In Vitro Fungicidal Activity of Acidic Electrolyzed Oxidizing Water

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ABSTRACT

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Acidic electrolyzed oxidizing (EO) water, generated by electrolysis of a dilute salt solution, recently gained attention in the food industry as a nonthermal method for microbial inactivation. Our objective was to determine if EO water has potential to control foliar diseases in greenhouses. Test fungi suspended in distilled water were combined with EO water (1:9 water:EO water) for various time periods, the EO water was neutralized, and germination was assessed after 24 h. Germination of all 22 fungal species tested was significantly reduced or prevented by EO water. All relatively thin-walled species (e.g., *Botrytis*, *Monilinia*) were killed by incubation times of 30 s or less. Thicker-walled, pigmented fungi (e.g., *Curvularia*, *Helminthosporium*) required 2 min or longer for germination to be reduced significantly. Dilution of EO water with tap water at ratios of 1:4 and 1:9 (EO:tap water) decreased efficacy against *Botrytis cinerea*. The presence of Triton X-100 (all concentrations) and Tween 20 (1 and 10%) eliminated the activity of EO water against *B. cinerea*. EO water did not damage geranium leaf tissue and inhibited lesion development by *B. cinerea* when applied up to 24 h postinoculation. EO water has a wide fungicidal activity which could facilitate its use as a contact fungicide on aerial plant surfaces and for general sanitation in the greenhouse.

The production of ornamental crops in both greenhouses and nurseries is one of the fastest-growing segments of agriculture in the United States, with an estimated value of over $6.1 billion in 1993 (4). In Georgia alone, the farmgate value of the greenhouse and nursery industry is over $350 million, with an estimated economic value of $991 million in 1999 (14).

Ornamental crops ranging from bedding plants (e.g., geranium, pansy) to cut flowers (e.g., lisianthus, zinnia) are produced in the greenhouse and are threatened by foliar plant pathogenic fungi, including *Botrytis cinerea* and powdery mildew (3). Effective greenhouse disease management includes sanitation, manipulating the environment, and use of preventive fungicides (3,5). Increasing concern about pesticides in the environment, potential worker safety issues, and fungicide resistance (9,15) indicate the need for alternative disease control measures.

A potential alternative to fungicides for control of foliar disease is the use of electrolyzed oxidizing (EO) water. EO water is generated by electrolysis of a dilute solution of sodium chloride in an electrolysis chamber where anode and cathode electrodes are separated by a nonselective membrane made from nonwoven polyester fabric (6). Water collected from the anode (EO water) has unique properties such as high oxidation-reduction potential, low pH, presence of hypochlorous acid, and strong bactericidal activity against most pathogenic bacteria (6,8,12). Exposing mixtures of *Escherichia coli*, *Salmonella enteritidis*, and *Listeria monocytogenes* to EO water for 5 min reduced populations by 7 log CFU/ml (12). Little is known about the exact mechanism of EO water for microbial inactivation. Extracellular oxidation-reduction potential (ORP) has been shown to modify metabolic fluxes and ATP production, probably due to the change in electron flow in the cell (11). Kim et al. (7) also suggested that ORP of the EO water might be the primary factor affecting microbial inactivation.

In a study to determine if EO water could be used to stimulate germination of *Tilletia indica*, Bonde et al. (2) observed that treatment of wheat seed for 20 min with EO water eliminated contamination by fungi such as *Aspergillus*, *Cladosporium*, and *Penicillium* spp. The objectives of this work were to (i) determine the range of fungicidal activity of EO water against several plant pathogenic fungi, including thin-walled species and thicker-walled pigmented fungi; (ii) determine the effects of dilution with tap water or presence of surfactants on EO water activity against *B. cinerea* and *Puccinia antirrhini*; and (iii) determine if EO water reduces in vitro lesion development by *B. cinerea* on geranium.

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MATERIALS AND METHODS

EO water. EO water was generated with a Hoshizaki ROX-20TA EO water generator using 2 M stock solutions of three test salts (NaCl, KCl, and $MgCl_2 \cdot 6H_2O$, certified A.C.S.; Fisher Scientific, Pittsburgh, PA). The pH and ORP of the EO water produced with each salt were measured with pH and ORP electrodes (model 50, ACCUMET meter; Denver Instrument Company, Denver) and ranged from pH 2.8 to 2.9 and 1,071 to 1,079 mV, respectively. The free-chlorine concentration was determined by an iodometric method using a digital titrator (model 16900; Hach Company, Loveland, CO) (12). Free-chlorine measurements were between 54 and 56 ppm for water generated with NaCl or KCl. Water generated from $MgCl_2 \cdot 6H_2O$ had free chlorine at 71 ppm. EO water generated from NaCl was used for all experiments except where noted. Water was generated and used within 1 h for each experiment.

In vitro germination assay. Fungal cultures were obtained from various symptomatic plant samples submitted for diagnosis to the Department of Plant Pathology, University of Georgia, Athens (UGA). Additional cultures were obtained from collections within the Department of Plant Pathology, UGA. Snapdragon rust (*P. antirrhini*) was maintained on snapdragon (*Antirrhinum majus*) cv. Tahiti Red. All other fungal isolates were stored on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) slants at 4°C or in 15% glycerol at –70°C. Conidia of each fungus were harvested from 2- to 3-week-old cultures on the appropriate agar medium (PDA, V8 agar, or water agar). Each culture was flooded with 10 ml of 0.05% Tween 20 (J. T. Baker, Phillipsburg, NJ), conidia were dislodged with a glass rod, and the resulting suspension was filtered through five layers of sterile cheesecloth. Rust urediospores on six to eight infected snapdragon leaves were dislodged from lesions by washing in 25 ml of 0.05% Tween 20. All spore types were pelleted, resuspended in sterile distilled water, and cell counts adjusted to 5×10^6 conidia ml^{-1}. A 100-µl volume of each conidial suspension was combined with 900 µl of EO water for 0, 30, 60, 90, 120, and 240 s. The activity of the EO water was stopped by the addition

of 9 ml of freshly prepared, filter-sterilized, neutralizing buffer (pH 7.2; Difco Laboratories) (12). A volume of 200 µl of spore suspension from each treatment was combined with 50 µl of yeast nitrogen base with 2% glucose (YNB, Difco Laboratories) and incubated in the dark at 24°C in 96-well microtiter plates for 24 h. After the 24-h incubation period, a volume of 25 µl of thimerosal (1 mg ml^{-1}; Spectrum Chemical MFG. Corp., Gardena, CA) was added to each well to stop fungal growth. Germination was assessed microscopically for a minimum of 200 conidia per treatment.

Due to the large variability observed in germination of each fungal species in the untreated checks (e.g., 98 to 100% of *B. cinerea* conidia germinated; less than 25% of *Colletotrichum* sp. conidia germinated), germination was recorded on a 0-to-5 scale relative to the untreated check, where 0 = none, 1 = 1 to 20%, 2 = 21 to 40%, 3 = 41 to 60%, 4 = 61 to 80%, and 5 = 81 to 100% of the germination observed in the untreated check.

Rust urediniospores were not treated with neutralizing buffer because the buffer interfered with germination (*data not shown*). A volume of 200 µl of urediniospores was combined with 3 ml of EO water in a sterile syringe, incubated, and added to 8 ml of distilled water. The resulting spore suspension was immediately filtered through 0.22-µm millipore filters, followed by passage of an additional 12 ml of distilled water to remove any residual EO water. Filters were placed in microcentrifuge tubes containing 1 ml of 0.05% Tween 20 solution and centrifuged (2,000 × *g*) to remove the urediniospores. Spores were resuspended in Tween 20 solution (0.05%), four 100-µl aliquots from each EO water incubation were plated onto water agar, and germination was assessed after 24 h. *Pantoea ananatis, Pseudomonas syringae* pv. *syringae*, *Pseudomonas syringae* pv. *glycinea*, and *Acidovorax avenae* subsp. *citrulli* were included in the in vitro assays as internal controls to confirm the bactericidal activity of EO water (6). Bacterial survival was determined by dilution plating onto nutrient agar (Difco Laboratories) and CFU recorded on a 0-to-5 scale (0 = no survival, 5 = 100% survival) compared with control treatments not receiving EO water treatment (100% survival).

Effect of dilution, surfactants, and electrolysis salt on efficacy of EO water. Conidia of *B. cinerea* were incubated as outlined above in EO water diluted with tap water in ratios of 1:1, 1:2, 1:4, and 1:9 (EO water:tap water). Conidia also were incubated with dilutions of Tween 20 and Triton X-100 (Fisher) combined with EO water to make concentrations of 0, 0.1, 1, and 10% surfactant. EO water was generated from 2 M solutions of each of three salts (NaCl, KCl, and $MgCl_2 \cdot 6H_2O$) and either *B. cinerea* or *P. antirrhini* were treated with the different waters. EO water activity was stopped for each experiment as outlined above. The percent germination was assessed after 24 h for a minimum of 200 spores from each of four 100-µl aliquots of treatment solution incubated on either PDA (*B. cinerea*) or water agar (*P. antirrhini*) at 24°C.

***Botrytis* disk assay.** Geranium (*Pelargonium* × *hortorum* L. H. Bailey cv. Dark Red) stock plants were grown in Metro-Mix 360 (The Scotts Co., Marysville, OH) in 11.4-liter pots and fertilized weekly with Peters 15-15-15 Geranium Special (The Scotts Co.) with N at 200 ppm. Leaves, 10 to 12 weeks old, were removed and surface sterilized by immersion in 10% bleach (60 s) and 70% ethanol (20 s), followed by three rinses in distilled water. Disks (12 mm in diameter) were cut and placed on sterilized filter paper moistened with sterile, distilled water in glass petri dishes. Leaf disks were inoculated with a 25-µl aliquot of *B. cinerea* conidial suspension (1×10^5 cells ml^{-1}) in dilute nutrient solution (40 mM glucose, 20-fold dilute YNB). Dishes were incubated at 24°C under cool white fluorescent bulbs (16 h dark, 8 h light). Disks were treated by pipetting 50 µl of EO water or 50 µl of distilled water onto the inoculum drops at 0, 6, 12, and 24 h postinoculation. Lesion development was rated after 96 h on a scale of 0 to 5 (0 = no lesion, 5 = complete browning of disk). Each treatment consisted of three replicate dishes with 15 disks per replicate.

Statistical analysis. All experiments were repeated at least two times. Data were analyzed by two-way analysis of variance (ANOVA) with means separated by Fisher's protected least significant difference (LSD) with $P = 0.05$.

RESULTS

In vitro germination assay. Treatment of fungal spores with EO water reduced or eliminated germination of all species tested (Table 1). No germination was observed for thin-walled species, including *B. allii*, *B. cinerea*, *Monilinia fructicola*, *Aspergillus* spp., and *Trichoderma* spp., after incubations of 30 s. *B. cinerea* did not germinate after EO water treatments as short as 5 s (*data not shown*). The yeast *Rhodosporidium toruloides* was killed by a 30-s exposure to EO water. Thicker-walled species, including *Epicoccum nigrum*, *Alternaria panax*, and *Curvularia* sp., re-

Table 1. Effect of length of acidic electrolyzed oxidizing water treatment on conidial germination and bacterial or yeast survival

	Treatment time and germination or survival[z]			
Test organism	**0**	**30**	**60**	**120**
Alternaria sp. #1	5	0	0	0
Alternaria sp. #2	5	0	0	0
Alternaria panax	5	1	0	0
Aspergillus flavus	5	0	0	0
Aspergillus niger	5	0	0	0
Botrytis allii	5	0	0	0
Botrytis cinerea	5	0	0	0
Cladosporium sp.	5	0	0	0
Colletotrichum sp. #1	5	0	0	0
Colletotrichum sp. #2	5	0	0	0
Curvularia lunata	5	3	2	0
Didymella bryonaie	5	0	0	0
Epicoccum nigrum #1	5	4	3	3
Epicoccum nigrum #2	5	4	3	3
Fusarium moniliforme	5	0	0	0
Fusarium sp.	5	0	0	0
Helminthosporium sp. #1	5	5	5	5
Helminthosporium sp. #2	5	4	1	1
Monilinia fructicola	5	0	0	0
Pestalotia sp.	5	0	0	0
Phomopsis longicolla	5	0	0	0
Rhodosporidium toruloides Y-1091	5	0	0	0
Rhodosporidium toruloides Rg-1	5	0	0	0
Stagonospora nodorum	5	0	0	0
Thielaviopsis basicola	5	0	0	0
Trichoderma spirale	5	0	0	0
Acidovorax avenae subsp. *citrulli*	5	0	0	0
Erwinia chrysanthemi	5	1	1	1
Pantoea ananatis	5	0	0	0
Pseudomonas syringae pv. *syringae*	5	0	0	0
Pseudomonas syringae pv. *glycinea*	5	0	0	0

[z] Germination of at least 200 conidia was assessed microscopically after a 24-h incubation in the dark in dilute nutrient solution. Fungal germination was recorded on a 0-to-5 scale relative to the untreated time 0 control, where 0 = no germination, 1 = 1 to 20% of control, 2 = 21 to 40% of control, 3 = 41 to 60% of control, 4 = 61 to 80% of control, and 5 = 81 to 100% of the control. Bacterial and yeast population sizes were determined by dilution plating and converted to a 0-to-5 scale, where 0 = no growth, 1 = 1 to 20% of control, 2 = 21 to 40% of control, 3 = 41 to 60% of control, 4 = 61 to 80% of control, and 5 = population size of time 0 control.

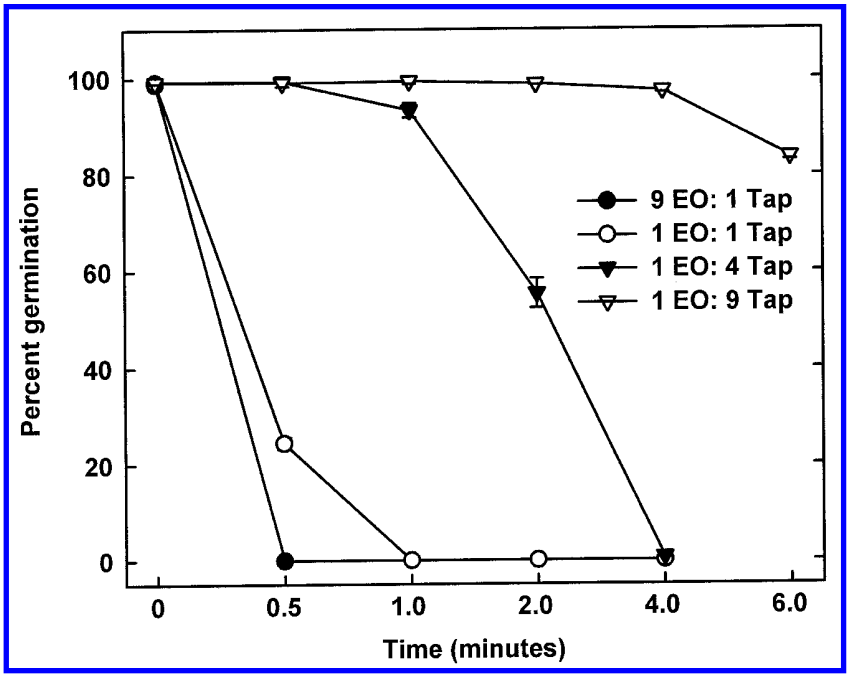

Fig. 1. Germination of *Botrytis cinerea* conidia after treatment with different dilutions of acidic electrolyzed oxidizing (EO) water and tap water. Germination was assessed microscopically after a 24-h incubation on potato dextrose agar. Each data point represents the mean and standard deviation of four groups of at least 200 conidia.

Table 2. Percent germination of *Botrytis cinerea* conidia on potato dextrose agar after incubation in acidic electrolyzed oxidizing water combined with different concentrations of either Tween 20 or Triton X-100

	Treatment time and germination[z]				
Surfactant, concentration (%)	**0**	**30**	**60**	**120**	**240**
Tween 20					
0.1	99.3 a	0.9 a	0.6 a	0.7 a	0.3 a
1	99.8 a	4.5 b	4.0 b	4.5 b	3.8 b
10	99.2 a	99.2 c	99.4 c	99.6 c	99.7 c
Triton X-100					
0.1	99.5 a	99.1 c	99.4 c	99.2 c	99.6 c
1	99.6 a	99.4 c	99.2 c	99.2 c	99.6 c
10	98.7 a	99.5 c	99.4 c	99.3 c	99.6 c

[z] The percentage of conidia that germinated was assessed microscopically after 24 h for a minimum of 200 spores from each of four 100-µl aliquots of each treatment solution. Data followed by a different letter within a column are significantly different ($P = 0.05$).

quired longer incubations to reduce germination (Table 1). All fungal hyphae were killed by exposure to EO water (*data not shown*). Epiphytic bacteria, including *Pantoea ananatis, Pseudomonas syringae* pv. *syringae*, *P. syringae* pv. *glycinea*, and the watermelon fruit blotch pathogen *A. avenae* subsp. *citrulli*, were killed by 30-s incubations with EO water (Table 1). However, small numbers (less than 1% of starting population) of the bacterium *Erwinia chrysanthemi* survived EO exposures of 120 s (Table 1).

Effect of dilution, surfactants, and electrolysis salt on efficacy of EO water. Dilution of EO water by 1:4 and 1:9 (EO water:tap water) significantly reduced efficacy against *B. cinerea,* with longer incubation times required to kill the conidia (Fig. 1). All of the *B. cinerea* conidia were killed by a 60-s exposure to EO water diluted 1:1 with tap water. A 4-min incubation was required to kill all conidia with a fivefold dilution of EO water (Fig. 1). Higher concentrations of Tween 20 (1 and 10%) significantly reduced the fungicidal activity of the EO water (Table 2). A concentration of 0.1% Tween 20 did not affect the fungicidal activity of the EO water. The presence of Triton X-100 at all tested concentrations eliminated activity of the EO water against *B. cinerea* (Table 2). There were no differences in pH or ORP among EO water concentrations made from the different salts, but significantly more free chlorine was present in the water made from $MgCl_2 \cdot 6H_2O$ (*data not shown*). EO water generated from the different salts did not differ in the ability to kill *B. cinerea* conidia (100% of conidia killed by 30-s exposures) or *P. antirrhini* urediniospores (Fig. 2). A 2-min exposure of *P. antirrhini* to EO water prevented germination (Fig. 2).

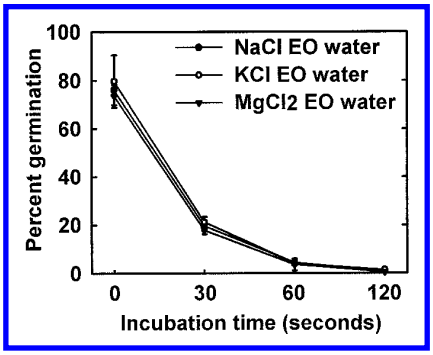

Fig. 2. Germination of *Puccinia antirrhini* urediniospores after treatment with acidic electrolyzed oxidizing (EO) water generated from three different salt solutions (NaCl, KCl, and $MgCl_2 \cdot 6H_2O$). Germination was assessed microscopically after a 24-h incubation on water agar. Each data point represents the mean and standard deviation of four groups of at least 200 urediniospores.

***Botrytis* disk assay.** Tissue discoloration, indicating initial lesion development, was visible on the geranium leaf disks at 24 h postinoculation. EO water prevented lesion development by *B. cinerea* on geranium leaf disks when applied 0, 6, and 12 h postinoculation (Fig. 3). EO water significantly reduced lesion development when applied to inoculation drops 24 h after inoculation. There was no damage to the geranium leaf material by the EO water.

DISCUSSION

Alternatives to conventional fungicides are desirable for control of foliar diseases of greenhouse-grown crops. These products ideally should fit into existing integrated pest management (IPM) programs and reduce risk to both workers and the environment. EO water has been demonstrated to sanitize food preparation surfaces and hospital equipment (2,6,12,13). In this study, acidic electrolyzed water quickly killed a wide variety of fungal spores and hyphae, confirming reports of broad fungicidal properties in addition to the bactericidal properties previously reported (2,12,13). This quick activity could facilitate the use of EO water as a contact fungicide on aerial plant surfaces and for general sanitation in the greenhouse.

EO water has greater potential for the control of foliar diseases than soilborne diseases because the activity of the water is neutralized by nonselective reducing agents (10) possibly found in soil or potting mix. Inactivation by organic compounds was suggested in our study by the reduced efficacy of EO water when combined with high concentrations of surfactant. Spray programs for foliar pathogens would have to limit or omit spreader stickers and apply sufficient volumes to wet the foliage. It is unknown what happens to the properties of EO water when in contact with the waxy cuticle on plant surfaces, but our in vitro studies suggest that contact

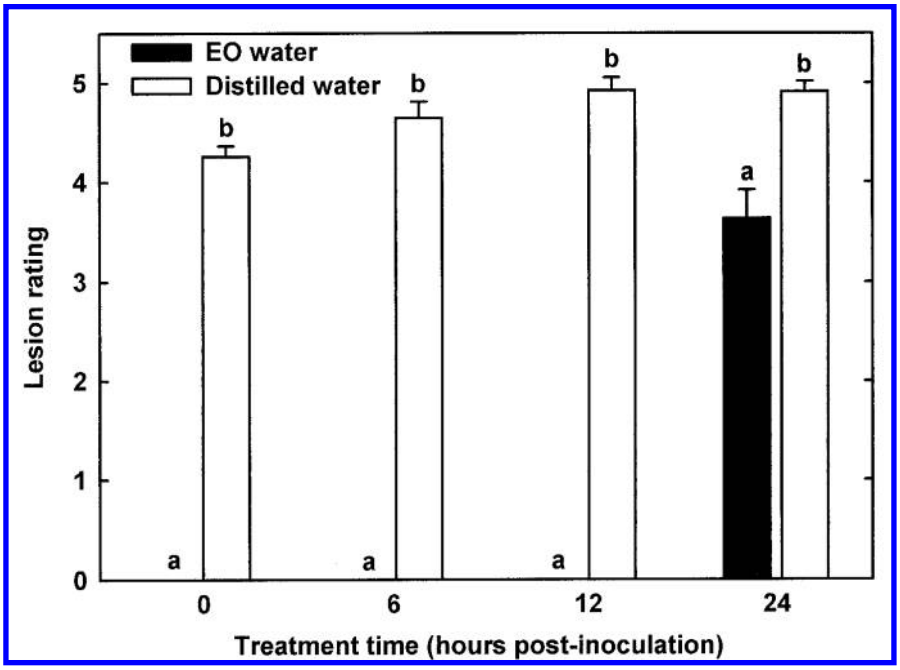

Fig. 3. *Botrytis cinerea* lesion development on geranium leaf disks treated with acidic electrolyzed oxidizing (EO) water or distilled water at time of inoculation or postinoculation. Lesion development was rated after 96 h on a scale of 0 to 5 (0 = no lesion, 5 = complete browning of disk). Each data point represents the mean and standard deviation of three replicate groups of 15 disks. Lesion ratings with different letters within each treatment time are significantly different ($P = 0.05$).

with geranium leaf surfaces does not reduce efficacy. The possibility of diluting EO water at least 1:1 (and perhaps more) with tap water further facilitates use in a greenhouse setting.

EO water did not cause phytotoxicity on the foliage of over 16 species of bedding plants, including alyssum, geranium, salvia, and vinca (*unpublished data*). Slight damage, but not sufficient damage to reduce marketability, was observed to the short-lived flowers of vinca. Damage may have been caused by the sodium, which is phytotoxic to a variety of plant species (1), or by the high ORP of the water. EO water generated from other chloride salts (KCl, $MgCl_2$) had similar pH, ORP, and fungicidal properties as EO water generated from NaCl. This ability to generate EO water from a variety of salt solutions suggests that any problems with phytotoxicity potentially could be avoided. We currently are investigating the effect of electrolysis salt on phytotoxicity to bedding plants.

Many types of electrolyzed water generators are currently manufactured commercially. The machine used in this study produces both acidic and basic electrolyzed water separately by electrolysis with a dilute salt solution. The acidic EO water has been demonstrated to have very strong bactericidal (6,12,13) and fungicidal effects (this study, 2). Other types of electrolyzed water generators include one that produces very mild acidic and basic solutions, a machine that produces a single type of electrolyzed water with a pH around 6.8, one producing strong acidic and basic solutions using an ion-selective membrane during electrolysis, and others.

EO water is safe to handle (6) and several hospitals in Japan routinely use EO water for surface sterilizing and hand washing (2). The safety of EO water and the ability to use existing spray equipment would allow for rapid integration of this technology into existing IPM programs for foliar disease control. The window of activity against foliar pathogens would probably be limited to the preinfection stages of fungal life cycles and general sanitation in the greenhouse. Repeated, preventive sprays would have to be utilized for continual disease control. In preliminary experiments, EO water eliminated visible powdery mildew symptoms on gerbera daisy when sprayed twice a week to leaf wetness (*unpublished data*). Additional greenhouse studies are being completed to determine spray volumes and timing for disease control with various host–pathogen systems.

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