Detection of High Concentrations of Organic Acids in Fish Emulsion and Their Role in Pathogen or Disease Suppression

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ABSTRACT

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Fish emulsion (FE) added to a sandy-loam soil at 1 and 2% rates reduced the viability of *Verticillium dahliae* microsclerotia by 39 and 74% in 1 day, 87 and 98% in 3 days, and 95 and 99% in 6 days, respectively. The immediate kill of microsclerotia indicated that FE contains toxic substances. We found in FE high concentrations (400 mmol/liter) of organic acids, including some known toxicants. Glycolic, acetic, formic, *n*-butyric, and propionic acids were the major organic acids detected in FE at the proportions of 52.5, 26.9, 7.9, 7.2, and 4.7%, respectively. In solution assays, the viability of *V. dahliae* microsclerotia treated for 24 h in 1, 2, 5, and 10% FE (pH 3.6 to 3.0) or a mixture of organic acids (pH 4.1 to 3.9) equivalent to the proportions in FE was reduced by 74, 94, 97, and 99% or 81, 91, 98, and 99%, respectively. The viability of microsclerotia was increased when the treatment solutions were buffered to pH

6.0. The organic acids mixtures and formic (0.025%) and acetic (0.1%) acids were toxic to *Pythium ultimum*. A mixture of organic acids (1, 2, and 4%) provided immediate protection of cucumber seedlings from damping-off in *P. ultimum*-infested muck and sandy-loam soils but not in peat-based mix. FE (1 and 2%) provided immediate protection of cucumber seedlings from damping-off in an infested muck soil, and disease protection was consistent when planting was delayed for 7, 14, and 28 days after adding FE. FE (1, 2, and 4%) did not provide immediate protection of cucumber seedlings from damping-off in a *P. ultimum*-infested peat-based mix; however, disease suppression was evident when planting was delayed for 7, 14, and 21 days after adding FE. Real-time polymerase chain reaction analyses of the peat-based mix indicated that the *P. ultimum* populations in the FE-amended mix declined over time. This study suggests that these organic acids in FE played a major role in pathogen or disease suppression, depending on the soil and substrate.

Additional keywords: organic soil amendment, volatile fatty acid.

Additions of organic materials to pathogen-infested soils or substrates have been reported to suppress plant diseases (8,25, 28,31–33) but the mechanisms of suppression were rarely investigated or understood. Any information on how organic amendments provide disease suppression is essential for optimizing and enhancing disease-reducing efficacy that these organic materials may provide (29). A general or long-term effect of organic soil amendments is their potential to enhance microbial activity, including biocontrol agents, leading to the establishment of natural disease suppressive conditions (8,25,32,33). Organic amendments increase the activity and diversity of resident microbial communities by increasing the organic matter content of the amended soil (30) and, thereby, may provide a climate more favorable to natural biological control of soilborne plant pathogens.

Short-term effects may be more specific to a particular type of amendment that results in an immediate reduction of pathogen inoculum (31,38,39). High nitrogenous and volatile fatty acid (VFA)-containing amendments are excellent examples displaying an almost immediate reduction in pathogen populations via short-lived toxic metabolites (18,29,37,39). Products such as soymeal, poultry manure, meat and bone meal, and so on, when applied to soil in high quantities, are broken down by soil microbes and liberate large quantities of ammonia which, at neutral or acid pH levels, is converted to ammonium. However, at pH levels above 8.0, ammonium is converted to the volatile gas ammonia (29,38)

and, whereas ammonium is not toxic, ammonia is very toxic (38). In the course of nitrification ammonium is converted to nitrite which is not toxic at neutral pH but, at low pH (≤ 5.0) is converted to nitrous acid, which is a very potent biocide (38). The presence of these active products is highly dependent on soil organic matter content and soil pH and its buffering capacity (38). Volatile toxic compounds can also be produced by creating anaerobic and reducing conditions in the soil by incorporating fresh organic material such as broccoli or grass (11). Covering of the amended plots with plastic sheets enhances the concentration and the longevity of the toxicants in the soil profile (11).

Liquid swine manure (LSM) was found to provide disease relief by virtue of the presence in the manure of high concentrations of toxic VFAs, such as acetic, butyric, propionic, valeric, and other acids (18,37). Only manures that contained sufficient quantities of these VFAs provided significant disease reduction and only in soils where the pH was < 5.0 , allowing for the biological activity of the acids (18,37). In soils with pH levels > 6.0 , the VFAs exist as salts that have no biological activity (37). The effects of organic amendment suggests that both chemical and biological components of the amended soils can contribute to disease suppression (1,14,19), and it is likely that several mechanisms contribute to activity simultaneously with some products in some soils.

Fish emulsions (FE) or fish-soluble nutrients are liquid by-products of the fish-processing industry and have been used mainly as fertilizers (10,15). FE was used in one study as a nutrient base for plant-growth-promoting rhizobacteria (21). We demonstrated that FE can also provide highly effective disease protection against some foliar (5) and soilborne diseases (2,3). A

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preplant amendment of FE in various peat-based substrates or soils suppressed damping-off diseases caused by *Rhizoctonia solani* and *Pythium aphanidermatum* (2) and potato scab (3). The suppressing effect of FE was immediate in soil but not in peat-based substrates, and the effective rate of FE was also different in peat-based substrates and soils. This suggested that the mechanisms of suppression by FE may be different in peat-based substrate, organic or muck soil, and sandy-loam soils. The current study was undertaken to analyze FE for the presence of any toxic substances and investigate mechanisms of pathogen or disease suppression.

MATERIALS AND METHODS

Plant material, soil, and FE. Fungicide-free cucumber (*Cucumis sativus* L. cv. Straight Eight) seed was purchased from William Dam Seeds Ltd., Flamborough, ON, Canada. A muck or organic soil (organic matter 50%, pH 6.8) naturally infested with *P. ultimum*, *P. irregulare*, and other *Pythium* spp. (6,7) was collected from commercial vegetable fields near Holland Marsh, ON. As reported earlier, the average propagule density of *Pythium* spp. in the muck soil was 1.4×10^3 CFU/g soil (7) as determined by soil dilution plating on PARP medium (corn meal agar amended with 5 mg of pimarin, 250 mg of ampicillin, 10 mg of rifampicin, and 100 mg of pentachloronitrobenzene), selective for isolating pythiaceus organisms (22). Sandy-loam soils (organic carbon 0.7 and 0.8%; pH 6.2 and 7.1) used in laboratory microcosm assays and growth-room bioassays were from commercial potato fields near Alliston, ON (sites M and V2). No native *Pythium* populations were found in these sandy-loam soils. FE and fish meal (FM) samples used in this study were prepared from menhaden fish (*Brevoortia tyrannus*) and provided by Omega Protein, Houston, TX.

***Verticillium dahliae* germination assay.** The toxicity of FE or FM to microsclerotia of *Verticillium dahliae*, the fungal pathogen that causes early dying of numerous plants (34), was determined in a sandy-loam soil (site M) in a laboratory microcosm assay (24,38). FE (1 and 2% or 1 and 2 g/100 g of dry soil) and FM (0.5 and 1% or 0.5 and 1 g/100 g of dry soil) were added to the soil at 10% moisture (mass/mass soil) in polyethylene bags and vigorously mixed. Aliquots (20 g) were then transferred to test tubes (22 by 150 mm) and closed with plastic caps that allowed for air exchange. Freshly prepared microsclerotia produced as described previously (24) were mixed with acid-washed quartz sand at a ratio of 15 mg of microsclerotia (106 to 250 µm in diameter) per gram of sand (106 to 250 µm in diameter). The sand:microsclerotia mixture was added to polyester mesh bags (8 to 10 mg/bag) and the bags were placed in the tubes prior to addition of soil in order to ensure that soil surrounded the bag. The tubes were gently tapped to settle the soil and incubated in the dark at 24°C. After 1, 3, and 6 days, three replicate bags for each treatment were removed and placed in a sandy-loam soil (site M) at 10% moisture for 1 week prior to plating onto a semiselective medium (24) using an Anderson air sampler. Plates were incubated at 24°C in the dark for 2 weeks. The number of germinating microsclerotia was counted under a dissecting microscope from the first 100 from each plate and percent germination

for each treatment was determined. Soil pH was determined by placing soil (8 g) and cold water (40 ml) in plastic sacks. The sacks were sealed and the slurry mechanically disrupted (30 s) using a Stomacher homogenizer (Seward Medical, London), and the sacks were placed on an orbital shaker at 200 rpm for 1 h at 4°C, then removed from the cold, mechanically disrupted again, allowed to sit at room temperature for 1 h, and the pH determined. The experiment was repeated once.

Analysis of organic acids in FE. The concentration of organic acids in FE samples was determined by chemical suppression ion exclusion chromatography and conductivity detection (Dionex model 100; Dionex Corp., Sunnyvale, CA) as described previously (37). FE samples were diluted 10 and 100 times with Millipore distilled water. The diluted samples were centrifuged for 10 min at $10,600 \times g$ to remove particulates and 40 µl of solution was injected to the ion chromatograph by a refrigerated autosampler. The chromatograph was equipped with an IonPac ICE-AS1 analytical column and AMMS ICE II chemical suppressor (Dionex Corp.). The identity of individual organic acids was confirmed by running standards.

Toxicity of organic acids from FE. A mixture of organic acids was prepared by adding individual organic acid components at the proportions found in FE (Table 1) to a final concentration of ≈ 400 mmol/liter. The stock mixture of organic acids was stored in a refrigerator at 4°C until use. Toxicity of the organic acids mixture to *P. ultimum* culture plugs and *V. dahliae* microsclerotia was determined in solution assays. The mixture of organic acids and FE was diluted with sterile double-distilled water (ddH₂O) to the final concentrations of 1, 2, 5, and 10% (vol/vol) immediately before use and their pH was determined with a pH meter. One set of FE and organic acid solutions was buffered to pH 6.0 with citric acid-NaOH buffer. A nonbuffered water control (pH 6.8) and a water control buffered to pH 4.0 with citric acid-NaOH were included as well. The treatment solutions (5 ml) were transferred to 12-ml sterile culture tubes (three per treatment) and three mesh bags containing *V. dahliae* microsclerotia were submerged in each of the three replicate tubes per treatment. In the case of *P. ultimum*, three 5-mm plugs from a 48-h-old culture were submerged only in organic acid solutions in each of the three replicate tubes per treatment. The tubes were capped and incubated for 24 h in the dark at 24°C. The culture plugs or mesh bags were retrieved from the solutions, rinsed twice with sterile distilled water, and dried in a laminar flow hood under sterile conditions for 2 to 3 h. The culture plugs were plated (one per plate) immediately in the center of potato-dextrose agar (PDA) plates and mesh bags were placed in a sandy-loam soil for 1 week prior to plating onto soil-pectate-tertitol agar as described above. The PDA plates were incubated at 24°C in an incubator and growth of *P. ultimum* was recorded every day for 1 week. The number of germinating microsclerotia was counted under a dissecting microscope from each plate and percent germination for each treatment was determined as described above.

Toxicity of organic acids mixture to *P. ultimum* was also tested in PDA medium. The filter-sterilized mixture of organic acids was mixed with autoclaved molten PDA medium at 45°C to final concentrations of 1, 2, and 4% (vol/vol), and the amended medium was poured into 9-cm petri plates and allowed to solidify. Plates

TABLE 1. Concentration of organic acids in fish emulsion (FE) samples determined with chemical suppression ion exclusion chromatography and conductivity detection^a

FE	Concentration of organic acids (mmol/liter)							Total
	Glycolic acid	Formic acid	Acetic acid	Propionic acid	n-Butyric acid	iso-Butyric acid	iso-Valeric acid	
2005	200.0	38.3	113.0	14.2	34.0	1.0	1.7	402.2
2006	225.0	25.3	104.8	24.0	24.6	1.4	2.3	407.4
Mean	212.5	31.8	108.9	19.1	29.3	1.2	2.0	404.8

^a FE samples were diluted 10 and 100 times before analyses and particulates were removed by centrifugation for 10 min at $10,600 \times g$.

were inoculated with 5-mm plugs of a freshly growing culture of *P. ultimum* and incubated at 24°C in an incubator, and colony diameter was measured every day for 1 week. Toxicity of individual organic acids such as acetic (0.025, 0.05, and 0.1%), formic (0.025 and 0.05%), and glycolic (0.05, 0.1, and 0.15%) acids to *P. ultimum* was also tested in PDA medium as described above.

Growth-room bioassays. The efficacy of FE added as a preplant amendment to a peat-based mix artificially infested with soil inoculum of *P. ultimum* or a muck soil naturally infested with *Pythium* spp. to suppress damping-off of cucumber seedlings was determined in a growth room as described previously (2). The soil inoculum of *P. ultimum* produced as described previously (2) had an average propagule density of 2×10^3 CFU/g of soil as determined by soil-dilution plating on PARP medium (22). The amount of inoculum required to give more than 80% disease incidence within 2 weeks after planting was determined in preliminary experiments. FE (1, 2, or 4% mass/mass) was incorporated into the infested peat-based mix or soil in plastic bags. The contents were mixed thoroughly, and loosely tied bags were incubated at 24°C in darkness. At day 1, 7, 14, 21, or 28, the amended mix or soil from the bags was dispensed into 10-cm-diameter plastic pots, and eight cucumber seed were planted in each of the five replicate pots per treatment. Pots were kept in a growth room (15 h of fluorescent light at 22°C and 9 h of darkness at 19°C) and arranged in a completely randomized design. They were watered as required to maintain appropriate moisture level for growth and disease development. Plants were rated 2 weeks after sowing for damping-off using a 1-to-4 scale where 1 = healthy, 2 = yellowing of stem or root, 3 = plant dead, and 4 = seedling not emerged. Data were also expressed as percent healthy seedlings. All experiments were repeated once.

In separate trials, the efficacy of the mixture of organic acids equivalent to the proportions found in FE to suppress *Pythium* damping-off of cucumber seedlings was determined in a muck soil, sandy-loam soil (site V2), and peat-based mix artificially infested with soil inoculum of *P. ultimum*. The mixtures of organic acids (1, 2, and 4% vol/mass) were thoroughly mixed with the infested soils or peat-based mix and incubated for 24 h before planting cucumber seed as described above. Plants were main-

tained in a growth room for disease development and rated for damping-off severity as described above.

Real-time polymerase chain reaction assay. After rating cucumber plants, the peat-based mix samples were analyzed by real-time polymerase chain reaction (PCR) for quantification of *P. ultimum* using species-specific primer set K1/K3 (23), which amplifies an amplicon of 670 bp specific for *P. ultimum*. Three subsamples (0.1 g each) of the freeze-dried peat-based mix from each of three replicate pots selected arbitrarily were taken for DNA extraction. DNA was extracted with Ultra Clean soil DNA purification kit (Mo Bio Laboratories Inc., Carlsbad, CA) according to the manufacturer's protocol.

External standards for quantification consisted of six serially diluted *P. ultimum* genomic DNA (23.1, 2.31, 0.231, 0.0231, 0.00231, and 0.000231 ng/μl). All standards were prepared in 10 mM Tris-HCl (pH 7.4) and included in triplicates in each real-time PCR quantification run to construct a standard curve and calculate quantities of *P. ultimum* DNA in the unknown samples. Quantification algorithms (Mx300PTM real-time PCR system software, version 2.0; Stratagene, La Jolla, CA) were used for the calculation of DNA amounts. Standard curves were constructed from duplicate standards using their cycle threshold numbers, PCR cycle numbers at which the fluorescence of the sample exceeds the background fluorescence, and the known starting DNA amounts.

Quantitative PCR was performed using Mx3000P (Stratagene, Cedar Creek) and SYBR Green QPCR master mix (Stratagene) following the manufacturer's recommendations. Each run included a negative control and a standard curve. Two technical replicates were performed using two different master mixes. Reactions were prepared in optical strip tubes Mx3000p using the following master mix: 10 μl of Brilliant SYBR Green PCR master mix, 0.30 μl of ROX reference dye, 1 μl of 1/10 diluted DNA template, 2 μl of each K1 and K3 primer (100 nM) and 2.2 μl of sterile ddH₂O. Thermocycling was performed with an initial denaturation at 95°C for 10 min, followed by 50 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s. The specificity of the quantitative real-time PCR single products was confirmed by the melting curve analysis and by gel electrophoresis on 1.5% agarose gel. Each run included a negative control and was repeated twice on two different days (two technical replicates).

Statistical analyses. The data were subjected to analysis of variance using MINITAB statistical software (version 13.0; Minitab Inc., State College, PA) and, if *P* values indicated a significant difference ($P \leq 0.05$), means were separated by Fisher's protected least significant difference test. Disease severity data were analyzed using Jandel SigmaStat statistical software (Jandel Scientific, San Rafael, CA). Data were subjected to one-way analysis of variance using Kruskal-Wallis nonparametric test statistics and means were separated according to Student-Newman-Keuls test. *P. ultimum* DNA quantification data were log₁₀ transformed and two-way analysis of variance was performed using the general linear model procedure of SAS (35).

RESULTS

Effect of FE on viability of *V. dahliae* microsclerotia. Addition of increasing rates of FE to a sandy-loam soil from a commercial potato field (site M) reduced the germination of *V. dahliae* microsclerotia compared with the control treatment (Fig. 1). The 1% rate of FE reduced MS germination by 39% after day 1, 87% after day 3, and 95% after 6 days of exposure over the control (Fig. 1). The 2% rate of FE significantly reduced microsclerotia germination by 74% over the control after 1 day of exposure (Fig. 1). After 3 and 6 days of exposure to the 2% FE treatment, more than 99% of microsclerotia did not germinate compared with the control (Fig. 1). On the other hand, exposure of microsclerotia for 1 and 3 days to the sandy-loam soil amended

Fig. 1. Effect of fish emulsion (FE) added to site M soil on germination of *Verticillium dahliae* microsclerotia (MS) and soil pH. MS were placed in the soil at day zero and removed after 1, 3, and 6 days. Percent germination was determined by plating MS onto semiselective medium. Means ($\pm$ standard error) are average of two experiments ($n = 6$). The numbers enclosed in brackets above the bars are pH values. Bars with the same letter within each sampling time do not differ significantly for MS germination according to Fisher's protected least significant difference test at $P \leq 0.05$.

with 0.5 and 1% rates of FM did not affect their germination; however, after 6 days of exposure, both rates significantly reduced microsclerotia germination by 42 and 47% over the control (Fig. 1). The 1 and 2% FE treatments reduced the soil pH by 0.8 and 1.1 log units over the control (from 5.9 in the control to 5.1 and 4.8) immediately after the amendment incorporation, and pH remained low at the same levels with both rates of FE after 1 day (Fig. 1). Both FE rates increased the soil pH to more than 1.0 log unit after 3 days. Soil pH was further increased from 5.6 in the control to 7.4 and 7.8 by 1 and 2% FE rates after 6 days. Both 1 and 2% rates of FM did not affect soil pH immediately after incorporation but increased pH by 0.5 log units after day 1, 2.0 to 2.7 log units after day 3, and 2.3 to 3.1 log units after day 6 over the control (Fig. 1).

Organic acids in FE. The average concentration of total organic acids in the FE was ≈ 400 mmol/liter (Table 1). A chromatogram of organic acids fingerprints of FE is shown in Figure 2. More than half of the total organic acids concentration was of glycolic acid. Acetic, formic, *n*-butyric, and propionic acids were other major organic acids present in FE at the proportions of 26.9, 7.9, 7.2, and 4.7% of total organic acids, respectively (Table 1). Minor concentrations (0.9 to 2.0 mmol/liter) of *iso*-butyric, *n*-valeric, and *iso*-valeric acids were also detected in FE samples. We also analyzed an FE sample from Brazil that had a similar

Fig. 2. Chromatogram of volatile and nonvolatile organic acids of 100 times diluted fish emulsion (FE) sample. FE samples were diluted 10 and 100 times before analyses. Particulates were removed by centrifugation (10 min at $10,600 \times g$) and the concentration of individual organic acid determined using chemical suppression ion exclusion chromatography and conductivity detection (Dionex model 100; Dionex Corp., Sunnyvale, CA). The chromatograph was equipped with an IonPac ICE-AS1 analytical column and AMMS ICE II chemical suppressor (Dionex Corp.).

chromatogram; however, the concentration of organic acids was much higher (1,000 mmol/liter) compared with our FE sample (data not shown).

Toxicity of organic acids from FE. In solution assays, the viability of *V. dahliae* microsclerotia treated for 24 h in 1, 2, 5, and 10% FE or a mixture of organic acids equivalent to the proportions in FE was reduced by 74, 94, 97, and 99% or 81, 91, 98, and 99%, respectively, over the control (Fig. 3). The viability of microsclerotia was similar in the citric acid-NaOH-buffered water control (pH 4.0) and the nonbuffered water control (pH 6.8). The pHs of organic acids or FE solutions were 4.0, 3.6, 3.2, and 3.0 or 4.4, 4.0, 3.9, and 3.8, respectively. In the pH 6.0-buffered FE and equivalent organic acid solutions, the viability of microsclerotia was increased compared with in the nonbuffered FE and organic acid solutions (Fig. 3). Only the 10% pH 6.0-buffered treatment solutions significantly reduced the viability of microsclerotia by 54 to 57% compared with the control (Fig. 3).

The culture plugs treated with organic acid solutions did not grow, and no growth was observed on PDA plates after 1 week, whereas control plugs submerged in sterile ddH₂O or buffer controls grew and fully covered the plates after 2 days. Formic acid and acetic acid were inhibitory to *P. ultimum* when amended in the PDA growth medium. There was no growth of the pathogen on plates after 7 days of incubation with the lowest concentration (0.025%) of formic acid compared with the control plates, which were fully covered after 2 days of incubation. The maximum linear growth attainable in these plates was 9 cm. There were 3.6 and 1.5 cm of growth of the pathogen on plates amended with 0.025 and 0.05% acetic acid, respectively, but no growth on plates amended with 0.1% acetic acid. Glycolic acid was not toxic to *P. ultimum* in the PDA medium at up to 0.15% concentration tested.

Effect of organic acids mixture on damping-off of cucumber seedlings. In muck and sandy-loam soils artificially infested with soil inoculum of *P. ultimum*, the mixture of organic acids equivalent to the proportions found in FE provided immediate protection of cucumber seedlings from damping-off in a dose-response manner (Fig. 4). Higher concentrations (2 and 4%) of organic acids mixtures were more effective than a lower concentration (1%). There were more than two-, three- to four-, or four- to five-fold increases in the percentage of healthy seedlings with 1, 2, or 4% mixtures of organic acids, respectively, over the control (Fig.

Fig. 3. Effect of fish emulsion (FE) and a mixture of volatile and nonvolatile organic acids (OA) on the germination of *Verticillium dahliae* microsclerotia (MS) in solution bioassays. The OA mixture was prepared with concentrations of individual OA components as found in FE. MS were submerged in the buffered (pH 6) and nonbuffered solutions for 24 h and percent germination was determined by plating MS onto semiselective medium. Means ($\pm$ standard error) are average of two experiments ($n = 6$).

4). The reduction in damping-off severity by increasing concentrations of organic acid mixtures followed a similar trend in both soils, with 20 to 53% reduction of disease severity over the control (Fig. 4). None of the concentrations of organic acids mixtures provided immediate protection of cucumber seedlings against damping-off in an infested peat-based mix (Fig. 4). No protection was observed even when the planting was delayed for 7 or 14 days after adding the organic acid mixtures to the infested peat-based mix (data not shown).

Effect of FE on damping-off of cucumber seedlings. The naturally infested muck soil from a commercial field had a high damping-off potential on cucumber seedlings. More than 75% of the cucumber plants in the nonamended control were diseased, with an average disease severity rating of 3.4 (Fig. 5A and B). After 1 day of incorporation in muck soil, both 1 and 2% rates of FE were effective in improving the healthy seedlings by 31 and 50%, respectively (Fig. 5A), and decreasing the disease severity by 1.2 to 1.6 units (Fig. 5B) over the control. The improvement in healthy seedlings and reduction in disease severity were consistent when planting was delayed for 1, 2, and 4 weeks after FE incorporation. When seed were planted after 1 week of FE incorporation, the percentage of healthy seedlings was increased by 63 to 71% (Fig. 5A) and disease severity decreased by 1.9 to 2.2 units (Fig. 5B) over the control. When planting was delayed for 2 week after FE incorporation, the percentage of healthy seedlings was increased by 56 and 65% (Fig. 5A) and disease severity decreased by 1.6 and 2.0 units (Fig. 5B) with the 1 and 2% FE treatments over the control, respectively. When planting was delayed for 4 weeks after FE incorporation, the percentage of healthy seedlings was improved by 53 to 59% (Fig. 5A) and disease severity decreased by 1.6 to 1.9 units (Fig. 5B) with the FE treatments over the control.

Peat-based mix artificially infested with soil inoculum of *P. ultimum* was very pathogenic to cucumber throughout the 4-week incubation period. More than 80% of the cucumber plants in the control were diseased, with an average disease severity rating of

3.4 (Fig. 6A and B). When the infested mix was amended with 1, 2, and 4% rates of FE and incubated for 1, 7, 14, and 21 days prior to planting cucumber seed, damping-off suppression appeared over time. There was no protection of cucumber seedlings from damping-off when seed were planted 1 day after FE incorporation in the infested mix. The percentage of the healthy seedlings and disease severity were similar to the control (Fig. 6A and B). When planting was delayed for 7, 14, or 21 days after incubation, damping-off suppression become evident in the FE-amended peat-based mix. When seed were planted after 7 days of incubation, the percentage of healthy seedlings was increased from <15% in the control to >75% in the FE treatments (Fig. 6A), with a decrease in disease severity ratings over the control (Fig. 6B). The FE-amended peat-based mix was consistently suppressive against *Pythium* damping-off after 14 and 21 days of incubation (Fig. 6A and B).

Real-time PCR analyses of the *P. ultimum* DNA extracted from the peat-based mix indicated that all FE concentrations significantly reduced the pathogen compared with the control (Fig. 7A). The amount of *P. ultimum* DNA was similar in the control and FE treatments after 1 day of incubation of the amended peat-based mix (Fig. 7B). The amount of *P. ultimum* DNA significantly decreased in the FE-treated peat-based mix compared with in the control after 7, 14, and 21 days of incubation (Fig. 7B). The K1/K3 primer set amplified a single expected amplicon of 670 bp and a melting point temperature of $81.6 \pm 0.5^\circ\text{C}$ as determined by the melting curve analysis. Quantification showed a high linearity ($R^2 = 0.97$) between $\log_{10}$ values of DNA and real-time PCR threshold cycles over the range of DNA concentration examined, demonstrating a high reproducibility among the replicates tested and the accuracy of the PCR quantification assay.

DISCUSSION

This is first study demonstrating that FE contains large quantities of organic acids that are toxic to pathogens and may have a

Fig. 4. Effect of a mixture of volatile and nonvolatile organic acids (OA) as a preplant amendment on **A**, percent healthy and **B**, damping-off severity of cucumber seedlings in a muck soil or sandy-loam soil or peat-based mix artificially infested with soil inoculum of *Pythium ultimum*. The OA mixture was prepared with concentrations of individual OA components as found in FE and mixed with the infested soil or mix. After incubation at 24°C in the dark for 24 h, cucumber seed were planted and incubated in a growth room. Plants were rated 2 weeks later for damping-off disease using a 1-to-4 rating scale. Means ($\pm$ standard error) are the average of two experiments. Means within each planting time followed by the same letter do not differ significantly according to **A**, Fisher's protected least significant difference test or **B**, Student-Newman-Keuls test at $P \leq 0.05$.

role in disease suppression by FE, depending on soils or substrates. The concentration of organic acids reported here in FE (≈ 400 mmol/liter) was significantly higher than the concentration of organic acids found in LSM (18). Even higher concentrations (1,000 mmol/liter) of organic acids were detected in FE samples received from a Brazilian company (*unpublished data*). It is likely that these organic acids are produced during anaerobic decomposition of sugars or proteins present in the FE. More than half of the total organic acid concentration in FE was of glycolic acid. Glycolic acid is not classified as a volatile organic acid but it has antimicrobial properties at higher concentrations. Glycolic acid has been patented as a plant-growth-promoting agent (27) and is widely used as an ingredient in skin care products. In this study, glycolic acid ($\leq 0.15\%$) did not inhibit the growth of *P. ultimum* in PDA agar medium; however, it enhanced the percentage of healthy cucumber seedlings in a muck soil infested with *Pythium* spp. (data not shown). Glycolic acid is found in sugary plants such as sugarcane and is also manufactured synthetically. Its presence in FE at very high concentrations needs further investigation.

Acetic, formic, *n*-butyric, and propionic acids are the major toxic volatile organic acids in FE. All these organic acids, except glycolic and formic acids, have also been found in LSM and were considered to be a major factor associated with disease reduction by LSM (18,37). FE, in contrast, contains high levels of formic acid (7.9% of the total organic acids) and formic acid was found to be seven times more toxic to pathogens such as *V. dahliae*

compared with acetic acid (37). Higher concentrations of these volatile acids can be toxic to crop plants (20), and some of these organic acids are also known to kill fungal plant pathogens (13, 17,26,36) and plant-parasitic nematodes (12,13). Organic acids from freshly composted municipal wastes have been implicated in suppression of infection of citrus seedlings by *Phytophthora nicotianae* (40). Although volatile organic acids are directly toxic to pathogens in solution or laboratory assays, their effectiveness for disease suppression under field conditions should be investigated.

A mixture of organic acids equivalent to the proportions found in FE and acetic and formic acids were toxic to *Pythium ultimum* in solution assays in this study. The increasing concentrations of a mixture of organic acids also suppressed damping-off of cucumber seedlings in a muck and sandy-loam soils but not in a peat-based mix. Similarly, comparable rates of FE also provided immediate protection of cucumber seedlings from damping-off within 1 day after incorporation of FE in an infested muck soil (2). However, damping-off suppression of cucumber seedlings in a peat-based mix was achieved only after planting was delayed for a week or more after adding FE to the pathogen-infested mix. Real-time PCR analyses of the peat-based mix also indicated no immediate effect of FE on *P. ultimum* populations. It is less likely that organic acids from FE played a role in delayed suppression of *P. ultimum* damping-off in a peat-based mix. This specificity of organic acids for disease suppression in various soils or substrates should be further investigated at various inoculum levels.

Fig. 5. Effect of fish emulsion (FE) as a preplant amendment on **A**, percent healthy seedlings and **B**, damping-off severity of cucumber seedlings in naturally-infested muck soil from Holland Marsh, ON. FE (mass/mass soil) was incorporated into the infested soil and incubated at 24°C in the dark. After 1, 7, 14, and 28 days, cucumber seed were planted and incubated in a growth room. Plants were rated 2 weeks later for damping-off disease using a 1-to-4 rating scale. Means ($\pm$ standard error) are the average of two experiments. Means within each planting time followed by the same letter do not differ significantly according to **A**, Fisher's protected least significant difference test or **B**, Student-Newman-Keuls test at $P \leq 0.05$.

Fig. 6. Effect of fish emulsion (FE) as a preplant amendment on **A**, percent healthy and **B**, damping-off severity of cucumber seedlings in a peat-based mix artificially infested with soil inoculum of *Pythium ultimum*. FE (mass/mass mix) was incorporated into the infested mix and incubated at 24°C in the dark. After 1, 7, 14, and 21 days, cucumber seed were planted and incubated in a growth room. Plants were rated 2 weeks later for damping-off disease using a 1-to-4 rating scale. Means ($\pm$ standard error) are the average of two experiments. Bars with the same letter within each planting time do not differ significantly according to Fisher's protected least significant difference test at $P \leq 0.05$.

The rapid reduction in the viability of *V. dahliae* microsclerotia in a sandy-loam soil with FE was likely due to the toxicity of these organic acids. More than 98% of *V. dahliae* microsclerotia did not germinate after exposure to 2% FE for 3 days. When *V. dahliae* microsclerotia were treated with the mixture of organic acids at the proportions found in FE in solution assays, the viability of microsclerotia was significantly reduced and was comparable with that seen with equivalent rates of FE. The viability of microsclerotia was increased when the treatment solutions (FE and mixture of organic acids) were buffered to pH 6.0 with NaOH-citrate buffer. This clearly suggests that organic acids in FE play an important role in pathogen suppression. The biological activity of VFAs is maximized under acidic conditions (37); therefore, it is possible that VFA toxicity may have been one of the mechanisms of disease reduction by FE in the low pH soils (3).

Disease suppression by FE in various soils or substrates as reported in this study or previous studies (2,3) was certainly not due to toxicities of ammonia or nitrous acid. The rates of FE used in sandy-loam soil were not high enough to generate ammonia at the toxic concentration, and even high rates of FE could not have generated toxic concentration of ammonia due to the high buffering capacity of peat-based mix and muck soil. The pH of the FE-treated peat-based mix or muck soil did not substantially increase or decrease compared with their respective controls (data not

shown). FM, on the other hand, did not immediately affect the germination of *V. dahliae* microsclerotia in a sandy-loam soil but the effect started to appear after 6 days. FM, which does not contain organic acids, is a high nitrogenous amendment and it is known that nitrogen transformation products, such as ammonia and nitrous acid, play a major role in reducing the viability of *V. dahliae* microsclerotia (38).

In conclusion, FE contains large quantities of organic acids that are toxic to plant pathogens and may have a role in disease suppression, depending on the soil and substrate. Immediate pathogen or disease suppression in sandy-loam or muck soils suggests that organic acids may play a major role. In addition, the role of biological control in the suppression of soilborne pathogens or diseases in the FE-amended soil or substrate cannot be ruled out. Biological control stimulated by the addition of organic material into potting substrates or agricultural soils has been attributed as a mechanism of disease suppression in some studies (2,4,8,9,16, 19). The rates of FE providing effective disease control in the field (3) may not be economical for every crop and every soil. However, it may be economical if the disease control effect lasts for multiple years after a single FE application. In order to maximize the pathogen or disease suppression effect of organic acids, FE should be applied to the soils with low pH and low buffering capacity.

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Fig. 7. A and B. Real-time polymerase chain reaction (PCR) analyses of a peat-based mix infested with *Pythium ultimum* and amended with fish emulsion (FE). FE (mass/mass mix) was incorporated into the infested mix and incubated for 1, 7, 14, and 21 days at 24°C in the dark before planting cucumber seed. After rating plants, three subsamples of peat-based mix from each of three replicate pots were taken for DNA extraction and PCR analyses. The DNA of *P. ultimum* was estimated based on external standards. Bars with the same letter (B, within each planting time) do not differ significantly according to Fisher's protected least significant difference test at $P \leq 0.05$.

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