Carbon Dioxide as a Potential Water Disinfectant for Phytophthora Disease Risk Mitigation

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Abstract

Kong, P. 2013. Carbon dioxide as a potential water disinfectant for Phytophthora disease risk mitigation. Plant Dis. 97:369-372.

The spread of *Phytophthora* spp. through irrigation systems and natural waterways can have a significant impact on plant health and requires mitigation. Pressurized carbon dioxide (CO₂) can inactivate *Phytophthora nicotianae* zoospores but its effectiveness at low pressure and on other species was unknown. This study evaluated the effect of injected CO₂ at 63 to 4,000 ppm in irrigation water on zoospore survival of four *Phytophthora* spp. and infectivity of *P. nicotianae* zoospores. Zoospore survival of *P. nicotianae*, *P. tropicalis*, and *P. pini* was reduced by over 90% at 4,000 ppm and was reduced by 40% at 125 to 2,000 ppm after a 2-h exposure. Survival of *P. megasperma* was less

affected by injected CO₂, with a reduction of 37.1% at ≤4,000 ppm. CO₂ treatments at 4,000 ppm for 30 or 120 min of water infested with *P. nicotianae* at zoospore concentrations of 1,000 and 5,000 ml⁻¹ reduced disease incidence of annual vinca (*Catharanthus roseus*) by 92 and 75%. Comparable efficacy was observed in the CO₂ treatment at 2,000 ppm. The CO₂ treatments at <2,000 ppm also significantly reduced disease caused by water infested at 1,000 zoospores ml⁻¹. These results indicate that CO₂ may have potential as a safe and effective water disinfectant for *Phytophthora* spp.

The spread of many plant pathogens, including the causal agent of sudden oak death in forestry and horticultural industries, has been associated with infested irrigation water sources (3,11,26). Chlorination is a widely used water treatment because of its effectiveness and relatively low cost at the recommended concentration of 2 ppm (13). However, chlorine gas is extremely hazardous and its effectiveness is influenced by pH, organic and inorganic chemicals in the water, and other factors (13,14,23). Chlorination is most efficacious at a pH of 5 to 6.5, and its efficacy drops with increasing pH (e.g., it can be as low as 20% at pH 8) (13,22). Such low efficacy may be common because pH in many irrigation water sources is high (up to 10.3), particularly during growing seasons (10).

It has been known for more than 100 years that carbon dioxide (CO₂) can exert an inhibitory effect on microbial growth (27). CO₂ has been used in the food industry and elsewhere for inactivation of microorganisms because of its safe, cost-effective, and straightforward application without extensive safety training and equipment. Pressurized CO₂ has been used as a fumigant or injected into juice or milk to kill microorganisms, preventing food spoilage and increasing shelf life (4,7,8,15,19,25). Its use has also been extended to sewage treatment for *Escherichia coli* contamination (20). For plant pathogens, pressurized CO₂ has been effective for inactivating *Phytophthora nicotianae* inocula (1,2). In recent studies, CO₂ was shown to work well at low pressures for inactivation of microorganisms in liquids (1,5,21,24). The treated liquid becomes weakly acidic due to dissolved CO₂ effectively killing pH-sensitive microbes (1,5). CO₂ as an acidifier could improve chlorination efficiency and also, by low pH itself, suppress *Phytophthora* spp., including *P. ramorum*, which are favored by a basic environment (17,18). However, little information is available regarding the effectiveness and cost of CO₂ as a disinfectant or acidifier. This study tested the efficiency of different concentrations of injected CO₂ in irrigation water on zoospore survival and resulting plant infection.

Materials and Methods

Pond water processing and CO₂ treatments. Water was collected from a nursery irrigation reservoir at a wholesale ornamental nursery located in eastern Virginia on 27 February, 6 April, and 7 June 2012 (Table 1, samples 1, 2, and 3, respectively). For each experiment, 10 liters of water were taken and filtered through membranes with pore sizes of 5.0 and 0.45 µm to remove algae, zoosporic microorganisms and most bacteria in the water, eliminating their interference. Processed water was stored at 4°C and equilibrated to room temperature before pH measurement and use.

Compressed (50 psi) CO₂ was bubbled through 2.5 liters of the filtered water in 3.8-liter plastic containers at a rate of 0.67 liter min⁻¹ for 8 min. This resulted in a CO₂ concentration of approximately 4,000 ppm; this estimate was based on the CO₂ mass flow rate with Boyle's law at 25°C and 1 atmosphere of pressure. The bubbled water was then immediately diluted with nonbubbled control water to obtain CO₂ concentrations of 2,000, 1,000, 500, 250, 125, 63, and 0 ppm. The dilutions were made three independent times for each experiment, and 100 ml of each dilution concentration was placed in 167-ml tissue culture containers (Greiner Bio) and used immediately. Duplicates were prepared for pH measurements with a UB10 pH/MV Meter (Denver Instrument) to estimate dissolved CO₂ levels. CO₂ injection reduced the pH of all three irrigation water samples. The pH of all samples injected with CO₂ at 4,000 ppm and their dilutions are listed in Table 1. The dissolved CO₂ levels were estimated with the aquarium CO₂ calculator CO₂ = 3 × KH × 10^(7-pH) (http://www.fishfriend.com/aquarium_co2_calculator.html). KH refers to carbonate hardness. It is measured in degrees and converted from CaCO₃ ppm (1° KH is equal to CaCO₃ at 17.9 ppm). Because CaCO₃ in the irrigation water reservoir was 63 ppm, KH of the water samples was 3.5°. The injected CO₂ levels were much higher than those estimated (Table 1) and many of the levels were outside the measurement range of water CO₂ sensors currently on the market. As such, injected CO₂ levels instead of dissolved CO₂ levels were used throughout in this study.

Phytophthora spp. and zoospore suspension preparation. Isolates of *P. megasperma* (42D2), *P. nicotianae* (45H1), *P. pini* (previously *P. citricola*; 43H1), and *P. tropicalis* (7G9) from irrigation water as well as an annual vinca isolate of *P. nicotianae* (3A12) from Virginia were used in this study. Zoospore production followed the method described previously (18). Briefly, agar plugs of

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Accepted for publication 28 September 2012.

1-week-old cultures were grown in 10% clarified V8 juice broth at room temperature for 7 days for *P. nicotianae* and *P. tropicalis* and 3 days for *P. megasperma* and *P. pini*. After the media were decanted, the cultures were rinsed with sterile distilled water (SDW), drained, and exposed to light for 24 to 48 h for *P. nicotianae* and *P. tropicalis* and 8 h for *P. megasperma*. The rinsed *P. pini* culture was flooded with SDW and incubated under light for 8 h to facilitate sporangium production. After the light exposure, plates were refilled with chilled, sterile soil water extract. Zoospores were harvested by passing suspensions through a layer of sterile Miracloth (Calbiochem). Zoospores were then counted with a hemocytometer after 30 min for *P. nicotianae* and *P. tropicalis* and after overnight incubation under light for *P. megasperma* and *P. pini*. Suspensions at greater than 10^4 zoospores ml^{-1} were used as stocks for working suspension preparation.

Agar plate assay for zoospore survival. A volume of stock zoospore suspension was added to the CO_2 water series in individ-

ual tissue culture containers to obtain a final zoospore concentration at 50 to 100 zoospores ml^{-1} . Three 1-ml samples were withdrawn from each container after 2 h of exposure and spread onto selective V8 agar medium plates (6). Emerging colonies were counted after a 2- to 3-day incubation at room temperature (22 to 25°C). Each treatment included three replicate containers and the experiment was repeated twice with different water samples. The average number of colonies in control plates was used as the denominator to calculate relative survival rate of zoospores for each treatment.

Infection assay. Annual vinca (*Catharanthus roseus* 'Little Bright Eye') was seeded and grown on a mist bench for 5 weeks. Four seedlings were transplanted into pine bark in 15-cm pots and grown for 8 to 10 weeks in the greenhouse before use. Zoospores of *P. nicotianae* (isolate 3A12) were exposed to CO_2 in the irrigation water sample from 7 June at designated concentrations for 30 min and 2 h before applying to the foliage. Zoospore concentra-

Table 1. Injected carbon dioxide (CO_2) concentrations and estimated dissolved CO_2 levels in irrigation water samples^a

CO_2 (ppm) ^b	Water sample 1		Water sample 2		Water sample 3	
	pH	D- CO_2	pH	D- CO_2	pH	D- CO_2
0	7.8	2	9.0	0	9.1	0
63	7.6	3	7.8	2	7.5	3
125	7.3	5	7.3	5	7.0	11
250	6.7	21	6.9	13	6.6	26
500	6.3	53	6.4	42	6.2	46
1,000	6.0	105	6.0	105	5.9	132
2,000	5.7	210	5.7	210	5.6	264
4,000	5.2	663	5.3	526	5.1	834

^a Dissolved CO_2 (D- CO_2) estimated based on measured pH and carbonate hardness of 3.5° .

^b Injected CO_2 concentration; 2.5-liter irrigation water samples were bubbled with compressed CO_2 at $0.67 \text{ liter min}^{-1}$ for 8 min which resulted in CO_2 at 4,000 ppm. The lower CO_2 concentrations were established through dilutions.

Fig. 1. Effect of injected carbon dioxide (CO_2) (0 to 4,000 ppm) on zoospore survival of *Phytophthora* spp. in irrigation water samples. Untreated (CK) and treated water at indicated concentration levels in containers was infested with zoospores and incubated for 2 h; then, 1 ml of the suspension was plated onto PARP medium. Emerged colonies in each plate were converted into relative survival (%) by dividing by the average number of colonies in control plates. Letters on the top of columns indicate significance levels among CO_2 treatments at $\alpha = 0.05$ by least significant difference test.

tions used were 1,000 and 5,000 zoospores ml⁻¹, and an average of 7 ml was sprayed onto plants in each container. To determine whether CO₂ may cause phytotoxicity on plants, a treatment without zoospores was also included. Each treatment contained three replicate pots, and experiments were performed on 1 and 6 June 2012, respectively. Disease development was monitored and the number of total shoots and diseased shoots were recorded after symptoms fully developed (3 to 5 days post inoculation).

Data analysis. Data from repeated experiments were first analyzed for homogeneity, then pooled for further analysis of variance among treatments. Means were separated based on the least significant difference at $\alpha = 0.05$ (SAS Institute, Inc.). The standard deviations among the experiments were calculated using Microsoft Excel.

Results

Effect of injected CO₂ on zoospore survival in irrigation water. Irrigation water with and without injected CO₂ from 63 to 4,000 ppm received zoospores of four *Phytophthora* spp., and the effect of CO₂ concentrations on zoospore survival was examined. In general, as CO₂ levels in irrigation water increased, zoospore survival decreased. Significant reduction of survival rates was ob-

Table 2. Significance levels of factors affecting annual vinca (*Catharanthus roseus* 'Little Bright Eye') disease incidence when inoculated with *Phytophthora nicotianae* zoospores sprayed onto foliage

Source	Mean	F	P
Experiment ^a	28.37	0.12	0.7343
Exposure time (T) ^b	1,362.19	5.56	0.0200
CO ₂ concentration (C) ^c	13,934.73	56.86	<0.0001
Zoospore density (Z) ^d	14,425.18	58.86	<0.0001
T × C	428.41	1.75	0.1439
T × Z	829.12	3.38	0.0684
C × Z	897.39	3.66	0.0075
T × C × Z	492.96	2.01	0.0900

^a Two experiments with irrigation water sample from June 2012.

^b Two exposures times at 30 min and 2 h.

^c Five carbon dioxide (CO₂) concentrations, including control and injected CO₂ at 250, 1,000, 2,000 and 4,000 ppm.

^d Two inoculum densities at 1,000 and 5,000 zoospores ml⁻¹.

served at as low as 63 ppm ($P < 0.0001$; Fig. 1). The reduction was much greater when injected CO₂ concentrations were 2,000 ppm or higher. Differences among species response to the treatments were detected, especially when CO₂ concentrations were low (Fig. 1). *P. megasperma* was the most tolerant of CO₂. The difference in zoospore survival between 250 and 4,000 ppm was not significant ($P = 0.9926$). In contrast, *P. nicotianae*, *P. pini*, and *P. tropicalis* were much less tolerant and most zoospores did not survive at high concentrations of CO₂. For *P. pini*, only 30 and 10% of the population survived at 2,000 and 4,000 ppm, respectively. *P. nicotianae* and *P. tropicalis* had similar responses but there was less than 20% population survival at 2,000 ppm and almost no survival at 4,000 ppm. At CO₂ concentrations of 1,000 ppm and below, all three species survived well although there were some differences among CO₂ concentrations ($P < 0.0001$).

Effect of injected CO₂ in irrigation water on infectivity of zoospores. To determine whether CO₂-treated water affects the ability of *P. nicotianae* (vinca isolate 3A12) zoospores to infect plants, infested irrigation water with and without injected CO₂ at 250, 1,000, 2,000 and 4,000 ppm for 30 min and 2 h was sprayed on annual vinca plants, and disease incidence was then recorded. Similar results were observed in two independent experiments and there was no difference between trials ($P = 0.7343$). There were significant differences among CO₂ treatments between exposure times and inoculum concentrations as well as interactions among the three factors (Table 2). The treated water with CO₂ concentrations of 2,000 and 4,000 ppm resulted in the most significant reduction of disease incidence (Fig. 2). Regardless of the length of exposure time, injected CO₂ at 1,000 ppm effectively lowered disease incidence at low density of inocula. However, longer exposure time (2 h) facilitated reduction of disease incidence resulted from the inoculum concentration at 1,000 ml⁻¹ when CO₂ was at 250 ppm (Fig. 2). No damage was observed on plants sprayed with CO₂-injected water even at 4,000 ppm, the highest concentration tested, indicating that CO₂-injected water was not phytotoxic.

Discussion

This study advances previous work on the efficacy of pressurized CO₂ (1,2) on controlling *Phytophthora* spp. and demonstrates that CO₂ at a low pressure can be used to treat *Phytophthora* spp.-

Fig. 2. Effect of injected carbon dioxide (CO₂) (0 to 4,000 ppm) in irrigation water on disease incidence of annual vinca (*Catharanthus roseus* 'Little Bright Eye') by *Phytophthora nicotianae* zoospores. Untreated (CK) and treated water at indicated concentration levels was infested with zoospores at inoculum densities of 1,000 and 5,000 ml⁻¹ for 30 min or 2 h and 7 ml was sprayed onto the foliage. Disease incidence is indicated with percentage of diseased shoots assessed 3 days post inoculation. Columns topped with the same letter are not significantly different according to the least significant difference test ($\alpha = 0.05$).

infested irrigation water, with the effectiveness dependent on the concentration, treatment time, pathogen species, and inoculum density. Injected CO₂ at 2,000 ppm or higher for 2 h was effective for treating irrigation water with a zoospore density of *P. nicotianae*, *P. pini*, and *P. tropicalis* as high as 5,000 zoospores ml⁻¹. Such a concentration was not effective for *P. megasperma*. Note that *P. megasperma* also tolerated a wide range of pH (18). The injection of CO₂ into water could be used to reduce viable inocula and protect plants from disease outbreaks in the field. Further, it may be possible to inject lower concentrations of CO₂ continuously into water in order to manage *Phytophthora* populations in irrigation systems where the pathogen is chronically present at low levels (9,16). Although injected CO₂ at 4,000 ppm can kill most zoospores within a few minutes (*data not shown*), levels of CO₂ as low as 250 ppm can reduce disease incidence. Such treatment in the present experiments resulted in a lowered inoculum level by 50% in 2 h. To effectively use CO₂ for waterborne *Phytophthora* spp. management, it is important to understand the differences in CO₂ tolerance among species present in the system.

Although the efficacy of injected CO₂ on zoospore pathogen survival and plant infection is evident, the practicality of using CO₂ for water treatment must be assessed in terms of cost. One pound of compressed CO₂ can treat 227 liters (60 gallons) of water to obtain CO₂ at 2,000 ppm for effective decontamination of severely infested water (5,000 zoospores ml⁻¹). The cost is \$8.3 per 3,785 liters (1,000 gallons) of water or \$2.2 per 1,000 liters, given that compressed CO₂ is \$0.5 per pound. Concentrations of *P. nicotianae* and other *Phytophthora* spp. are normally low (33 to 570 CFU/liter in the case of *P. nicotianae*; 12,16); thus, injected CO₂ concentration for regular water treatment could be as low as 250 ppm, consequently reducing the cost to \$0.26 per 1,000 liters (\$1 per 1,000 gallons) of water. This cost is greater than chlorine gas at the effective concentration of 2 ppm (0.025 per 1,000 gallons or \$6.6 per million liters; 13). However, the hazards inherent in chlorine gas handling, the cost of training workers to handle chlorine safely, the cost of the equipment needed, and its low effectiveness at high pH must be considered. Operation with compressed CO₂ does not require extensive training. A compressed CO₂ leak is not hazardous unless it occurs in an enclosed space. Also, CO₂ has other advantages over chlorine gas. For example, it reduces the pH of water with minimal impact on its effectiveness. CO₂ treatment could be used in combination with chlorine gas, reducing the water pH to levels optimal for chlorination.

Acknowledgments

This study was supported, in part, by the Virginia Nurserymen's Horticulture Research Foundation and by the United States Department of Agriculture National Institute of Food and Agriculture Specialty Crop Research Initiative (agreement number 2010-51181-21140). I thank P. Richardson, J. Zhang, M. Pistininzi, C. Hong, and G. Moorman for taking water samples, assistance in this study, or comments and suggestions on this manuscript.

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