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Research Paper

Chlorine and mefenoxam sensitivity of *Phytophthora nicotianae* and *Phytopthora citrophthora* from South African citrus nurseries

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Summary. *Phytophthora nicotianae* and *P. citrophthora* isolates were subjected to mefenoxam and chlorine sensitivity evaluations at different concentrations, and for chlorine, different exposure times. Based on mefenoxam sensitivity, the isolates of the two species were divided in six sensitivity groups with EC_{50} values ranging from sensitive (0.04 ppm mefenoxam) to highly insensitive (greater than 123.69 ppm mefenoxam), with 86% of isolates being sensitive to mefenoxam. Chlorine sensitivity testing indicated strong interactions between chlorine concentration and exposure time for both species. Increased mortality was observed with increased concentration and exposure time to chlorine. For some isolates, close to 100% mortality was only reached at 6 ppm active chlorine and at an exposure time of 60 min. Because highly mefenoxam-insensitive isolates were detected from South African citrus nurseries, this fungicide should be used with care as a curative method for management of diseases caused by *Phytophthora* spp. It is recommended that chlorination of irrigation water, at 6 ppm active chlorine and exposure of more than 60 min, is used to eliminate *P. nicotianae* and *P. citrophthora* propagules from irrigation water as a preventative measure for these diseases.

Keywords. Irrigation water, soilborne pathogens.

INTRODUCTION

The South African citrus industry annually produces approx. 1.3 million tons of citrus fruit, of which about 1.1 million tons are exported, and these fruits are produced on 77,708 ha (Edmonds, 2018). New plantings and replacement of old orchards are important to maintain this level of production. This places heavy demand on continuous production of good quality, disease- and pest-free nursery produced young trees.

Soilborne pathogens, especially *Phytophthora nicotianae* (Breda de Haan) and *P. citrophthora* (R.E. Sm. & E.H. Sm.) Leonian, have been shown to cause economic losses in citrus plantings in many countries (Meitz-Hopkins *et al.,* 2013). In established orchards, *P. nicotianae* causes collar and root rots of trees as well as infections of low hanging fruit, resulting in brown rot. This fruit rot can potentially spoil entire cartons of fruit during export if it spreads among the fruit in each carton (Graham and Feichtenberger, 2015). *Phytophthora citrophthora* can also attack aerial parts of citrus trees, such as trunks and limbs, ultimately causing tree death (Graham and Feichtenberger, 2015).

Phytophthora nicotianae has been reported to occur sporadically in citrus nurseries in many countries, including South Africa (Wehner *et al.,* 1986; Ahmed *et al.,* 2012). Diagnostic results from the Citrus Research International Diagnostic Centre (Nelspruit, South Africa) also showed that *P. citrophthora* occurs sporadically in South African citrus nurseries. These findings are important, because infected nursery trees are sources of infection of new citrus orchards (Ippolito *et al.,* 2004).

Water is a potential source of infections caused by *P. nicotianae* and *P. citrophthora* in nursery environments (Grech and Rijkenberg, 1992; Ippolito *et al.,* 2004). As a result, preventative control measures are employed to ensure that irrigation water is free from these pathogens. One of these measures is chlorination of irrigation water. This is routinely used in citrus nurseries in South Africa, and other citrus producing countries, to eradicate pathogen propagules that might be present. This practice is also employed by nurseries in other industries (Hong *et al.,* 2003; Ghimire *et al.,* 2011). In cases where nurseries are infested with one of these *Phytophthora* spp., curative fungicide treatments are applied. One such fungicide is mefenoxam, a systemic compound which has been widely studied for control of *Phytophthora* spp. in citrus, and in other tree and ornamental crops (Farih *et al.,* 1981; Davis 1982; Matheron and Matejka, 1988; Matheron *et al.,* 1997; Morales-Rodríguez *et al.,* 2014; Aiello *et al*., 2018).

Despite the application of preventative and curative measures by South African citrus nurseries, *P. nicotianae* and *P. citrophthora* continue to occur sporadically, which indicates ineffective control measures. Hong *et al.* (2003) reported that although zoospores of *P. nicotianae* in water did not survive exposure to 2 mg kg^{-1} active chlorine, mycelium fragments could, in some cases, survive exposure of up to 8 mg kg^{-1}. This is significant, as zoospores and mycelium fragments have been isolated from irrigation water (Hwang and Benson, 2005), with current treatment of citrus nursery water in South Africa carried out with active chlorine concentrations between 3 and 6 ppm. Mefenoxam resistance, has been reported for a number of *Phytophthora* spp., including *P. nicotianae* and *P. citrophthora*, occurring on a wide variety of crops (Hwang and Benson, 2005; Hu *et al.,* 2008, 2010).

The aims of the present study were to obtain *P. nicotianae* and *P. citrophthora* isolates from South African citrus nurseries, and characterize them with regards to sensitivity to chlorine and mefenoxam. The results obtained in this study could be used to develop effective preventative and curative control measures for these pathogens in citrus nurseries.

MATERIALS AND METHODS

Collection and purification of isolates

Isolates of *P. nicotianae* and *P. citrophthora* (Table 1) were collected from South African citrus nurseries, in different provinces of South Africa, by sampling plant propagation substrate from pots of young citrus trees. These samples were placed into different compartments

Table 1. Numbers of *Phytophthora citrophthora* and *Phytophthora nicotianae* isolates from different South African provinces used for mefenoxam and chlorine sensitivity testing.

Species	Province	No. of isolates per species	No. of isolates used for mefenoxam sensitivity testing	No. of isolates used for chlorine sensitivity testing
Phytophthora citrophthora	Eastern Cape	52	48	26
	Western Cape	8	7	4
Phytophthora citrophthora	Eastern Cape	27	27	11
	Limpopo	10	10	7
	Mpumalanga	9	8	5
	North West	4	4	3
	Western Cape	11	11	6

of ice trays, with one ice tray allocated per sample. The substrate in each compartment was covered with distilled water before placing two citrus leaf discs (each 5 mm diam.) in each compartment (Grimm and Alexander, 1973). Before cutting the leaf discs from the citrus leaves, they were washed thoroughly with distilled water. The leaves had been collected from trees not subjected to any fungicide treatments. The ice trays were covered to prevent light infiltration, and were incubated at ambient room temperature on a laboratory bench for 48 h. Following incubation, the leaf discs were removed, blotted dry on absorbent paper toweling, and plated onto 90 mm Petri dishes containing PARPH medium (Jeffers and Martin, 1986). Inoculated plates were then incubated in the dark at 29°C for 48 h before being inspected for *Phytophthora* spp. colonies. Isolates were selected from the inoculated plates and transferred to water agar (WA, Biological agar, Biolab), followed by additional incubation at 29°C for 48 h. Colonies were purified from WA by hyphal tipping onto 90 mm Petri dishes containing V8 agar (Galindo and Gallegly, 1960). Purified isolates were stored in molecular grade water in 2 mL capacity micro centrifuge tubes at 25°C.

Molecular identification of isolates

Selected isolates were grown on V8 agar at 29°C for 7 d before mycelia were harvested for genomic DNA extraction, using a modified CTAB-based extraction protocol (Allen *et al.,* 2006).

PCR-RFLP analyses. The ITS region of the isolates was amplified using the primers ITS 6 (Cooke and Duncan, 1997) and ITS 4 (White *et al.,* 1990). The PCR reaction consisted of 20.0 µL of GoTaq® G2 Hot Start Green Master Mix (Promega Corporation), 1.0 µL of each primer (concentration of 10 µM), 16 µL PCR grade water and 2 µL of genomic DNA, for a total volume of 40 µL. Amplifications were conducted in a 2720 thermal cycler (Applied Biosystems). Initial denaturation was at 94°C for 5 min, followed by 32 cycles of 94°C for 30 s, annealing for 30 s at 55°C, extension at 72°C for 30 s, with a final extension step at 72°C for 5 min. PCR products were resolved in a 1% agarose gel, and DNA fragments were visualized by staining with an ethidium bromide solution. The resulting PCR products were restriction digested with enzymes *Hin*fI and *Hha*I in a single reaction, according to manufacturer's instructions (Fermentas Inc.). The PCR-RFLP products were run on a 3% agarose gel, and isolates with the same RFLP banding patterns were assigned to each RFLP group.

ITS sequencing. The ITS regions of at least two isolates of each PCR-RFLP group were sequenced, and double stranded consensus sequences were obtained. The consensus sequences were subjected to BLAST analyses in Genbank (https://blast.ncbi.nlm.nih.goc/Blast.cgi), and were identified to species level based on similarity of at least 99% to existing *P. nicotianae* or *P. citrophthora* ITS sequences on Genbank (https://www.ncbi.nlm.nih.gov/genbank/).

Mefenoxam sensitivity testing

In vitro sensitivity testing

Sensitivity testing was conducted according to a slightly amended protocol described in Timmer *et al.* (1998). A total of 60 *P. citrophthora* and 61 *P. nicotianae* isolates, from different nurseries in different citrus production areas in South Africa (Table 1), were selected and were on 90 mm Petri dishes containing corn meal agar (CMA; Sigma-Aldrich). Plates were incubated at 29°C for 5 d. After incubation, 5 mm plugs were cut from the edges of the actively growing cultures and plated onto 90 mm Petri dishes containing CMA amended with mefenoxam (Ridomil Gold® 450 EC; Syngenta) at 0, 1.0, 2.0, 5.0, 10.0, 25.0, 50.0 or 100.0 ppm. These plates were then incubated at 29°C for 2 d.

Each isolate and concentration combination was repeated using two plates, while the whole trial was repeated twice at the same time. Colony diameters of growing isolates were each measured in two directions and the average colony diameter was calculated for each isolate at each concentration. The percentage inhibition for each plate at each concentration for all the isolates was calculated, and data were subjected to statistical analyses to group isolates, and determine EC_{50}, EC_{80} and EC_{90} values for each isolate. The percentage inhibition was calculated using the following equation:
Percentage inhibition (%) = mean colony diameter of control (0 ppm) – mean diameter ÷ mean diameter of control (0 ppm) × 100.

Data analyses

The Mitscherlich function [$y = a(1\text{-}e^{-bx})$] or % Inhibition = Maximum Inhibition [$1\text{-}e^{-(Rate)(Concentration)}$] fitted the data well, and was used throughout the study. Hereafter, percentage (%) inhibition will be referred to as %Inhb, and maximum inhibition, as MaxInhb. The function was fitted for the two Petri dishes representing each isolate mefenoxam concentration combination within each of the two trials.

EC_{50}, EC_{80}, and EC_{90} values were calculated from the estimated regression parameters (MaxInhb and Rate of

inhibition) for each isolate. Wherever MaxInhb < EC, these respective values could not be calculated according to the appropriate equations (EC_{50} = (-log (1 - (50 ÷ a))) ÷ b; EC_{80} = (-log (1 - (80 ÷ a))) ÷ b; EC_{90} = (-log (1 - (90 ÷ a))) ÷ b). MaxInhb did not always give realistic values, especially where the Rate of inhibition was very slow, because MaxInhb is a value at a theoretical concentration. An additional value, PInhbConc100 = $a[1\text{-}e^{-b(100)}]$, was therefore calculated. This represents the %Inhb at a fungicide concentration of 100 ppm, which gave a more realistic interpretation within the boundaries of the data than just MaxInhb. Regression parameters and EC_{50}, EC_{80} and EC_{90} values were subjected to analysis of variance (ANOVA), and cluster analysis using Ward's clustering method to cluster isolates. Principle component analysis (PCA) was also carried out for the 115 isolates and numbers of isolates in clusters as labels, to see if the grouping or clustering obtained from the cluster analyses made sense.

Chlorine sensitivity testing

Mycelium suspension preparation

Hong *et al.* (2003) found that *Phytophthora* spp. mycelium fragments were more insensitive to chlorine than zoospores. Mycelium fragment suspensions were therefore used in chlorine sensitivity tests. Ten percent V8 broth was prepared by adding 0.5 g $CaCO_3$ (Calcium carbonate; Merck) and 50 mL V8 juice (V8 Original Vegetable Juice, Campbell Soup Company) to each Schott bottle containing 450 mL filtered water, and bottles were then autoclaved at 121°C for 15 min. *Phytophthora* isolates of the two species (32 *P. nicotianae* and 30 *P. citrophthora*; Table 1), randomly selected from the populations used for mefenoxam sensitivity testing, were plated onto 90 mm Petri dishes containing CMA and incubated for 7–10 d at 29°C. After sufficient growth, the agar from each Petri dish was divided into smaller pieces using a scalpel, and placed into the prepared 10% V8 broth. The inoculated V8 broth was then placed on an orbital shaker (SHKO 20; FHM Electronics) running at 100 rpm and 29°C, for 21 d in the dark.

To prepare the mycelium broth, autoclaved filtered water was adjusted to pH 6.5, Adjustment of pH was achieved using sodium hydroxide (NaOH 40 g mol^{-1}, Merck; 2 g in 250 mL autoclaved filtered water) and hydrochloric acid (HCl 32%, Merck; 5 mL in 250 mL autoclaved filtered water). The mycelium masses harvested from the 10% V8 broth were drained using a 180-µm sieve before being each washed twice with 100 mL autoclaved filtered water. Excess water was then pressed out of the remaining fungal mycelium mass using two sterile stainless steel teaspoons. For trial purposes, a measured *Phytophthora* suspension was prepared by blending 1 g (wet mass) of mycelium in 100 mL filtered water (pH 6.5) for 30 s, followed by filtration (1,000 µm sieve) into a Schott bottle, which was then filled to 500 mL using deionized water (pH 6.5).

Chlorine sensitivity testing

Trial variables included chlorine concentration (0, 1.5, 3 or 6 ppm) and a range of chlorine exposure times (0, 5, 10, 30 or 60 min). A chlorine stock solution (SS) was prepared by adding 0.15 g chlorine granules (HTH®, South Africa) to 100 mL filtered, autoclaved water (pH 6.5). In order to achieve 0, 1.5, 3 and 6 ppm concentrations of active chlorine, 0, 0.75, 1.5 or 3.0 mL chlorine SS was added to different 500 mL Schott bottles. As a positive control, a 1.5 and 6 ppm active chlorine solution was tested using a chlorine photometer (Total Chlorine Ultra High Range Portable Photometer, HI 96771; Hanna Instruments Inc.) before the commencement of each trial set. These chlorine control solutions were also de-activated with sodium thiosulfate stock solution ($Na_2S_2O_3$*$5H_2O$; 248.21 g mol^{-1}; Merck) containing 1.47 g $Na_2S_2O_3$*$5H_2O$ in 1,000 mL of filtered, autoclaved water, and tested with Insta-Test® low range 90–10 ppm) free chlorine test strips (LaMotte) to determine whether the stock solution was still functional.

Additionally, the prepared *Phytophthora* mycelium suspension of each isolate was mixed on a magnetic stirrer plate for 10 min before being used to inoculate two PARPH plates as positive controls. Each mycelium suspension (two separate suspensions per *Phytophthora* isolate) was added (treated) to 0, 1.5, 3 or 6 ppm active chlorine and mixed for a further 30 s. Following each exposure time (0, 5, 10, 30 or 60 min), 40 mL of solution was dispensed into two containers and de-activated using the sodium thiosulfate SS. For deactivation, 0, 0.3, 0.6 or 1.2 mL of sodium thiosulfate SS was required to de-activate, respectively, 0, 1.5, 3 and 6 ppm active chlorine. De-activated solution (1 mL) from each container was used to inoculate two PARPH plates and was subsequently spread using a hockey stick and incubated for 2 d at 29°C. Free chlorine test strips were used to confirm de-activation.

Following incubation, *Phytophthora* spp. colonies were counted and percentage mortality determined using the following formula:
[(Cn-Tn)/Cn)]*100, where Cn is the number of colonies on control plates and Tn the number of colonies on treated plates.

The percentage mortality data were subjected to statistical analyses using SAS (SAS Institute Inc.). Fisher's LSD was calculated at the 5% level to compare means.

RESULTS

Molecular identification of isolates

The 121 isolates were divided into two distinct groups based on the ITS-RFLP analysis. ITS sequence analyses of representative isolates from each group identified 61 of the isolates obtained from citrus nurseries as *P. nicotianae*. The remaining 60 isolates were identified as *P. citrophthora*. The species identity was based on a 100% nucleotide homogeneity with *P. nicotianae* and *P. citrophthora* isolates lodged from previous studies on Genbank (https://www.ncbi.nlm.nih.gov/genbank/).

Mefenoxam sensitivity testing

Plotting of the mean EC_{50}, EC_{80} and EC_{90} values of all the isolates indicated that for a group of five isolates no EC_{50}, EC_{80} or EC_{90} values could be calculated. These isolates were grouped together in sensitivity group 1 (Table 2). A further two isolates had EC_{50} values that were greater than 100 ppm. These two isolates were placed in sensitivity group 2 (Table 2). The remaining isolates were subjected to Ward's cluster analysis and PCA to group them into sensitivity groups. Both the Ward's cluster analysis and PCA showed that the remaining *P. citrophthora* and *P. nicotianae* isolates could be aligned into four distinct sensitivity groups (Figure 1). This brought to six the total number of sensitivity groups (Table 2). The analysis of variance (ANOVA) comparing the groups using the regression parameters and EC_{50}, EC_{80} and EC_{90} data showed highly significant ($P < 0.0001$) sensitivity group effects for these three parameters. The ANOVA of the PInhbCon100 and Rate of inhibition data showed a significant sensitivity group × species interaction ($P < 0.0001$ for PInhbCon100 and $P = 0.0021$ for Rate of inhibition).

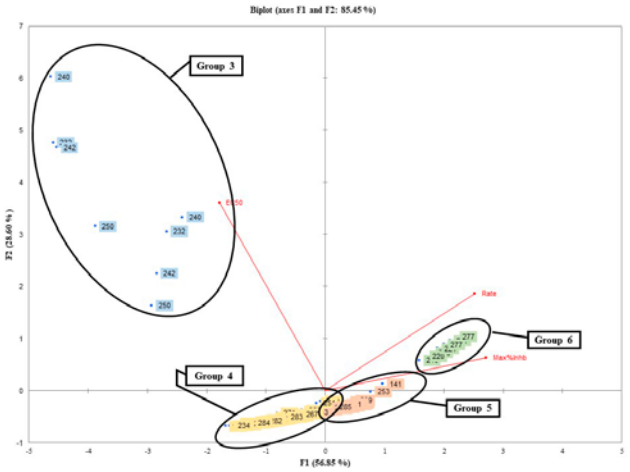

Figure 1. Principal component analysis (PCA) indicating separation of *Phytophthora nicotianae* and *Phytopthora citrophthora* isolates into four distinct mefenoxam sensitivity groups based on the regression parameters EC_{50}, rate of inhibition and Max%Inhb.

Table 2. Mean PInhbConc100 and rate of inhibition values for *Phytophthora citrophthora* and *Phytophthora nicotianae* isolates, grouped into mefenoxam sensitivity groups 1–6, following *in vitro* exposure to different mefenoxam concentrations.

Sensitivity group	Species	PInhbConc100 (%)	Rate of inhibition
1	*P. citrophthora*	39.36 h[1]	0.068 e
	P. nicotianae	30.74 i	0.036 e
2	*P. citrophthora*	44.30 g	0.002 e
	P. nicotianae	---	---
3	*P. citrophthora*	54.88 f	0.027 e
	P. nicotianae	---	---
4	*P. citrophthora*	70.29 e	1.811 d
	P. nicotianae	80.94 d	1.307 d
5	*P. citrophthora*	92.35 c	2.616 c
	P. nicotianae	96.02 b	1.858 c
6	*P. citrophthora*	100.00 a	17.752 a
	P. nicotianae	100.00 a	15.820 a
LSD		3.430	0.7200
P		< 0.0001	0.0021

[1] Means followed by the same letter are not statistically different at a 95% confidence level.

Within group 6, the isolates of *P. citrophthora* and *P. nicotianae* were 100% inhibited by a concentration of 100 ppm mefenoxam. Within group 5 the *P. nicotianae* isolates had a mean of 96.02% inhibition that was significantly more than the *P. citrophthora* isolates in the same group (92.35% inhibition) (Table 2). Both these means of group 5 were significantly less than those of group 6. Also, within group 4 the mean inhibition of *P. nicotianae* isolates was 80.94% and was statistically greater than the mean inhibition (70.29%) of the *P. citrophthora* isolates in this group (Table 2). These two means were less than that observed for the two species in groups 5 and 6. Only *P. citrophthora* isolates were grouped into group 2 and 3. In these groups the mean inhibition of isolates was 54.88% (group 3) and 44.30% (group 2), significantly less than the mean inhibition percentages for isolates in the other groups (Table 2). Isolates of both

species were placed in group 1 that had the least sensitivity to mefenoxam. In this group, mean inhibition of the *P. citrophthora* isolates was 39.26% compared to 30.74% for the *P. nicotianae* isolates. The mean percentage inhibition for both species in group 1 were also the least observed in any of the groups for either pathogen species (Table 2).

The rate of inhibition results for the group × species interaction showed similar trends to those for the PInhbCon100 results. The groups with the greater PInhbCon100 means, also had the greatest rates of inhibition (Table 2). Group 6 had the greatest mean rate of inhibition of all the groups. The mean rate of inhibition of *P. citrophthora* isolates was 17.75, significantly greater than the rate for the *P. nicotianae* isolates (15.82) in this group. The mean inhibition rate of the *P. citrophthora* isolates in group 5 was 2.62 which was significantly greater again than the rate of the *P. nicotianae* isolates (1.86). This mean rate for *P. nicotianae* isolates was comparable to the rates observed for the two species in group 4 (Table 2). In this group, the mean rate of inhibition for *P. citrophthora* was 1.81 compared to 1.31 for *P. nicotianae*. In groups 1, 2 or 3, the mean rates for the two species (0.002 to 0.068) were statistically similar (Table 2).

For the isolates of both species sensitivity group 1, at 100 ppm mefenoxam, the maximum inhibition only reached a mean of 39.36% and the rate of inhibition was only 0.068 (Table 2). Consequently, no EC_{50}, EC_{80} or EC_{90} values could be determined for this group (Table 3). For group 2, the mean EC_{50} value was 123.69 ppm, which was statistically greater than the mean EC_{50} of any other group. Similarly, the mean EC_{80} (214.12 ppm) and EC_{90} (250.25 ppm) values of this group were the greatest of all the groups (Table 3). For group 3, the calculated mean EC_{50} was 76.12 ppm, which was the second greatest of all the groups. Again, no EC_{80} or EC_{90} means could be determined, possibly also due to the slow rate of inhibition of this group (Tables 2 and 3). Group 4 had mean EC_{50} (0.82 ppm) and EC_{90} (2.86 ppm) values that were statistically similar to those for groups 5 and 6. However, an EC_{90} could also not be calculated for this group (Table 2). Groups 5 and 6 had mean EC_{50} (0.45 ppm), EC_{80} (0.10 ppm) and EC_{90} (1.68 ppm) values that were statistically similar to those of group 6. For group 6, the mean EC_{50} (0.04 ppm), EC_{80} (0.10 ppm) and EC_{90} (1.15 ppm) values were the least of all the groups (Table 3). This indicated that the isolates in this group were the most sensitive to mefenoxam.

Table 3. Mean EC_{50}, EC_{80} and EC_{90} values for different mefenoxam sensitivity groups identified after *in vitro* exposure of *Phytophthora citrophthora* and *Phytophthora nicotianae* isolates to different mefenoxam concentrations.

Sensitivity group	EC_{50}	EC_{80}	EC_{90}
1	---	---	---
2	123.69 a[1]	214.12 a	250.25 a
3	76.11 b	---	---
4	0.82 c	2.86 b	---
5	0.45 c	1.11 b	1.68 b
6	0.04 c	0.10 b	0.15 b
LSD	5.101	8.545	12.608
P	< 0.0001	< 0.0001	< 0.0001

[1] Means followed by the same letter are not statistically different at a 95% confidence level.

Distribution of isolates in sensitivity groups

Within the different sensitivity groups, the number of isolates of *P. citrophthora* and *P. nicotianae* varied greatly. For *P. citrophthora*, most of the isolates (90%) were in groups 1, 2, 3 or 4, where the mean percentage inhibition at 100 ppm mefenoxam ranged from 39.36% to 70.29% (Table 4). Based on the classification of Hu *et al*. (2008), these isolates were intermediately insensitive or sensitive to mefenoxam. However, for *P. nicotianae* only 5% of isolates were in the groups 1 to 4, while 95% were in groups 5 and 6. These isolates would be classified as mefenoxam sensitive (Table 4).

Table 4. Numbers (and proportions) of *Phytophthora citrophthora* and *Phytophthora nicotianae* isolates occurring in the different mefenoxam sensitivity groups.

Species	Mefenoxam sensitivity group	No. of isolates in group
Phytophthora citrophthora (n= 60)	1	5 (8%)
	2	2 (3%)
	3	4 (7%)
	4	43 (72%)
	5	4 (7%)
	6	2 (3%)
Phytophthora nicotianae (n = 61)	1	1 (2%)
	2	0 (0%)
	3	0 (0%)
	4	2 (3%)
	5	36 (59%)
	6	22 (36%)

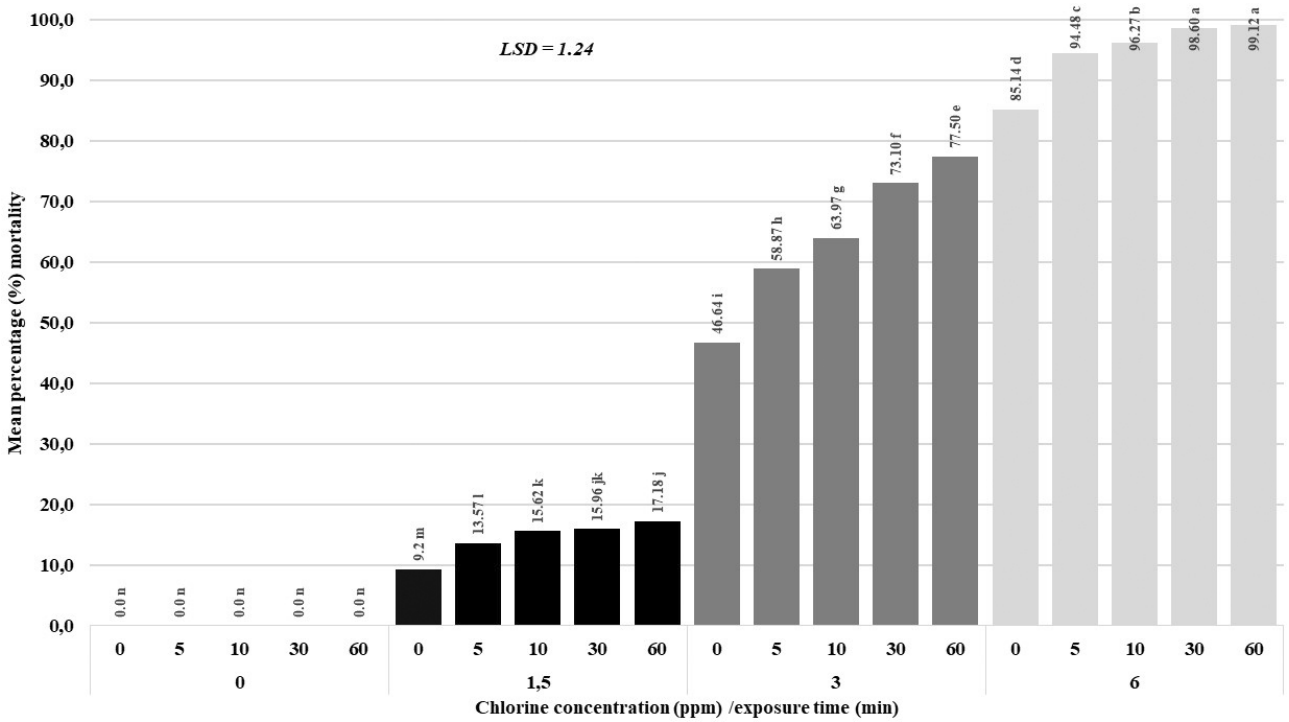

Figure 2. Mean percentage (%) mortality of *Phytophthora* spp. propagules exposed to 0, 1.5, 3 or 6 ppm active chlorine for exposure times of 0, 5, 10, 30 or 60 min.

Chlorine sensitivity testing

The analysis of variance (ANOVA) of the percentage mortality data indicated a highly significant ($P < 0.0001$) experimental repetition × species × isolate × chlorine concentration interaction, and an experimental repetition × chlorine concentration × exposure time interaction. These multifactor interactions are attributed to the significant ($P < 0.0001$) variation seen in the percentage inhibition data between the two experimental repetitions, which could be due to different mycelium suspensions used for each repetition, combined with the significant ($P < 0.0001$) variation seen in mean percentage inhibition between the different chlorine concentrations. Between isolates in the two *Phytophthora* spp., the ANOVA also indicated that there were statistical ($P < 0.0001$) differences between percentage mortalities obtained. Mean mortality of *P. citrophthora* isolates due to chlorine ranged from 27.09 to 73.47%, whereas those for *P. nicotianae* isolates were from 19.69 to 62.30%.

The results from the significant ($P < 0.0001$) chlorine concentration × exposure time interaction indicated no mortality of the *Phytophthora* spp. for 0 ppm chlorine. With chlorine concentration of 1.5 ppm, the mean percentage mortality at no exposure (chlorine deactivated immediately) was 9.20%. This then increased with each increase in exposure time to reach a maximum mean of 17.18% after 60 min exposure to 1.5 ppm active chlorine (Figure 2). When the 3 ppm chlorine treatment was deactivated immediately, the mean mortality was 46.64% and was significantly greater to be 77.50% after 60 min exposure. At 6 ppm chlorine the initial mean mortality was 85.14% with immediate deactivation. Increase in exposure time led to greater mortality at 99.12% after 60 min exposure (Figure 2).

DISCUSSION

Production of citrus trees in nurseries that are free from the soilborne pathogens *Phytophthora nicotianae* and *P. citrophthora* is regarded as essential for establishment of new orchards. This has been concluded from previous studies indicating that infected nursery trees can be sources of infection of new citrus orchards (Ippolito *et al*., 2004). As a result, nurseries need to treat irrigation water with chlorine, because the water is a potential source of infection (Ghimire *et al*., 2011). As a curative measure, infected plants are often treated in these nurseries with drenches of metalaxyl or mefenoxam (Hu *et al*., 2008).

In the present study a total of 121 *P. nicotianae* and *P. citrophthora* isolates were subjected to mefenoxam sensitivity testing at eight different mefenoxam concentrations. Results indicated that the isolates were divided in six sensitivity groups. The mean percentage inhibition calculated at 100 ppm mefenoxam for the different groups varied from 30.74 to 100.00% (Table 2). In the groups with the least inhibition (groups 1, 2 and 3), the rate of increase in inhibition was also very low and could explain the low maximum inhibition achieved at 100 ppm mefenoxam (Table 2). Hwang and Benson (2005) demonstrated that isolates of *P. cryptogea, P. nicotianae* and *P. palmivora*, occurring on floriculture crops in North Carolina, were also divided into different mefenoxam sensitivity groups.

In groups 1, 2 and 3 the mean percentage inhibition at 100 ppm mefenoxam was less than 60%, indicating (Hu *et al*., 2008), that the isolates of *P. citrophthora* and *P. nicotianae* in these groups were insensitive to mefenoxam. Compared to this, the isolates of these two species in groups 4 and 5 were intermediately sensitive, while the isolates in group 6 were sensitive to the compound. There were more *P. citrophthora* isolates than *P. nicotianae* isolates in the groups with the least sensitivity to mefenoxam. Within the groups, *P. citrophthora* isolates often had lower percentages of inhibition compared to *P. nicotianae*, indicating less mefenoxam sensitivity. This is similar to the results of Farih *et al.* (1981) and Coffey and Bower (1984), who found *P. citrophthora* isolates from citrus were less sensitive to mefenoxam than *P. nicotianae* isolates from citrus.

Mean $EC_{50,}$ EC_{80} and EC_{90} values for the isolates in groups 1 to 4 could not be calculated (Table 3). This was probably because at 100 ppm mefenoxam, the highest concentration used in this study, the calculated percentage inhibition for *P. citrophthora* and *P. nicotianae* isolates in these groups was less than 50–90%. For these isolates, 100% inhibition would only be achieved at mefenoxam concentrations much greater than 100 ppm. This was shown by the results for the group 2 isolates, where the mean mefenoxam EC_{90} value was 250.25 ppm.

Timmer *et al.* (1998) published similar results, where they found that some isolates of *P. nicotianae* from citrus had EC_{50} values greater than 100 ppm. Similarly, Farih *et al.* (1981) reported that some isolates of *P. citrophthora* and *P. nicotianae* from citrus had 100% inhibition of mycelium growth at mefenoxam concentrations greater than 100 ppm. High levels of insensitivity to mefenoxam or metalaxyl among isolates of these two citrus pathogens are therefore not unknown. Even among *P. nicotianae* isolates from ornamental crops, Ferrin and Kabashima (1991) showed that highly insensitive isolates had EC_{50} values greater than 100 ppm. In groups 5 and 6 in the present study, the isolate EC_{50} values were well below 2 ppm, and in the case of group 6 below 0.2 ppm. These values were similar to those for isolates described in the reports cited above. These levels of mefenoxam insensitivity have also been determined for *P. infestans* from potato (Goodwin *et al*., 1996) and *P. cinnamomi* from avocado (Coffey and Bower, 1984) and ornamental crops (Hu *et al*., 2010).

For *P. infestans* isolates, the basis of mefenoxam or metalaxyl insensitivity or resistance, and differences in sensitivity between isolates within a species, and between species, were found to be due to genotypic differences between the isolates and species (Goodwin *et al*., 1996). The particular level of sensitivity within a genotype was determined by insensitivity loci present (Fabritius *et al*., 1997). Childers *et al*. (2015) also discovered that sensitive isolates of *P. infestans* can acquire mefenoxam resistance after repeated *in vitro* exposure to the fungicide. However, isolates developing resistance *in vitro* lost some resistance when they were repeatedly plated onto media not amended with mefenoxam.

It is therefore possible that sensitive isolates of *P. nicotianae* and *P. citrophthora* could acquire resistance to mefenoxam when repeatedly exposed to this fungicide in nurseries or orchards. Careful use of this fungicide in citrus nurseries is therefore important, to prevent development of highly insensitive isolates in nurseries that may reach newly established orchards. This was emphasized by the results of Timmer *et al*. (1998), who showed that insensitive isolates from citrus nurseries competed with sensitive isolates as causes of root rot. Furthermore, these insensitive isolates maintained their insensitivity after use of the fungicide ceased.

No differences in sensitivity to chlorine were observed between isolates of *P. citrophthora* and *P. nicotianae*. Increasing exposure time at particular chlorine concentrations increased percentage mortality of both pathogens. However, mean percentage mortality only came close to 100% when the isolates (32 *P. nicotianae* and 30 *P. citrophthora*) were exposed to 6 ppm chlorine for 60 min (Figure 2). Hong *et al*. (2003) tested limited numbers of isolates of *P. nicotianae, P. capsici, P. cinnamomi, P. citricola, P. citrophthora, P. cryptogea* and *P. megasperma*, from irrigation water from ornamental nurseries, for their sensitivity to chlorine. They also observed that with increasing active chlorine concentrations, mean percentage mortality of these pathogens increased. However, they did not detect a chlorine concentration and exposure time interaction. This interaction recorded in the present study could have been due to the higher numbers of isolates of the two

species tested. Chlorination was introduced in South African citrus nurseries based on the study of Grech and Rijkenberg (1992). They indicated that chlorination eliminates soilborne pathogen propagules from irrigation water, consequently reducing the level of *Phytophthora* infection in roots of citrus rootstock seedlings irrigated with treated water. This treatment was also shown to not cause phytotoxic effects on the irrigated seedlings.

The present study is the first to focus on chlorine sensitivity of multiple *P. citrophthora* and *P. nicotianae* isolates subjected to a range of chlorine concentrations and exposure times. Practically, the results indicate that for complete elimination of *Phytophthora* spp. propagules from citrus nursery irrigation water, treatment with 6 ppm active chlorine for 60 min or longer, is required. Mefenoxam as a curative soil drench treatment should also be used with care, as low numbers of highly resistant *P. citrophthora* and *P. nicotianae* isolates were found in this study. For the elimination of these pesticide-resistant pathogens from nurseries, it is therefore important to make use of alternative fungicides (e.g. captan) for curative soil drenches.

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