3) Inoculation: The deliquescent beef extract medium at the dose 10 mL / petri dish were poured into each of sterile Petri dishes, then, bacteria solution with a solution of 1.0 ml was also added into each of the Petri dishes. A small circular filter paper (CFP, dia. 5 mm), made by a hole puncher, was dipped in copper-based bactericide solutions for one minute; then were moved out to the rampart of sterile Erlenmeyer flask for dripping. Four pieces of CFP filter papers dipped in the each of five concentrations from each bactericide were carefully placed onto each of Petri dishes at diagonal pair, and one CFP filter paper dipped in sterile water was placed on the center as control (see Figure 1). All Petri dishes were maintained in germiculture-box in the laboratory at the temperature of 27-28 °C for 48 h before examined. Ruler Cross -measuring method was used to measure the diameter of each of the colonies.

2.4 Field Trial Evaluation

The goal of the field trials was to further examine control efficacy of the copper products against *X. campestris*. The spraying method was based on China national standard (GB-T 17980). The field experiment was located in the Liangfeng farm, Guangxi province (N 25°02'38"~25°09'35", and E 110°17'12"~110°20'10"). Citrus variety was a two-year old Hamlin orange (*Citrus sinensis* L. Osbeck), planted on a 7.0×4.0 m spacing of each plant. The climate in the region is sub-tropical, with annual average temperature 18.2 °C. Precipitation falls mostly during summer (600–700 mm) and winter is dry (225–250 mm). The experiment was initially carried out from Jul 7, 2006 to Aug 20, 2006 on citrus plants which were nearly fully infested with heavily disease in the citrus orchards. The weather condition was favor for both *X. campestris* development and bactericide spraying, and survey during trial period.

2.5 Experimental Design

The field experiment had three different copper-based bactericides with different doses to evaluate their efficacy against citrus canker, caused by *X. campestris*. The copper-based bactericides were diluted to seven treatments: CG (1/400, 1/600, 1/800 or 1/1000), DT (1/700), CH (1/500), Mancozeb WP (1/1000) (1 unit chemical v/v water), next to pure water as a control treatment. Each concentration treatment contained five plots, representing five replications with each of five trees, these selected infested plots were homogeneous, a total of 200 trees were used in the experiment trial. Before spraying bactericide solutions, the Imidacloprid (Anhui Huaxing Chemical Industry Co., Lid.) was first used to suppress citrus leaf miner one time after during fall seasonal flush. The first spraying of these bactericides was carried out on Jul 7, 2006 in new flush (~3.0 cm) growth periods, and the second spraying was Jul 20 2006. A total of 2.0 L diluting solutions of each concentration of the three bactericides were sprayed by Hand-held Knapsack Sprayers to each selected tree. The inner and outer canopies were sprayed thoroughly until dripping. The experimental arranged with randomized completely blocks design.

Citrus plants infested citrus cankers were sampled on Aug 10 and Aug 20, 2006 after spraying of the bactericides, respectively. Each treatment had five plots, a sampling tree from each plot was selected, a total of 40 trees were sampled. For each sampling tree, a young flush was collected from all quadrants (E, W, S, N) of the tree canopy, then the proportion of damaged leaves was quantified to determine the incidence of citrus canker on the leaves. At the same time, orchard weather conditions and citrus phytotoxicity were also recorded. Estimation of leaf severity of citrus canker of the four branches was observed based on a diagrammatic scale (Leite et al., 1987, Belasque et al., 2005). Whenever possible, the most recent flushes on the branches (approximately 3–6 weeks old) were evaluated following same standard scales (0 = no leaf spots; 1.0 =1-5 leaf spots; 2.0=6-10; 3.0=11-15; 4.0=16-20; 5.0 = more than 21).

2.6 Statistical Analysis

Data collections were used to build regression equation of toxicity. The effective median concentrations (EC_{50}) of the different treatments was calculated based on Index of disease following the formula: Id = [Σ (value of grade × number of leaves with spot)]/[number of leaves examined × the maximum value of grade] ×100; Effectiveness of the copper bactericide also called control efficacy = (Id of control plot) - (Id of treatment plot) / (Id of control plot) × 100; Percentages were subjected to arcsine transformation, and the data was analyzed with one-way analysis of variance (ANOVA) followed by Tukey-Kramer honestly significant difference (HSD) test to determine significant differences among the different concentrations or different copper-based bactericides ($P < 0.05$, SAS Institute Inc. 2009).

3. Results

3.1 Laboratory Bioassay on X. campestris

There was significant higher parallel inhibited zone around each of the four pieces of CFP filter papers on petri dish in the CG treatment groups, compared with control CFP filter paper in the center zone (Figure 1). The average diameter of inhibited zone and the inhibited rate showed significant increased as the increasing concentration of CG's (Table 1). Similar results were also recorded with DT and CH treatments. Among three bactericides, CG had a almost same inhibited zone of CFP as CH, showing both having strong inhibiting effect with different concentrations (Figure 2), however, CG produced the highest significant inhibition zone against the pathogen compared with DT treatment (Figure 3). Probit analysis indicated that the effective median concentration (EC_{50}) of CGs that suppressed *X campestris* was 1.57 g/L.

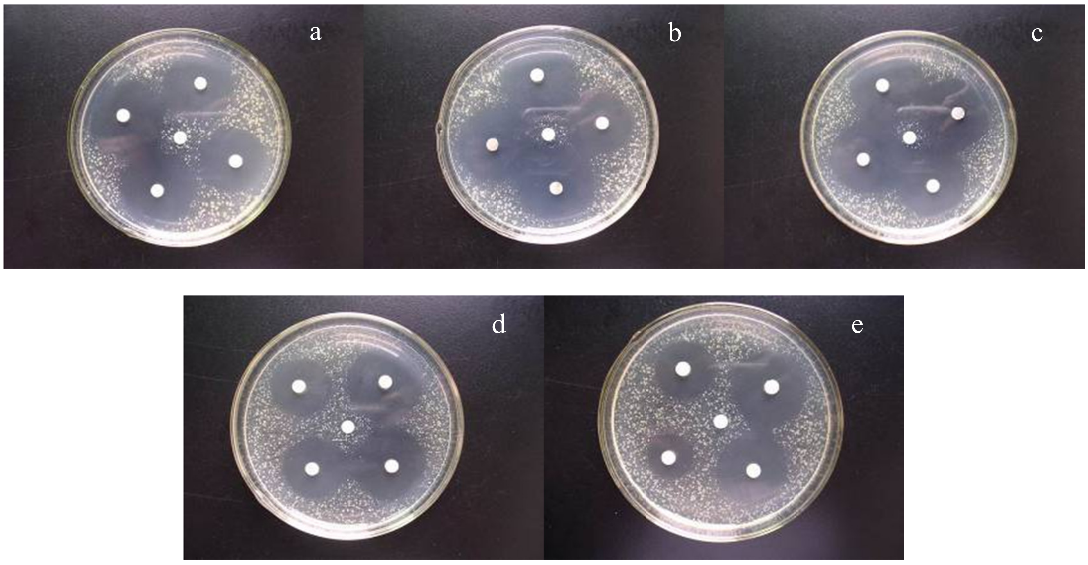

Figure 2. The inhibiting effect of copper hydroxide (CH) on *X. campestris* (a: 100; b: 80; c: 60; d: 40; e: 20g/L)

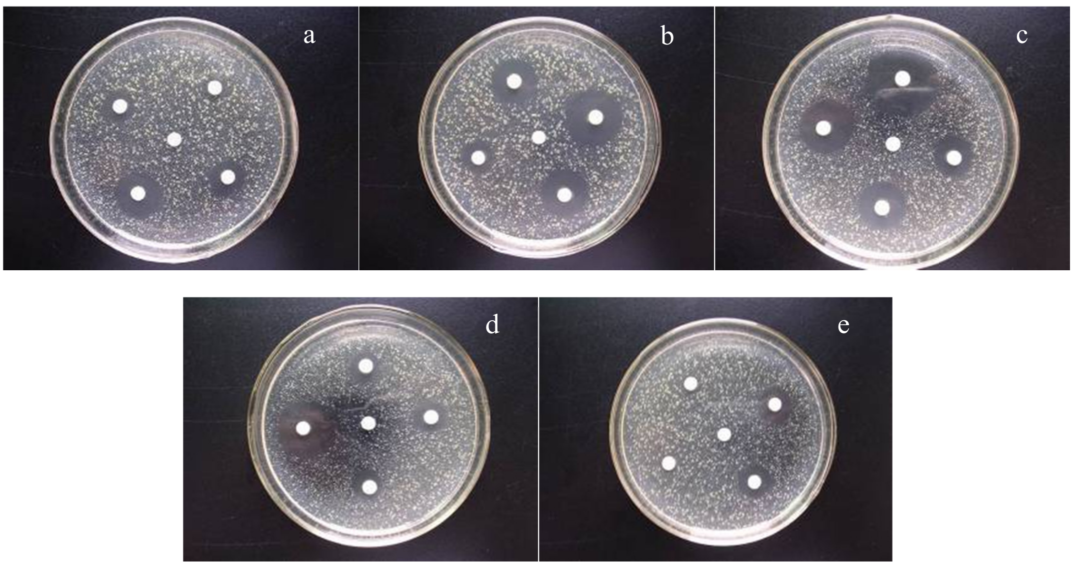

Figure 3. The inhibiting effect of bactericide DT on *X. campestris* (a: 100; b: 80; c: 60; d: 40; e: 20g/L)

Table 1. Toxicity of copper gluconate (CG) on bacteria, *X. campestris* in laboratory

Concentration (g/L)	Actual dosage (g/L)	Mean dia. inhibited zone (mm)	Inhibited rate
100.0	1.56	29.9 ± 5.6	55.0 %
80.0	1.25	25.5 ± 5.1	40.0 %
60.0	0.94	21.4 ± 4.6	27.0 %
40.0	0.63	20.2 ± 4.9	24.0 %
20.0	0.31	13.2 ± 2.3	9.0 %

Note: the Toxicity curve of CG's was $y = 1.94X + 4.62$, $r^2 = 0.95$ ($p = 0.0044$), where y is the probability value of CG's, x is the logarithmic value of CG's concentration.

3.2 Field Evaluation

Two surveys were sampled in the field trials 20d and 30 days post spraying. For first survey 20 d post spraying, each of four concentrations of CG had significant inhibition effect on *X. campestris*. Mean efficacy of the CG ranged was from 67.2 to 87.5%. The new bactericide CG was almost equally effective against the citrus cankers compared with three other common commercial bactericides (CH, DT, and Mancozeb), which produced 68.7 up to 76.9% (Table 2). CG showed significant lower three parameters, such as the rate of infested twig (RT), rate infested leaves (RL), and disease index (DI) in comparison with control (water spray) and no significant difference compared with those of other three commercial common bactericides. A similar result was surveyed 30 d post spraying (Table 3).

Table 2. Effectiveness of the four bactericides on *X. campestris* 20 d post field spraying

Treatments	Dose	RT (%)	RL (%)	DI	Control efficiency (%)
CG	1/400	56	20.7±9.6 a	4.1 ± 2.0a	87.5 ± 6.0a
	1/600	60	40.8±15.4a	8.5 ± 3.6a	74.2 ± 10.9a
	1/800	56	38.3±15.6a	8.2 ± 3.5a	75.3 ±10.6a
	1/1000	92	66.2±7.5ab	10.7 ± 2.2a	67.6 ± 6.5a
DT	1/700	60	40.4±8.4a	10.6 ± 4.8a	68.1 ± 14.7a
CH	1/500	64	38.8±7.6a	7.6 ± 1.6a	76.9 ± 4.8a
MC	1/1000	92	43.6±2.1ab	9.9 ± 3.1a	70.0 ± 9.3a
Control	Water	96	89.6±4.3b	32.2 ± 4.8b	-

Note: CG: 95% Copper Gluconate; CH: 77% copper hydroxide WP; M: 80% Mancozeb WP; Pure water as control; RT: Infected twigs ratings; RL: infected leaves rating; DI: disease index; Means within the same columns followed by the same letter were not significantly different (P>0.05) using Tukey test.

Table 3. Effectiveness of the four bactericides on *X. campestris* 30 d post spraying

Treatments	Doses	RT (%)	RL (%)	DI	Control efficiency (%)
CG	1:400	76	31.1±7.4a	6.9±2.7a	83.6±6.4a
	1:600	60	45.6±17.2a	10.3±4.3a	75.5±10.2a
	1:800	56	52.3±14.8a	11.3±4.8a	73.4±11.5a
	1:1000	100	72.3±7.6ab	13.7±2.9a	67.6±6.8a
DT	1:700	68	52.4±12.2a	14.4±5.7a	66.2±13.6a
CH	1:500	64	40.8±17.1a	9.5±2.1a	77.4±5.1a
MC	1:1000	100	46.4±2.3a	14.9±4.0a	64.7±9.4a
Control	Water	96	96.8±1.9b	41.1±4.2b	-

Note: CG: 95% Copper Gluconate; CH: 77% copper hydroxide WP; M: 80% Mancozeb WP; Water as Control: RT: Rating of Infected twigs; RL: Ratings of infected leaves; DI: disease index; Means within the same columns followed by the same letter were not significantly different (P>0.05) using Tukey test.

However, we were aware that some leaf undersides were found to have phytotoxic spots 20 d after spraying the CG at the concentration (> 1/1000 v/v), even some trees become deciduous at the concentration of CG (1/400 v/v). When the concentration of both CG and CH was below (1/1000 v/v) , only slight phytotoxicity in trees was observed. Second survey 30 d after spray application indicated that the phytotoxicity had a significant reduction. No phytotoxicity was observed in the treatment of 80% mancozeb tech. The field trials further confirmed that bactericide CG had the same efficacy against citrus canker as CH, DT and Mancozeb.

4. Discussion

The paper disc-agar diffusion was considered to be a feasible technique to assess toxicity of fungicides and / or bactericides, and a critical for potential field application (Conner, 1983). The antifungal effect of CG was linearly correlated with their concentration gradients, whereas other two copper bactericides were not. The possible reason was that calcium carbonate in DT might influenced the effective ingredient diffusion.

Based on Timmer et al. (2007) report that two or three times of sprayings of copper-based bactericide were recommended to be effective against early canker during citrus tree flush growth period. In our study, as two times of a copper-based bactericide were sprayed on Jul 7 and Jul 20, 2006, the severity of canker disease had a significant decline at 20 d and 30 d after spraying the bactericides, respectively. Several similar reports had been documented as previous studies (Krishna & Nema 1983; Timmer 1988; Leite and Mohan 1990). Our results further confirmed that CG showed equally or similar results of CH and DT at the recommended doses. We also aware that CG with higher concentration [1/ 800 (v/v)] or over produced some degree of phytotoxicity on citrus tree leaves 20 d after spraying. However, CG with the concentration (1/1000 v/v) had no or only slight phytotoxicity, even the lower phytotoxic than that of CH, which is common commercial bactericide, possible cause may be variety of Hamlin orange sensitive to Cu^{2+}, or other possible mechanism.

In summary, based on above study, bactericide CG with concentration of (1/1000 (v/v) was recommended for effective against the early citrus canker. We should understand that copper gluconate (CG) may not only need to reduce its cost through the improvement of synthetic technology, but also to explore suitable applied formulation for canker control and for avoiding the phytotoxicity.

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