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Efficacy test of commercial formulations against *Phytophthora* root rot of citrus

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ABSTRACT

Phytophthora root rot is one of the most important and destructive soil borne disease of citrus in all major citrus growing regions worldwide. The goal of this study was to investigate the efficacy of commercial formulations that contain the biocides peracetic acid, hydrogen peroxide and phosphorous acid for management of *Phytophthora* root rot infections of citrus seedlings under greenhouse conditions. One-month old sour orange seedlings were inoculated with *P. nicotianae* zoospore suspension (2.5×10^4 zoospores/ml) for 4 h and then treated with commercial biocides by soil drench. One week after treatment, seedling mortality reached 80% in inoculated mock-treated seedlings whereas seedlings treated with biocides showed only 5-10% mortality, similarly to healthy control. Plating of roots showed that inoculated mock-treated seedlings had 95% of root segments infected with *P. nicotianae,* whereas roots from inoculated seedlings treated with biocides showed significantly lower infection rates ($P<0.05$). Average plant height and root growth of inoculated seedlings treated with the different biocide products were significantly higher ($P<0.05$) than inoculated mock-treated seedlings. Moreover, root length, root surface area, root diameter and number of root tips were significantly higher in inoculated seedlings treated with biocides than inoculated mock-treated seedlings ($P <0.01$). The results from this study indicate that formulations containing peracetic acid, hydrogen peroxide and phosphorous acid help to reduce root infections caused by *P. nicotianae* under greenhouse conditions and that could be used as alternative chemistries to manage *Phytophthora* spp. infections in the field and greenhouse production systems.

Additional index words: Citrus diseases, *Phytophthora nicotianae*, fungicides

Citrus occupies an important place in the economy of United States with $3.28 billion value of production for 2017-2018 (USDA NASS, 2018). Texas is the third largest citrus producing state after California and Florida where citrus production is concentrated in the Lower Rio Grande Valley (LRGV). Phytophthora root rot is a serious and economically important disease affecting citrus producing areas of the world (Gade and Lad 2018; Thomas et al., 2010; Alvarez et al., 2008). In the LRGV of Texas, Phytophthora foot and root rot are widely spread diseases affecting orchard productivity (Chaudhary et al., 2016). Yield and production losses due to Phytophthora root rot are difficult to assess, however studies conducted in Florida have estimated that Phytophthora foot rot and root rot reduce yields by 3-6% annually (Graham and Menge, 1999). Root rot of citrus can be caused by several *Phytophthora* species including *P. nicotianae* Breda de Hann, *P. citrophthora* (R. E. Smith & E. H. Smith) Leonian, and *P. palmivora* (E. J. Butler) (Erwin and Ribeiro, 1996). In Texas, as in the majority of subtropical regions in the world, citrus root rot has been associated with *P. nicotianae*, the most prevalent species found in Texas citrus orchards (Timmer, 1973; Kunta et al., 2007; Chaudhary et al., 2017). This pathogen can infect almost every citrus variety, causing death of seedlings, gummosis in mature trees in orchards, foot rot of the trunk and branches and root rots in field and nursery trees (Menge and Nemec, 1997).

Management strategies for the control of root rot are especially important throughout the initial years of citrus orchard establishment (Hao et al., 2019). Different control practices for managing root rot include the use of resistant rootstocks, planting disease-free seedlings, biological control, fungicide applications and proper management of irrigation water (Guest et al., 2004). Trifoliate and trifoliate hybrids (e.g., C-35 and Swingle) can be used as *Phytophthora*-tolerant rootstocks in order to reduce disease severity, however all commercial scions are susceptible to the disease (Ferguson et al., 1990; Roose, 2014). Systemic fungicides, such as metalaxyl and fosetyl-Al, have been used for many years as management strategies for foot rot and root rot of citrus. Applications of both fungicides in Florida have shown to reduce propagules of *P. nicotianae* and increase fibrous root weight and improve citrus yields (Sandler, 1989; Timmer et al., 1989). However, lack of fungicide rotation in citrus nurseries has led to the development of metalaxyl-resistant isolates of *P. nicotianae* (Timmer et al., 1998). Although new oomycete specific fungicides ethaboxam, fluopicolide, mandipropamid, and oxathiapiprolin have demonstrated effective control against Phytophthora root rot of citrus (Hao et al., 2019), there are reports of resistance development in *P. capsici* to oxathiapiprolin (Miao et al., 2016). Therefore, there is a continuous need for more commercial fungicides products to incorporate into an integrated disease management program to prevent fungicide-resistant *Phytophthora* strains.

Peracetic acid or peroxyacetic acid (PAA) is strong oxidizing agent, biodegradable product and a very effective biocide against a wide array of microorganisms particularly bacteria and fungi (Baldry, 1983; Kitis, 2004). PAA solutions for sanitation are commercially available in the form of equilibrium mixture containing acetic acid, hydrogen peroxide (HP), PAA and water (Alasri et al., 1992; Gehr et al., 2002). PAA and other peroxide based sanitizers disinfect through oxidation (Block, 1991). PAA oxidizes the outer cell membrane of vegetative bacterial cells, endospores, yeast, and mold spores, making it an effective sanitizer against all microorganisms, including bacterial spores (Lambert et al., 1999). The reason for the excellent and rapid antimicrobial effects of PAA is its specific capability to penetrate the cell membrane (Baldry and Fraser, 1988; Leaper, 1984). Once inside the cell, PAA plays a role in denaturing proteins, disrupting cell wall permeability, and oxidizing sulfhydryl and sulfur bonds in enzymes and other proteins (Block, 1991; Leaper, 1984). PAA irreversibly disrupts enzyme systems, which destroys the microorganism (Fraser et al., 1984). Treatments with PAA have been used to control postharvest decay caused by *Penicillium digitatum* on tangerines (Pukdee and Sardsud, 2007), *Rhizopus stolonifer* on raw salad vegetables (Alvaro et al., 2009), *Botrytis cinerea* on tomato (Ayoub et al., 2017), and *Monilinia laxa* and *R. stolonifer* on stone fruits (Mari et al., 2004).

Hydrogen peroxide (HP) is a biodegradable antimicrobial that is generally recognized as safe for food sanitation and sterilization (Sangchote, 2008; Baldry, 1983). The antibacterial activity of HP on fresh fruits and vegetables has been reported (Sapers et al., 2000; Venkitanarayanan et al., 2002). Lin et al. (2002), found that 2% HP solution reduced the population of *Escherichia coli* O157: H7 and *Listeria monocytogenes* on lettuce from 4 to 3 logs. Sapers et al. (2000), also showed that a 5% HP solution reduced the population of *E. coli* on inoculated apples. PAA solutions are readily mixed with HP (Gehr et al., 2002) as several studies have suggested the potentiation of antimicrobial efficiency in PAA and HP mixtures (Alasri et al., 1992; Flores et al., 2016).

Phosphorous acid (PhA) or phosphite based fungicides have become a common practice in agricultural or horticultural tree crops which are severely affected by *Phytophthora*-induced diseases (Darvas et al., 1983; Garbelotto et al., 2007; Orbovic et al., 2008). Phosphite may act either directly on the pathogen (Cohen and Coffey, 1986; Dolan and Coffey, 1988), indirectly by stimulating host defenses (Dunstan et al., 1990; Guest and Grant, 1991) or by both modes of action (Grant et al., 1990; Smillie et al., 1989). Concentrations of phosphite in the plant may determine which mode of action is of greater importance (Afek and Sztejnberg, 1989). Phosphite fungicides are excellent candidates for treatments in natural ecosystems because they have extremely low toxicity to terrestrial and aquatic organisms, including human health (Garbelotto et al., 2007). Several studies have shown that phosphite have high efficacy against soilborne pathogens including *P. palmivora* on citrus (Orbovic et al., 2008), *P. tropicalis* on macadamia (Lisa et al., 2014) and *P. cinnamomi* on avocado (Darvas et al., 1983).

New commercial formulations that contain PAA, HP and PhA are available in different concentrations and mixtures and could serve as alternative treatment for the management of *Phytophthora* spp. diseases. In this study, efficacy of PAA, HP and PhA mixtures has been evaluated under the commercial names of OxiPhos®, TerraStart® , TerraClean® 5.0 (BioSafe Systems, East Hartford, CT; Table 1) for the management of *P. nicotianae* root rot infection of citrus under greenhouse conditions.

MATERIALS AND METHODS

Evaluation of biocide treatments. Ten, one-month-old sour orange seedlings with similar height were selected per treatment for inoculation with *P. nicotianae* zoospores. The zoospore suspension was prepared as previously described (Ribeiro et al., 1978). Briefly, two 5-mm-diameter plugs of *P. nicotianae* isolate from 14-day-old culture were grown on 10% V8 agar medium (100ml V8 juice, 1 g $CaCO_3$, 100 µg ampicillin per liter) in darkness for 10 to 14 days at 23 ± 2°C until abundant sporangia was formed. Then, six 5-mm diameter agar plugs containing sporangia were flooded with sterile distilled water in a petri dish. Zoospore release were induced by chilling at 4°C for 20 min. Zoospore concentration was quantified using a hemocytometer and the inoculum suspension was prepared at 2.5×10^4 zoospores/ml. Seedlings were carefully uprooted from the tray and bare roots were gently rinsed with sterile water until all soil particles were removed. Cleaned roots were then immersed in a *P. nicotianae* zoospore suspension for 4-5 hours. Inoculated seedlings were then transplanted into plastic pots filled with potting soil (Sunshine mix #2, Sun Gro Horticulture, Agawam, MA) and treated with biocides listed in Table 1 as follows: 1) OxiPhos® solution was applied to seedling roots at the time of transplant and

Table 1. List of products tested for their efficacy at protecting citrus seedlings against *P. nicotianae*.

Product name	Rate/1000 sq ft*	Active Ingredients (%)	Manufacturer
OxiPhos®	36.5-73 fl.oz	Mono- and di-potassium salts of phosphorous acid 27.1% and Hydrogen Peroxide 14%	Biosafe Systems
TerraStart®	54.7 fl.oz	Hydrogen peroxide 18.5% and Peroxyacetic acid 12%	Biosafe Systems
TerraClean® 5.0	7.29 fl.oz	Hydrogen dioxide 27% and Peroxyacetic acid 5%	Biosafe Systems
TerraGrow®	0.56 fl.oz	Microbial inoculants 6%	Biosafe Systems
Ridomil Gold®	1.5 fl.oz	Mefenoxam	Syngenta

*OxiPhos®, TerraStart®, TerraClean® 5.0 and TerraGrow® rates were mixed in water and applied as a soil drench equivalent to 57 gallons of total solution per 1000 sq.ft of area.

then applied as a soil drench every 4 weeks at 0.5 X solution strength; 2) TerraStart® was applied as a soil drench during transplant; 3) TerraStart® applied as a soil drench, followed by two application of TerraClean® 5.0 at 7 and 14 days after transplant, followed by TerraGrow® on day 15. Mefenoxam (Ridomil® Gold, Syngenta) was used as treated-positive control in *P. nicotianae* inoculated seedlings. Controls included *P. nicotianae* inoculated mock-treated with water and healthy seedlings. One week after inoculation, seedling mortality per treatment was assessed. Each treatment had ten biological replicates (ten seedlings) and two independent experiments were performed. Pots were arranged in a completely randomized design on a bench in a greenhouse at 23 to 25°C, with 16 h photoperiod. Plants were watered to saturation twice a week and fertilized with 24-8-16 fertilizer (Miracle-Gro®, Scotts Co.) according to manufactures instructions, at 15 and 30 days after transplanting.

Quantification of root infection. The root system from three seedlings per treatment were cut out from the shoots, washed with tap water and surface sterilized with 1% sodium hypochlorite for 5 min. Then, the roots were rinsed twice with sterile water cut into 1 cm segments and plated onto CMA-PARPH plates (17 gm of corn meal agar, 5 mg pimaricin, 250 mg ampicillin, 10 mg rifamycin-, 50 mg PCNB, 50 mg hymexazol and 4 mg benomyl per liter) (Jeffers and Martin 1986). After 4 days of incubation at 25°C in the dark, the total number of *P. nicotianae* infected root cuttings was observed under a dissecting microscope and the results were expressed as percentage of infected roots per cm of root length using the following formula: Infected roots (%) = (number of infected root segments) / (total number of root segments plated) x 100

Seedling growth assessment. Seedling height was measured at 15, 30 and 45 days post treatment and transplanting. At the end of each independent experiment, the root systems of ten seedlings per treatment were washed with tap water, placed under a Epson Perfection V700 scanner (Epson America, Inc., Long Beach, CA)) and analyzed using the WinRhizo Pro software (Regent Instruments Inc., Canada) to measure root length, root diameter, surface area and number of root tips.

Data analysis. Statistical analysis was performed by one-way analysis of variance (ANOVA). Significant differences between treatments were calculated according to Levene's t-test at $P \leq 0.05$. All calculations were performed with SPSS software (SPSS Inc., Chicago, IL).

RESULTS

Effect of biocides on seedling infection. Seedlings inoculated with *P. nicotianae* showed significantly higher mortality (t= 6.102; df= 38; P = <0.0001) than healthy control seedlings, reaching 80% mortality one week post inoculation. Seedlings treated with OxiPhos®, TerraStart® and TerraStart® +TerraClean® 5.0 +TerraGrow® exhibited 10%, 5% and 5% seedling mortality respectively, which was comparable to the seedling mortality obtain from the mefenoxam treatment (5%) and not statistically significant from the healthy control (Fig. 1A).

Percentage of root infection was not significantly different among biocides treatments OxiPhos® (35%), TerraStart® (30%) and TerraStart® +TerraClean® 5.0 +TerraGrow® (30%), and as compared to the mefenoxam control (25%). All of the treated seedlings had significantly lower root infections as compared to the inoculated mock-treated seedlings, which showed 95% of root infection (t= 8.48; df= 2; P = 0.014) (Fig. 1B).

Effect of biocides on seedling growth. Analysis of seedlings height indicate that inoculated mock-treated seedlings were significantly smaller than inoculated seedlings treated with biocides (t= -7.983; df= 38; P= <0.0001) (Fig. 2). There were no significant differeces

Table 2. Efficacy of biocides on seedling root development. The means of twenty roots per treatment from two independent experiments with the standard errors are presented. Different letters denote a statistical differences (P<0.01) among treatments according to independent T-test.

Treatments	Root length (cm)	Root surface area (cm2)	Root diameter (mm)	No. of root tips
Healthy control	41.04 ± 2.8 a	9.79 ± 0.6 a	1.82 ± 0.01 a	107.6 ± 7.76 a
Inoculated- mock treated	9.37 ± 1.2 c	2.2 ± 0.33 c	0.52 ± 0.04 c	36.3 ± 6.03 c
Inoculated- mefenoxam	19.25 ± 1.9 b	4.8 ± 0.46 b	0.86 ± 0.03 b	87.3 ± 4.54 b
Inoculated- OxiPhos®	19.03 ± 2.8 b	4.99 ± 0.7 b	0.89 ± 0.04 b	81.3 ± 8.17 b
Inoculated-TerraStart®	14.4 ± 1.77 b	4.2 ± 0.45 b	0.96 ± 0.02 b	78.7 ± 5.35 b
Inoculated-TerraStart® followed by TerraClean® 5.0 and TerraGrow®	15.54 ± 2.1 b	4.47 ± 0.52 b	0.99 ± 0.03 b	83.9 ± 7.13 b

on seedling height among biocide treatments and healthy control.

Efficacy of biocides on seedling root development. To evaluate the efficacy of biocides on root growth, the root systems from ten seedlings per treatment from two independent experiments were harvested 45 days post inoculation and transplanting and evaluated for root length, root surface area, root diameter and number of root tips (Fig. 3). Statistical analysis showed that inoculated seedlings treated with biocides had significantly higher (t= -3.57; df= 38; P = 0.001) root growth than inoculated mock-treated seedlings (Table 2). However, biocides treated seedlings had significantly lower root growth than the healthy control. This observation may have been due to the roots treated with biocides were first exposed to the *P. nicotianae* which affected root growth prior to treatment.

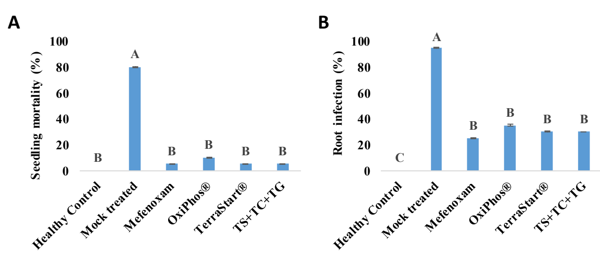

Fig. 1. Percentage of (A) seedling mortality and (B) root infection in citrus seedlings caused by *P. nicotianae*. Different letters above bars denote a statistical difference (P<0.05) among treatment according to independent T-test. Bar represent standard errors of the mean. Healthy Control= non-inoculated seedling, Mock treated= *P. nicotianae* inoculated but untreated, Mefenoxam= Mefenoxam + *P. nicotianae*, OxiPhos®= OxiPhos® + *P. nicotianae*, TerraStart®= TerraStart® + *P. nicotianae*, TS+TC+TG= TerraStart® + TerraClean® 5.0 + TerraGrow® + P. *nicotianae*.

DISCUSSION

Chemical pesticides have been widely used to treat *Phytophthora* spp. infections in many crops. Systemic

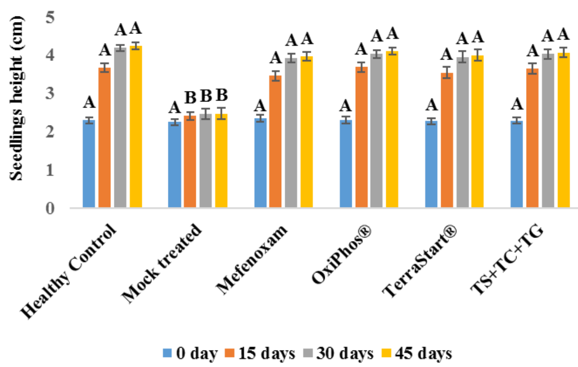

Fig. 2. Effect of biocides on seedling development were measured at 0 day=pre application and 15, 30 and 45 days post application. Different letters above bars denote a statistical difference *(P<0.05)* of seedling height among treatment according to independent T-test. Bar represent standard errors of the mean. Healthy Control= non-inoculated seedling, Mock treated= *P. nicotianae* inoculated but untreated, Mefenoxam= Mefenoxam + *P. nicotianae*, OxiPhos®= OxiPhos® + *P. nicotianae*, TerraStart®= TerraStart® + *P. nicotianae*, TS+TC+TG= TerraStart® + TerraClean® 5.0 + TerraGrow® + *P. nicotianae*.

fungicides such as mefenoxam and fosetyl-Al are effective, however their inappropriate use could lead to several problems such as development of fungicide-resistant *Phytophthora* strains, environmental pollution and increased pesticide residues in produce (Fisher et al., 2012; Harris et al., 2001). Therefore, there is need to evaluate the effectiveness of alternative pesticides that have reduced environmental impact and low residual effect (Carrasco and Urrestarazu, 2010). The present study showed that mixtures of PAA, HP and PhA are effective at suppressing *P. nicotianae* root infections and mortality of citrus seedlings in the greenhouse. Several studies on PAA and HP have focused on its use as postharvest treatments for fruits and vegetables to reduce spoilage from bacteria and fungi because is it a disinfectant with broad spectrum activity against microorganisms (Alla et al., 2011; Alvaro et al., 2009; Beuchat et al., 2004; Lin et al., 2002; Sapers et al., 2000; Venkitanarayanan et al., 2002).

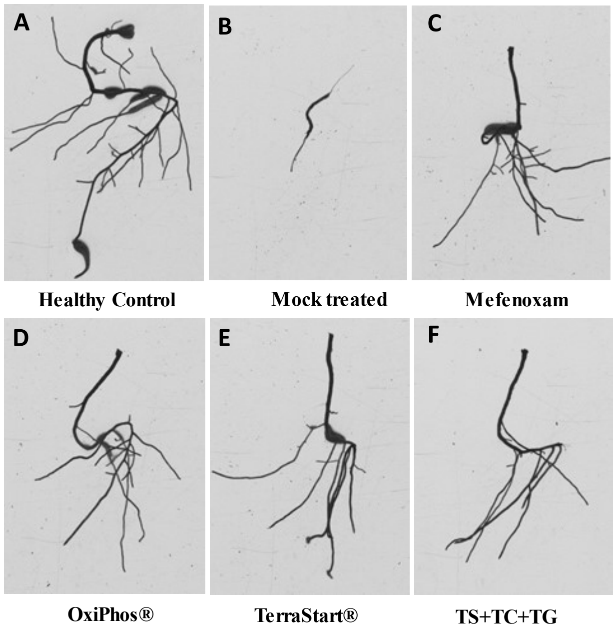

Fig. 3. Images of *P. nicotianae* inoculated roots at 45 days post treatment with biocides. A) Healthy control = uninoculated roots; B) Mock treated = *P. nicotianae* inoculated – water treated control; C) Mefenoxam = inoculated roots-Mefenoxam treated; D) OxiPhos®= inoculated roots- OxiPhos® treated; E) TerraStart® = inoculated roots-TerraStart® treated; F) TS+TC+TG= inoculated roots-TerraStart® +TerraClean® 5.0 +TerraGrow® treated.

Mixed peracetic systems are made with PAA, HP and acetic acid (Gehr et al., 2002). These systems are strong oxidant, ecofriendly, and can be used for antimicrobial activity (Hei, 2000; Mari et al., 2004). Significant (>5-log) microbial reductions have been found on hydroponic alfalfa sprout treatments with PAA and mixed peracetic systems against natural bacterial flora (Hei et al., 2001). Also, these formulations have been used to treat fruit trees (apple, apricot and pear) against fire blight, a disease caused by *Erwinia amylovora*, with significant effectiveness (Hei, 2000). Furthermore, peracetic systems have been used to treat narcissus bulbs for control basal rot (*Fusarium oxysporum* f.sp. *narcissi*) and steam and bulb nematode (*Ditylenchus dipsaci*) with 100% effectiveness (Hanks and Linfield, 1999). Results from this study showed that peracetic acid mix could effectively reduce *P. nicotianae*-induced disease incidence and mortality of citrus seedlings growing under greenhouse conditions.

In accordance to previous studies, our results show that Phytophthora root rot of seedlings is reduced by phosphite treatment (Barrett et al., 2003; F¨orster et al., 1998; Guest et al., 1991; Timmer et al., 1998). Our results are also similar to Orbovic et al. (2008), who showed that soil application of phosphite significantly decreased susceptibility of root rot of citrus seedling caused by *P. palmivora.* In this study, PhA mixtures did not promote seedling growth relative to untreated control or any other treatments. Similar results have been reported previously in hydroponically grown tomato and green pepper where phosphite was provided as the only source of P with no effect on growth (Förster et al., 2007). Nevertheless, our result contrast with previous reports that phosphite applications into a P-deficient soils induces growth of sour orange seedlings (Orbovic et al., 2008). The observed difference could be associated to the lack of P-deficiency in this study as all treatments were receiving phosphorous supplementation through fertilization. This work highlighted that phosphite may have strong fungicidal potential against *Phytophthora* root rot of citrus seedlings.

There are new commercial biocides that contain PAA, HP, PhA and acetic acid are available in different concentrations as stabilized matures. These mixtures are strong oxidizer, biodegradable and a very effective against a wide array of microorganisms particularly bacteria and fungi. They can be effective treatment of living or growing plant tissues including seeds, roots, tubers, seedlings, cuttings, rooting stock, growing plant , fruits and vegetables from the undesirable effects of microbial attack. This study indicated that PAA, HP and PhA could be used as alternative chemistries for controlling *Phytophthora* spp. infections in greenhouse production, not just for citrus but for other vegetable crops. However, specific recommendations need to be developed for greenhouse production of vegetables and target diseases.

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