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Monitoring Mortality of *Pythium* Zoospores in Chlorinated Water Using Oxidation Reduction Potential

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Abstract
Pythium species are frequently recovered from recycled irrigation water in greenhouse production systems and may cause damping off and root rot if the water is not disinfested properly. Chlorination is often employed as a disinfesting agent, but can be difficult to monitor accurately because its activity is pH-dependent. Oxidation reduction potential (ORP) is a reliable, real-time measurement of the oxidizing potential of a chlorine solution. We exposed zoospores of *Pythium aphanidermatum* and *P. dissotocum* municipal water where the ORP was increased by adding 0.125, 0.5, and 2 mg/liter chlorine (pH 7.6 to 8.1) or to the same water source where the pH was lowered to 6.0 prior to the addition of chlorine, resulting in a final pH of 6 to 7.3. Some zoospores of *P. aphanidermatum* and *P. dissotocum* survived treatment to the highest chlorine concentration for 4 min in water where pH was not lowered. When the water pH was lowered to 6.0 prior to chlorine addition, 100% of the *P. aphanidermatum* zoospores were killed after 0.5 min exposure to 0.5 mg/liter chlorine, where ORP ranged from 748 to 790 mV and pH 6.3. Lowering the initial water pH improved disinfestation of *P. dissotocum* zoospores at the highest chlorine concentration tested and a mean ORP of 790 mV.

Introduction
Recycled water is increasingly used in commercial greenhouse and nursery operations because of concerns over sustaining production during periods of limited water supply, increasing costs, and environmental impacts of pesticide and nutrient residues in wastewater. However, recycled water may contain a diversity of *Phytophthora* and *Pythium* species and this may increase the disease incidence or severity caused by these water-dispersed pathogens if disinfestation is not adequate (2). Certain *Pythium* species, such as *P. aphanidermatum* and *P. dissotocum*, produce motile zoospores that may be rapidly dispersed through recycled greenhouse water or hydroponic systems (14), thus creating a management challenge.

Hong and Moorman (5) reviewed several methods for disinfesting water of plant pathogens, including filtration, heat, ozone, ultra-violet irradiation, non-ionic surfactants, and fungicides. Some methods, such as ozone and ultra-violet irradiation, may be prohibitively expensive for smaller greenhouse operations. Surfactants rapidly lyse zoospores of oomycetes in irrigation water, but are not effective in eliminating inoculum of other plant pathogens (9,12,13,14). Fungicides can be added to irrigation water, but fungicide resistant populations of *Pythium* may develop quickly after treatment (8) when the fungicide becomes diluted to a point lower than the lethal dose.

Chlorine is a strong oxidizer and an excellent sanitizing agent. Chlorinating water by adding sodium hypochlorite, calcium hypochlorite, chlorine gas, or chlorine dioxide is an economical method for managing plant pathogens including oomycetes. Hong et al. (6) reported that zoospores of six *Phytophthora* species were killed following exposure to 2 mg/liter chlorine for 0.25 min. Similar findings were reported for some *Pythium* species (7). However, factors other than total chlorine concentration may affect the disinfestation potential of a chlorine solution. These factors include temperature, pH, and organic content in water. During the process of oxidation, hypochlorous acid is reduced to hypochlorite, which has weaker disinfestation properties. Therefore, it is important to maintain and monitor the concentration of the oxidizer in the water to ensure sanitation (10).

The oxidation reduction potential (ORP), measured in mV, is an alternative method for expressing the sanitizing ability of a chlorine solution. ORP is a straightforward and real-time assessment of a solution's potential disinfestation properties (15). ORP is the potential (voltage) at which oxidation occurs at the anode (positive) and reduction occurs at the cathode (negative) of an electrochemical cell. In other words, an oxidizing chemical, such as chlorine, pulls away electrons from the cell membrane causing leaking and/or lysis (16). ORP of a chlorine solution increases with a decrease in pH. We used ORP to measure the disinfestant potential of various chlorine treatments against *P. aphanidermatum* and *P. dissotocum* zoospores. Since ORP is inversely related to pH at a given chlorine concentration, the water pH was lowered to observe its effect on ORP and subsequent *Pythium* zoospore mortality.

Zoospore Production

P. aphanidermatum zoospores were produced as previously described (11). Agar plugs of cultured *P. dissotocum* grown for 5 days on 10% V8 juice agar were removed and placed in a sterile Petri dish, then flooded with sterile municipal water and incubated at 25°C in the dark for 24 h. Zoospores were harvested by straining the water through several layers of cheesecloth. Zoospores were counted with a hemocytometer and adjusted to 2700 zoospores/ml in sterile tap water (pH 7.7 ± 0.4).

Monitoring ORP, pH, and Chlorine Treatment

A stock solution of 40 mg/liter chlorine was prepared from a 6% sodium hypochlorite solution, then diluted to 0.25, 1, and 4 mg/liter chlorine in municipal water in which the pH was not adjusted. The solution was stirred on a stir plate while the pH (pH 510 Benchtop Meter, Oakton Instruments, Vernon Hills, IL) and ORP (sensION 1 Portable pH Meter, Hach Company, Loveland, CO; SOTA-ORP-PS Electrode, Pulse Instruments, Van Nuys, CA) were measured immediately prior to addition of zoospore treatment suspensions.

To determine the effect of pH on ORP and disinfestation potential of chlorinated water, a second set of experiments was conducted in which the water pH was lowered to 6.0 with 1N hydrochloric acid prior to addition of chlorine. Chlorine assays were designed similar to Hong et al. (6), where 0.5 ml of chlorinated water was mixed with 0.5 ml of each zoospore solution. This resulted in the addition of 0, 0.125, 0.5, and 2 mg/liter chlorine from the sodium hypochlorite to the zoospore solution and provided a range of ORP values (Table 1). Zoospores were exposed to each treatment for 0.25, 0.5, 1, 2, and 4 min at 25° C. Treatments in which the initial water pH was lowered were subjected to an additional exposure of 8 min. After exposure, 12 µl of 0.2 N sodium thiosulfate (LabChem Inc., Pittsburgh, PA) was added to neutralize the reaction (6), and 100 µl of each zoospore suspension was pipetted and uniformly dispersed onto modified PARP-corn meal agar (3) amended with 1 mg rifampicin (Sigma Aldrich, St. Louis, MO) dissolved in 1 mL dimethylsulfoxide (DMSO, Sigma Aldrich, St. Louis, MO) and 0.4 ml of a 2.5% pimaricin aqueous solution (Sigma Aldrich, St. Louis, MO) per liter deionized water. The number of *Pythium* colonies on each plate was counted after incubation at 25°C in the dark for one or two days, depending on growth rate. Counts from the two plates were averaged for each concentration and exposure period. Zoospore mortality for each chlorine treatment was calculated relative to the non-chlorinated control. Experiments were repeated three times and data were combined for presentation. Statistical analysis of the effects of chlorine concentrations on pH and ORP were performed using the Proc GLM procedure of SAS statistical software (SAS Institute Inc., Cary, NC).

Table 1. Mean pH and ORP values in water in which the pH was not adjusted or lowered to 6.0 prior to adding chlorine.

	Pythium aphanidermatum				***Pythium dissotocum***			
	Unadjusted pH		Lowered pH		Unadjusted pH		Lowered pH	
Added chlorine	**pH**[x]	**ORP** (mV)	**pH**	**ORP** (mV)	**pH**	**ORP** (mV)	**pH**	**ORP** (mV)
Water[y]	7.6c	275d	6.0c	357d	7.8b	281d	6.0c	340d
0.25 mg/liter	7.7bc	633c	6.2bc	673c	7.8b	622c	6.0c	670c
1.0 mg/liter	7.8b	691b	6.3b	764b	7.9b	690b	6.3b	766b
4 mg/liter	8.0a	719a	6.6a	802a	8.1a	730a	7.0a	790a

[x] Means within a column followed by the same letter are not statistically different based on Fisher's protected least significant difference (P = 0.05).

[y] Municipal water prior to chlorine addition.

Relationship of ORP, pH, and Mortality of Zoospores

The pH of the municipal water used in the experiments ranged from 7.5 to 8.1 with ORP values between 262 and 311 mV. We did not detect any free chlorine in the municipal water using a colorimetric assay (Free and Total Chlorine Test Kit, Hach Company, Loveland, CO) even though this water had been chlorinated at the source. Addition of 0.125 mg/liter chlorine to the water increased the ORP to a mean value of 623 mV. This ORP value was similar to that of distilled water in which the same chlorine concentration was added (ORP 614 mV). The final level of chlorine in these treatments is not reported, but nevertheless, may have been slightly higher than the concentrations we added to achieve increasing ORP values. In preliminary experiments we determined that diluting zoospore suspensions with chlorinated water did not alter final pH and ORP values compared to the undiluted chlorinated water. Therefore we report the pH and ORP values of the chlorinated water prior to dilution with the zoospore suspension.

ORP values were dependent on pH and chlorine concentration of the water. Increasing the chlorine concentration and/or lowering the pH to 6.0 prior to chlorination resulted in higher ORP values (Table 1). In general, increasing the chlorine concentration, and thus ORP, resulted in higher *P. aphanidermatum* zoospore mortality in experiments where water pH was not lowered prior to addition of chlorine (Fig. 1). However, none of the ORP values or exposure periods resulted in complete zoospore mortality. We did not make statistical comparisons among these treatment means since none of them were completely effective in disinfesting the zoospore-contaminated water. Results were similar for *P. dissotocum* although exposure to a mean ORP of 730 mV for as little as 0.25 min resulted in an average zoospore mortality of > 90% (Fig. 2). Still, exposure to these treatments did not provide complete zoospore mortality.

Lowering the municipal water pH to 6.0 prior to the addition of 0.5 mg/liter chlorine decreased the final pH of the chlorinated water by an average of 1.5 and increased the mean ORP by 73 mV compared to the same chlorine concentration where water pH was not lowered (Table 1). This resulted in > 95% mortality of *P. aphanidermatum* zoospores at 0.25 min and 100% mortality at 0.5 min or longer (Fig. 3). The ORP of this treatment was an average of 45 mV higher and provided superior disinfestation to the treatment where the initial water pH was not lowered and a higher chlorine concentration (2 mg/liter) was added. Thus, lowering the initial pH reduced the amount of chlorine necessary for complete disinfestation.

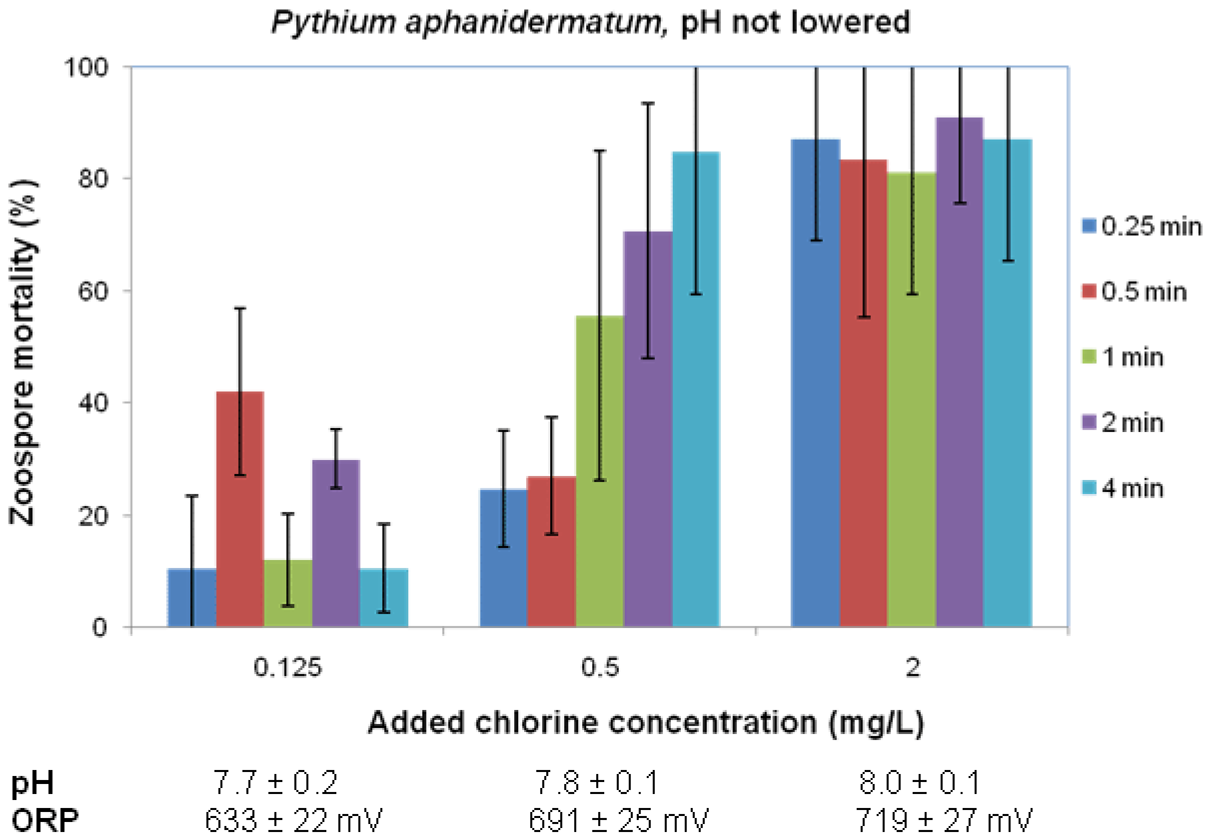
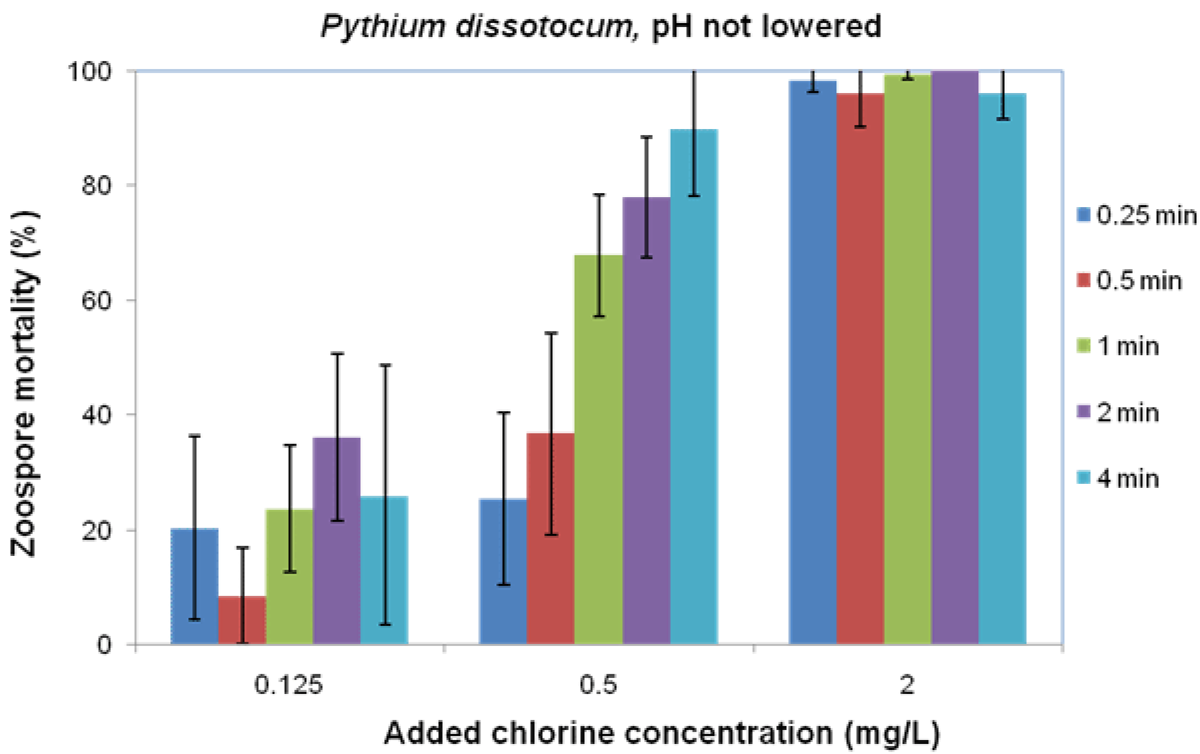


Fig. 1. Mortality of *P. aphanidermatum* zoospores following exposure to chlorine treatments prepared with municipal water in which the pH was not lowered. Each column represents the mean (with standard error bar) of 3 experiments. The pH and ORP values at the bottom of the graph are the means and standard errors associated with each chlorine concentration.



pH	7.8 ± 0.3	7.8 ± 0.2	8.1 ± 0.1
ORP	622 ± 35 mV	690 ± 19 mV	730 ± 6 mV

Fig. 2. Mortality of *P. dissotocum* zoospores following exposure to chlorine treatments prepared with municipal water in which the pH was not lowered. Each column represents the mean (with standard error bar) of 3 experiments. The pH and ORP values at the bottom of the graph are the means and standard errors associated with each chlorine concentration.

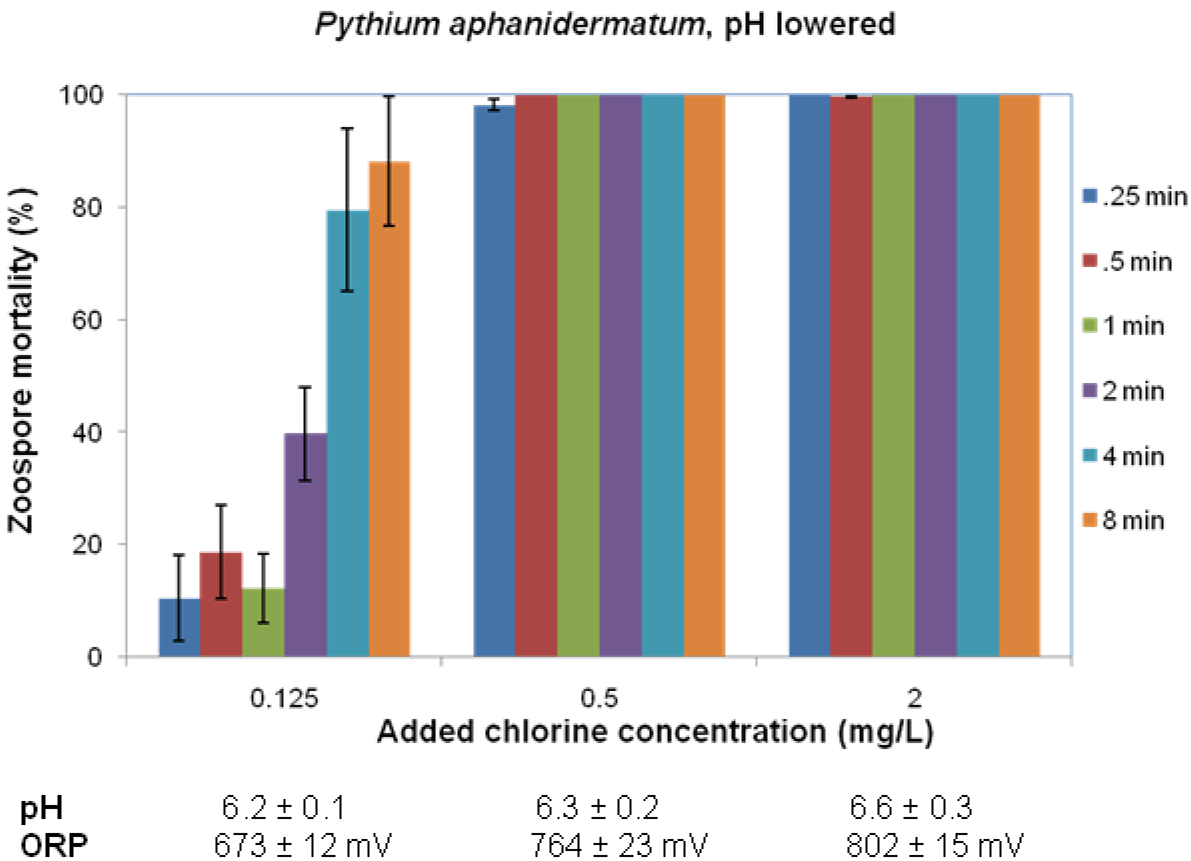
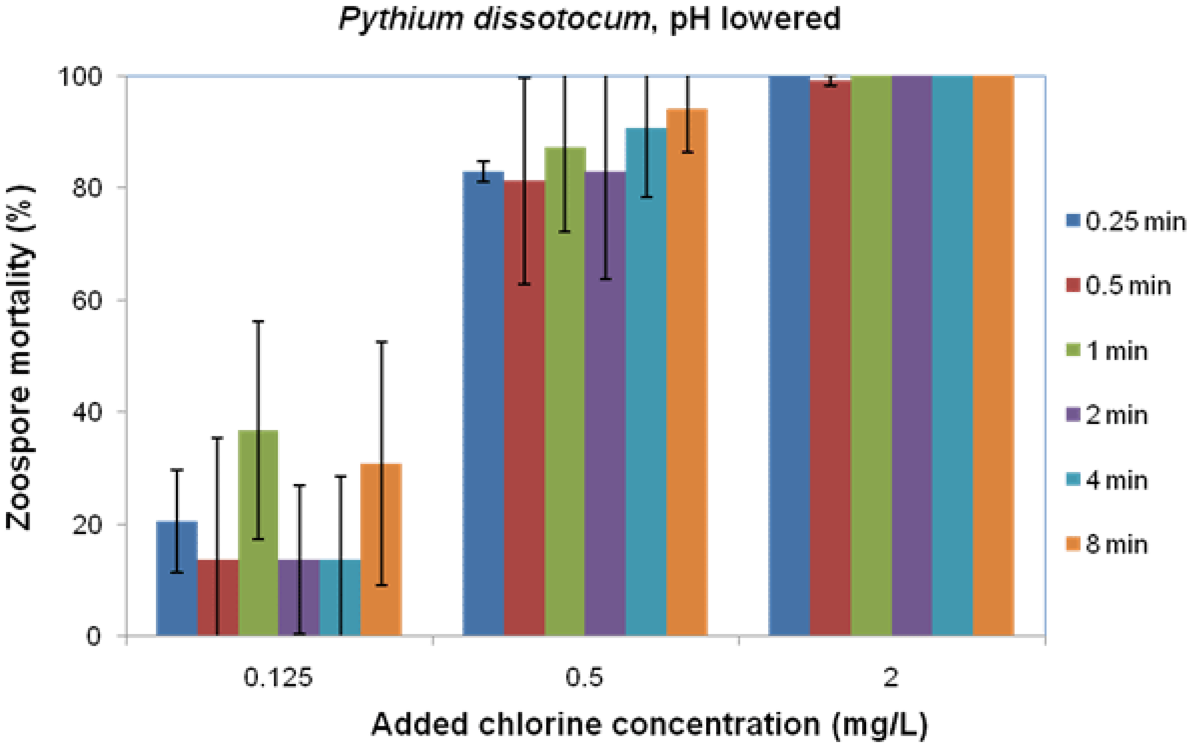


Fig. 3. Mortality of *P. aphanidermatum* zoospores following exposure to chlorine treatments prepared with municipal water in which the pH was lowered to pH 6.0 prior to addition of sodium hypochlorite. Each column represents the mean (with standard error bar) of 3 experiments. The pH and ORP values at the bottom of the graph are the means and standard errors associated with each chlorine concentration.

For *P. dissotocum*, lowering the initial water pH to 6.0 before adding 2 mg/liter chlorine reduced the final pH by an average of 1.0 and increased the average ORP by 60 mV compared to the non-pH adjusted treatment. It also resulted in complete mortality of *P. dissotocum* zoospores at all exposure times except 0.5 min (> 98% mortality) (Fig. 4). However, the benefits of lowering the water pH prior to addition of chlorine were not as evident at the lower chlorine levels. For example, adjusting the pH and then adding 0.5 mg/liter chlorine was less effective in killing *P. dissotocum* than *P. aphanidermatum* zoospores at all exposure times, even though ORP values were comparable in the treatments (Figs. 3 and 4). Furthermore, higher ORP values (> 780 mV) were needed for complete or near complete zoospore mortality in treatments where the water pH was initially lowered (Fig. 5). At 737 mV, the level of mortality in treatments which the pH was lowered (pH 6.6 with 0.5 mg/liter chlorine added) was 48% that of the complete mortality found in the non-pH adjusted treatment (pH 8.0 with 2.0 mg/liter chlorine added).



pH	6.0 ± 0.1	6.3 ± 0.2	7.0 ± 0.3
ORP	670 ± 21 mV	766 ± 26 mV	790 ± 13 mV

Fig. 4. Mortality of *P. dissotocum* zoospores following exposure to chlorine treatments prepared with municipal water in which the initial pH was lowered to pH 6.0 prior to addition of sodium hypochlorite. Each column represents the mean (with standard error bar) of 3 experiments. The pH and ORP values at the bottom of the graph are the means and standard errors associated with each chlorine concentration.

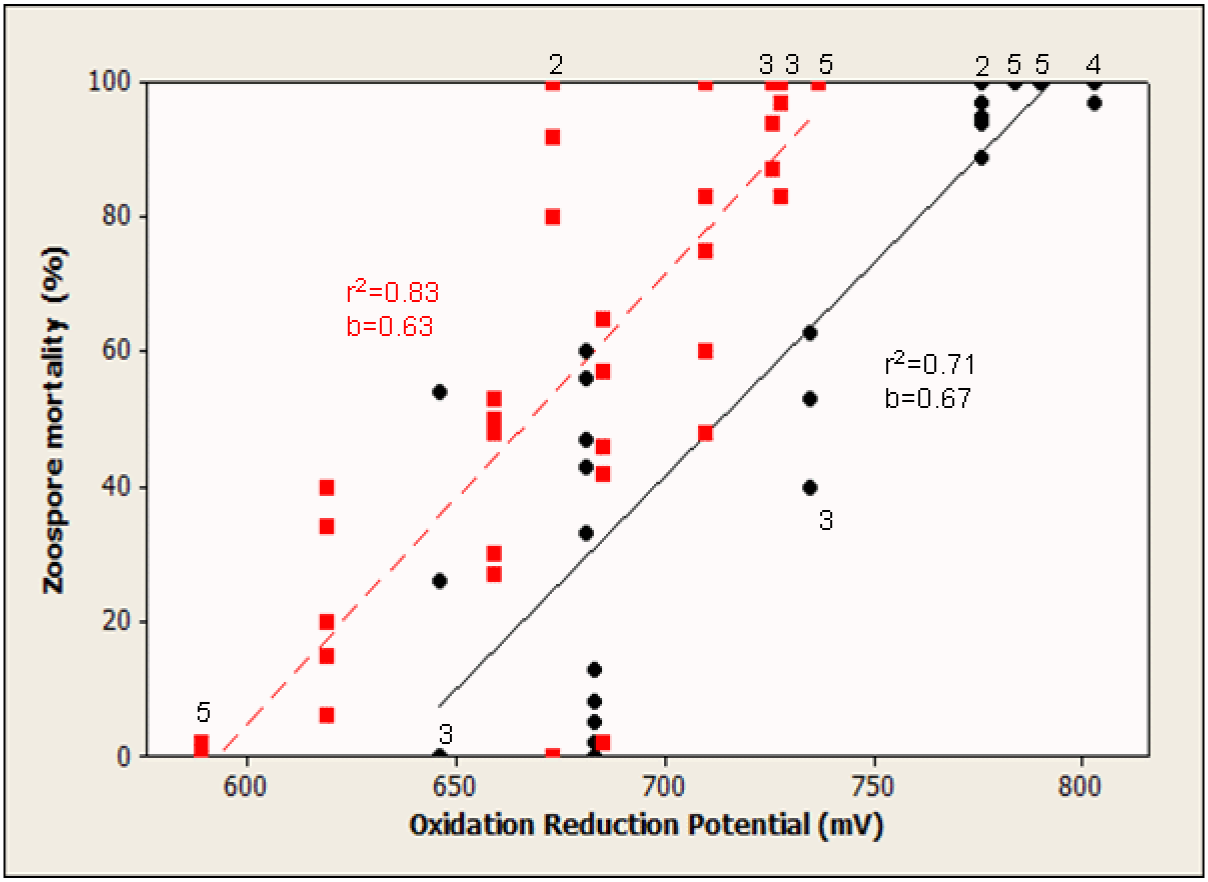


Fig. 5. Relationship of *P. dissotocum* zoospore mortality to ORP in chlorine treatments prepared in municipal water in which the initial pH was not lowered (red) and chlorine treatments in municipal water in which the initial pH was lowered to pH 6.0 (black). Data points represent values from all chlorine concentrations and exposure times for three experiments. Numerals indicate total number of observations represented by each data point. Slopes (b) of the two regression equations are not significantly ($P < 0.05$) different.

Discussion

In summary, *P. aphanidermatum* and *P. dissotocum* zoospores can be killed relatively quickly (0.25 to 2 min) by maintaining ORP values > 780 mV in chlorinated water. Hong and Richardson (7) reported complete mortality of *P. aphanidermatum* and *P. sulcatum* zoospores after 2 min exposure to 2.0 mg/liter chlorine. However they did not report the final pH and ORP measurements of chlorinated water treatments, so results cannot be directly compared. Maintaining an ORP > 780 mV may also be effective in killing other biological contaminants in water (6,15). This value is well above the ORP of 650 to 700 mV recommended to kill bacterial contaminants, such as *Escherichia coli*, and *Salmonella* species in water used in postharvest processing operations (16).

As mentioned previously, the oxidizing ability of a chlorine solution is pH dependent so lowering the pH of the water prior to chlorination increases the ORP. Suslow (15) recommended maintaining water pH between pH 6.0 and 7.5 to ensure adequate chlorine activity and to avoid the formation of toxic chlorine gas which occurs at pH < 6.0. Lowering the water pH before the addition of chlorine may be very important for growers using a municipal water supply where water pH may exceed 8.0. It is clear from our results that lowering the pH of a solution increased ORP and resulted in the mortality of *Pythium* zoospores. Buck et al (1) examined potential phytotoxicity caused by chlorine derived, acidic electrolyzed oxidizing (EO) water in bedding plants and found at pH of 2.8 to 2.9 and ORP of 1071 to 1079 mV only slight damage on certain plants, with severity ratings of 3 or less on a scale of 10. These values are much greater than experiments required for complete mortality of *Pythium* zoospores. Similarly, Grech et al. (4) did not witness significant phytotoxicity in citrus trees in field and greenhouse conditions when treated with up to 500 µg/ml of electrolytically generated chlorine, while *Phytophthora* sp., *Fusarium* sp. and bacteria were completely eliminated.

We believe measuring ORP values rather than total chlorine content is a simpler and more accurate measure of the disinfestation potential of a chlorine solution. ORP meters are relatively inexpensive and easy to operate and should be an essential piece of equipment for managers using a chlorination system for disinfestation.

Literature Cited

1. Buck, J. W., van Iersel, M. W., Oetting, R. D., and Hung, Y. –C. 2003. Evaluation of acidic electrolyzed water for phytotoxic symptoms on foliage and flowers of bedding plants. Crop. Prot. 22:73-77.
2. Bush, E. A., Hong, C., and Stromberg, E. L. 2003. Fluctuations of *Phytophthora* and *Pythium* spp. in components of a recycling irrigation system. Plant Dis. 87:1500-1506.
3. Ferguson, A. J., and Jeffers, S. N. 1999. Detecting multiple species of *Phytophthora* in container mixes from ornamental crop nurseries. Plant Dis. 83:1129-1136.
4. Grech, N. M., and Rijkenberg, F. H. J. 1992. Injection of electrolytically generated chlorine into citrus microirrigation systems for the control of certain waterborne root pathogens. Plant Dis. 76:457-461.
5. Hong, C. X., and Moorman, G. W. 2005. Plant pathogens in irrigation water: Challenges and opportunities. Crit. Rev. Plant Sci. 24:189-208.
6. Hong, C. X., Richardson, P. A., Kong, P., and Bush, E. A. 2003. Efficacy of chlorine on multiple species of *Phytophthora* in recycled nursery irrigation water. Plant Dis. 87:1183-1189.
7. Hong, C., and Richardson, P. A. 2004. Efficacy of chlorine on *Pythium* species in irrigation water. Proc. SNA Res. Conf. 49:265-267.
8. Moorman, G. W., and Kim, S. H. 2004. Species of *Pythium* from greenhouses in Pennsylvania exhibit resistance to propamocarb and mefenoxam. Plant Dis. 88:630-632.
9. Moorman, G. W., and Lease, R. J. 1999. Effects of fertilizer supplements and a biological control agent on Pythium root rot of geranium, 1998. B&C Tests 14:58-59.
10. Newman, S. E. 2004. Disinfecting irrigation water for disease management. Proc. of the 20th Ann. Conf. on Pest Management on Ornamentals. Soc. of Am. Florists, San Jose CA.
11. Raftoyannis, Y., and Dick, M. W. 2002. Effects of inoculum density, plant age and temperature on disease severity caused by pythiaceous fungi on several plants. Phytoparasitica 30:67-76.
12. Stanghellini, M. E., and Miller, R. M. 1997. Biosurfactants: Their identity and potential in the biological control of zoosporic plant pathogens. Plant Dis. 81:4-12.
13. Stanghellini, M. E., and Rasmussen, S. L. 1994. Hydroponics: A solution for zoosporic pathogens. Plant Dis. 78:1129-1138.
14. Stanghellini, M. E., Rasmussen, S. L., Kim, D. H., and Rorabaugh, P. A. 1996. Efficacy of nonionic surfactants in the control of zoospore spread of *Pythium aphanidermatum* in a recirculating hydroponic system. Plant Dis. 80:422-428.
15. Suslow, T. V. 2001. Water Disinfestation: A practical approach to calculating dose values for preharvest and postharvest applications. ANR Catalog Publ. no. 7256. Univ. of California, Davis, CA.
16. Suslow, T. V. 2004. Oxidation-reduction potential (ORP) for water disinfection monitoring, control, and documentation. ANR Catalog Publ. no. 8149. Univ. of California, Davis, CA.