Characterization of *Erwinia* Populations from Nursery Retention Ponds and Lakes Infecting Ornamental Plants in Florida

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ABSTRACT

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Water shortages in Florida are occurring due to intense utilization of the aquifer system by municipalities and agriculture, and because of continued deficits in annual rainfall. Water districts therefore, are, recommending the use of recycled irrigation water, stormwater runoff, reclaimed municipal sewage water, and lakes for agricultural use. With recycled water, however, there is potential for both introducing and concentrating plant pathogens. In Florida, *Erwinia* soft-rot bacteria (synonym *Pectobacterium*) cause extensive crop losses in ornamental plant production. To determine *Erwinia* spp. population levels, samples were taken monthly for 1 year from four hypereutrophic lakes and eight nursery retention ponds. Seventy-seven *Erwinia* strains were collected by both direct plating and by an enrichment process. With the direct plating method, 0 to 29 CFU/ml were detected on sodium polypectate medium. Significantly higher populations of *Erwinia* were detected in retention ponds of nurseries that were actively reutilizing their water. *Erwinia* strains were identified to species by fatty acid analysis and biochemical tests. Strains were further characterized by repetitive element-polymerase chain reaction (rep-PCR) and compared with 120 strains of *Erwinia* collected from ornamentals over a 17-year period in Florida. Using rep-PCR, most strains were clustered into two heterogeneous populations of *E. chrysanthemi* and *E. carotovora* subsp. *carotovora* in a 1:2 and a 1:4 ratio for isolates from ornamentals and from water, respectively. Within each population of *E. chrysanthemi* and *E. carotovora*, genetically different subpopulations could be identified that contained high percentages of *Erwinia* strains from water sources. Even though genetic differences exist, 99% of the strains from water sources were found to be pathogenic on dieffenbachia. Without water treatment of irrigation and stormwater runoff, there is a potential for both introducing and concentrating *Erwinia* populations within these water sources.

Additional keywords: FAME, ornamental foliage, soft rots

During warm summer months, bacterial soft rots caused by *Erwinia* spp. commonly occur in tropical foliage production. Tropical foliage crops (family *Araceae*) such as *Dieffenbachia* spp., *Syngonium* spp., and *Aglonema* spp. are especially susceptible. Within an infested production facility, spread of *Erwinia* spp. can be rapid and crop losses extensive. Symptoms may appear anywhere on the plant but, commonly, water-soaked lesions appear on leaves and soft rot damage on stems and plant crowns. Most growers are familiar with the foul, fishy smell of the volatile compounds released as *Erwinia* spp. break down plant tissues.

The primary mechanism of pathogen dispersal within the nursery is splashing

water and movement of contaminated soil and tools. Contaminated soil is not considered to be the primary mechanism of *Erwinia* spp. introductions into many nurseries in Florida, because synthetic soils containing Canadian peat, vermiculite, and perlite usually are used. Many species of ornamental plants in Florida are propagated via tissue culture in order to avoid common bacterial pathogens. Due to the cost of tissue culture, many propagative cuttings used in Florida are produced in nurseries in Central America. Introductions of pathogens into Florida via this propagative material is common. Once introduced into the nursery, *Erwinia* spp. usually enter susceptible plants through small wounds caused by plant handling or by insects. There are no bactericides that can rid infected plants of *Erwinia* spp., but copper compounds can help slow the spread of this pathogen. Thus, control of *Erwinia* spp. is based almost exclusively on sanitation and cultural recommendations. Therefore, knowing how *Erwinia* spp. can gain access, persist, and move about within a production facility is important to their control.

Over the past three decades, numerous studies have been conducted on *Erwinia*

spp. within water bodies such as rivers (4,5,10,12,15), lakes (8,15), and irrigation water (3). Most of these studies have dealt with relatively clean water sources, often in relationship to potato production. Most ornamental plants produced in Florida are watered with either municipal water or from wells drilled into the Florida aquifers. Due to rapid population growth of cities in Florida, there is direct competition with agriculture for the limited clean water supply. Surface water is considered to be a possible alternative for ornamental plant producers. The objective of our study was to examine hypereutrophic lakes and nursery retention ponds in Florida to determine (i) populations of *Erwinia* spp. within these water bodies and (ii) genetic relatedness of recovered strains to those found infecting ornamental plants in Florida.

MATERIALS AND METHODS

Isolation. *Erwinia* strains were collected over a 12-month period from 12 water sources, comprising four large hypereutrophic lakes in Central Florida (surface area 150 to 12,500 ha) and eight nursery retention ponds (Table 1). Water samples were collected monthly within 10 cm of the bottom of both lakes and retention ponds. Samples were collected by wading into the retention ponds and retrieving 4 liters of water or, in the case of lakes, using a boat and pooling several water samples. Retention ponds were characterized by whether they were lined with plastic and whether water was recycled back onto the nursery crop. Retention ponds were located either within the nursery and surrounded with plant production, or next to the nursery with water runoff through cement-lined ditches or drainage pipes.

Strains of *Erwinia* were recovered by direct plating of 1 ml of water onto sodium polypectate (CVP) medium (6), and by a polypectate enrichment procedure (2). In the enrichment procedure, 50 ml of the water sample was combined with 50 ml of (2×) enrichment broth. After incubating broth mixtures for 72 h at 28°C, strains of *Erwinia* were recovered via a dilution series on CVP medium. Strains isolated by both techniques were stored at -80°C in Difco nutrient broth amended with 50% glycerol.

Over the past 17 years, *Erwinia* strains have been collected from ornamental crops in Florida and cryogenically preserved. A total of 120 strains were revived for this study (Table 2).

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Strain identification. All *Erwinia* strains collected from plants and water samples were identified to species level via fatty acid (FAME) analysis and standard biochemical tests. FAME analyses were performed following protocols of Miller (17) and Sasser (21,22) with the use of the MIDI Microbial Identification System and software (version TSBA 3.90; Microbial ID, Inc., Newark DE). All strains also were characterized with the following biochemical tests: oxidase, catalase, motility, and oxidation and fermentation of glucose following protocols of MacFaddin (13). Six delineating tests (indole, phosphatase, lecithinase, erythromycin sensitivity, acid from trehalose, and malonate utilization) to distinguish between *E. carotovora* and *E. chrysanthemi* were performed following protocols of Schaad (23) and MacFaddin (13).

Pathogenicity tests. All strains of *Erwinia* were assessed for pathogenicity and hypersensitivity (HR). Strains were grown for 24 h at 29°C on nutrient agar medium (Difco) amended with 5% sucrose, harvested, suspended in sterile saline (8.5 g of NaCl/liter), and adjusted spectrophotometrically (absorbance at 600 nm [A_{600}]) to 5×10^8 CFU/ml. Two leaves each from *Diefenbachia exotica* 'Camille' and *Nicotiana tabacum* 'Hicks' were infiltrated in two different areas of the parenchymatous tissue.

Plants were kept in a fiberglass shade-house with maximum irradiance of $125 \mu\text{mol s}^{-1} \text{m}^{-2}$, temperature range of 15 to 34°C with natural photoperiod, and high relative humidity. Evaluations were done after 1 week for pectolytic collapse of dieffenbachia leaves and after 48 h for HR response on tobacco. When negative HR occurred, the injections were repeated on tobacco plants with a concentration of 5×10^9 CFU/ml.

Genomic comparison. A genomic comparison of the strains was done using three repetitive element-polymerase chain reaction (rep-PCR) primers: BOX, ERIC and REP. DNA was extracted (GenomicPrep Kit; Amersham Pharmacia Biotech, Piscataway, NJ), and PCR was done using Ready-To-Go RAPD Analysis Beads (Amersham Pharmacia Biotech) in a PTC-100 thermocycler (M. J. Research Inc., Watertown, MA). A modified version of the cycling profile suggested by Amersham Pharmacia Biotech was used with the rep-PCR primers to obtain profiles similar to those obtained using the original rep-PCR master mix (20). The following profile was used for the Box primer: 1 cycle at 95°C for 5 min; followed by 45 cycles of: 95°C for 1 min, 53°C for 1 min, and 72°C for 2 min; and held at 4°C when cycling was completed. For the ERIC and REP primers, the above profile was modified by replacing the

53°C temperature with 52 and 40°C, respectively.

After electrophoresis (20), banding patterns were recorded under UV light using a Kodak Digital Documentation System 120 (Rochester, NY). Pearson correlation and unweighted pair group means analysis were performed using the BioNumerics program (ver. 2.1; Applied Maths, Kortrijk, Belgium). For isolates with unique banding patterns, DNA was extracted again, PCR was redone, and the analysis was repeated. Using the BioNumerics program, a combined comparison was made of banding patterns from rep-PCR primers. Cluster cut-off values also were calculated using the BioNumerics software in each comparison. The following ATCC type strains were used as standards: *E. carotovora* subsp. *carotovora*, ATCC 15713; *E. chrysanthemi*, ATCC 11663; and *E. chrysanthemi* subsp. *atroseptica*, ATCC 33260.

RESULTS

Seventy-seven *Erwinia* strains were isolated from nursery retention ponds and lakes during the 12-month sampling period. With the direct plating of water samples onto CVP medium, 0 to 29 CFU/ml were detected. Average CFU/ml for each water source are listed in Table 1. No seasonal variation in population sizes within water sources was observed over the 12-month period (*data not shown*). However, significantly larger populations of *Erwinia* spp. were detected in most nurseries that were actively using recycled water (Table 1). Populations in the hypereutrophic lakes were generally lower than those found in nursery retention ponds.

All 197 strains (120 plant, 77 water) were identified to species by FAME analysis (Table 3). Populations of *E. chrysanthemi* and *E. carotovora* subsp. *carotovora* were found in a 1:2 and a 1:4 ratio in isolates from plants and water, respectively. Strains were characterized further by biochemical tests. The six delineating biochemical tests aided in distinguishing species of *E. carotovora* from *E. chrysanthemi*. However, there were a number of atypical strains that had one or more tests that were not consistent with the species descriptions.

When comparing strains by rep-PCR, most strains clustered into two large heterogeneous populations of *E. chrysanthemi* and *E. carotovora*. Within these large clusters, distinct subpopulations were observed (Fig. 1). *E. chrysanthemi* strains fell into clusters A and B, while the rest of the clusters consisted of *E. carotovora* strains. Of these *E. chrysanthemi* isolates, 71% were from plant samples and only two of the water samples were from lake water. Three clusters (A, C, and E) had large percentages of strains from water sources (64 to 86%), whereas other clusters were made-up primarily of isolates from plants. Clusters containing strains from water sources

Table 1. Water sources (four hypereutrophic lakes and eight nursery retention ponds) where *Erwinia* spp. were collected

Water source	Crops	Water recycled	Lined	CFU/ml ^y	LSD ^z
Ponds					
1	Perennials, annuals, woody tropical	Yes	No	6.0	c
2	Perennials, annuals, woody	Yes	Yes	5.3	bc
3	Perennials, annuals, woody tropical	Yes	No	2.3	abc
4	Perennials, annuals, woody	Yes	No	2.1	ab
5	Perennials, annuals, woody tropical	Yes	No	1.4	a
6	Woody	No	Yes	1.9	ab
7	Perennials, annuals	No	No	0.8	a
8	Tropical	No	No	0.6	a
Lakes					
Apopka	...	...	...	2.3	abc
Wauberg	...	...	...	1.0	a
Dora	...	...	...	0.9	a
Yale	...	...	...	0.2	a

^y Mean number of pectolytic CFU isolated directly onto sodium polypectate medium.

^z Water sources followed with different letters have significantly different numbers of pectolytic bacteria. Significant differences in means were determined using the least significant difference method (LSD, $P = 0.05$).

Table 2. Sources of 120 *Erwinia* strains, isolated from ornamentals, over a 17-year period in Florida

Host plants	Number of strains	Laboratory source ^z
Tropical aroids (<i>Araceae</i>)	84	1, 2, 3
Tropical (dracaena, palms, fern, etc.)	20	1, 2, 3
Woody ornamentals	5	1, 2, 3
Bedding plants	3	2, 3
Succulents (cactus, haworthia, etc.)	8	1, 2, 3, 4

^z Strains were supplied by the following laboratories: 1 = D. Brunk, Plant Disease Diagnostics, Inc. Apopka, FL 32703; 2 = Mid-Florida Research and Education Center, University of Florida, Apopka, FL 32703; 3 = J. Miller, Division of Plant Industry, Florida Dept. of Agriculture and Consumer Services, Gainesville, FL 32602; and 4 = G. W. Simone, Dept. of Plant Pathology, University of Florida, Gainesville, FL 32611.

had mixtures of strains from both hypereutrophic lakes and retention ponds. Cluster F contained only two strains: *E. chrysanthemi* subsp. *atroseptica* (ATCC 33260) and an *E. carotovora* isolate from water. Twelve outlier strains with similarity coefficients less than 15% are not displayed in Figure 1. These 12 strains were identified by MIDI FAME analysis as *E. carotovora* spp., although 6 strains had atypical biochemical tests that did not fit within described subspecies profiles.

Of the 197 *Erwinia* strains from water and plant samples, 99% were HR positive on tobacco within 48 h and also were pathogenic on dieffenbachia.

DISCUSSION

In this study, there was no observed variation in population size throughout the 1-year sampling period in either the lakes or nursery retention ponds (*data not shown*). These results differ from those reported from Colorado, where *Erwinia* populations were found to fluctuate seasonally with cropping cycles (12). Results for Florida may be influenced by the mild climate or continual drought throughout the sampling period or, perhaps, the year-round cropping cycle of ornamental plants. Populations of *Erwinia* spp. within lakes and retention ponds in Florida (0 to 29 CFU/ml) were substantially smaller than those found in Northern states. Jorge and Harrison (12) detected up to 144 CFU/ml in Colorado River water. Cappaert et al. (3) found populations of *Erwinia* spp. as high as 575 CFU/ml in Oregon irrigation water. Cappaert also noted that populations varied greatly from year to year in surface water from both Oregon and Colorado. The lower populations found in Florida might be attributed to the hypereutrophic water conditions in Florida, where high microbe loads could influence both survival and isolation. Cother and Gilbert (5), studying *E. chrysanthemi* populations in river systems in Australia, noted that populations either were masked or lower in water with a high quantity and complexity of microflora.

Based on the FAME analysis (MIDI), strains were in a 1:2 and a 1:4 ratio of *E.*

chrysanthemi to *E. carotovora* subsp. *carotovora* for strains from ornamentals and from water, respectively. However, within these populations, there were a number of atypical strains that did not agree with typical species criteria (Table 3). Specific species of *Erwinia* found in water sources have been found to vary with geographic location and water source. Harrison et al. (10) collected 819 *Erwinia* strains from lakes, rivers, ponds, and reservoirs in 16 states, including Florida. Of these strains, 98% were *E. carotovora* subsp. *carotovora* and the rest were *E. chrysanthemi* subsp. *atroseptica*. Similar results were obtained by McCarter-Zorner et al. (15), who examined surface water in Colorado and southern Scotland and found that 95% of the water samples contained *E. carotovora*

subsp. *carotovora*, whereas 9.8% also contained *E. chrysanthemi* subsp. *atroseptica*. McCarter-Zorner also examined seawater from Oregon, California, and the Gulf of Mexico and found only *E. carotovora* subsp. *carotovora*. No *E. chrysanthemi* strains were isolated in these studies. Cappaert et al. (3) examined irrigation water in Colorado and Oregon and found that 90 and 95%, respectively, of the strains recovered were *E. carotovora* subsp. *carotovora*, but the rest of the strains in his study were identified as *E. chrysanthemi* subsp. *atroseptica* and *E. chrysanthemi*; *E. chrysanthemi* was found only in a few samples from Oregon. Other studies have shown that *E. chrysanthemi* is a natural component of aquatic microflora in alpine water sources (4,5). The high percentage of *E.*

Fig. 1. Unweighted pair group means analysis was used to generate clusters based on rep-polymerase chain reaction profiles of *Erwinia* strains isolated from ornamental plants and surface water. The higher the similarity coefficient, the more genetically similar the isolates within the cluster. Within each of the major clusters, there exist subclusters (*data not shown*) with higher similarity coefficients.

Table 3. Percent of strains, isolated from ornamental plants and water, with biochemical characteristics typical of *Erwinia* spp.

Source ID [†]	Oxidase	Catalase	Motility	O/F D-glucose	Indole	Phosphatase	Erythromycin Lecith [‡] sensitivity [§]	Trehalose [¶]	Malonate [¶]	HR [¶]	Path [¶]
Plant (n = 120)											
1 (n = 35)	100	100	100	100	60	94	94	89	17	83	97
2 (n = 85)	100	100	100	100	5	3	0	0	94	15	98
Water (n = 77)											
1 (n = 15)	100	100	100	100	93	100	100	100	13	100	100
2 (n = 62)	100	100	100	100	6	2	2	10	85	19	98

[†] Species identification based on fatty acid analysis using MIDI Identification System; 1 = *E. chrysanthemi* and 2 = *E. carotovora* subsp. *carotovora*.

[‡] Lecithinase activity on egg yolk after 7 days at 28°C.

[§] Sensitivity to 15-µg dish of erythromycin.

[¶] Acid production from trehalose.

[¶] Utilization of malonate.

[¶] HR = hypersensitivity reaction on tobacco.

[¶] Path = pectolytic collapse of dieffenbachia leaves.

chrysanthemi strains in Florida water cannot be attributed to an alpine water source. However, of the *E. chrysanthemi* isolates obtained from water, only two were from local lakes. The rest were obtained from nursery retention ponds. This indicates that the frequent presence of *E. chrysanthemi* might be related to agricultural contamination of the water rather than an endemic natural population.

A number of techniques have been employed to genetically identify and fingerprint *Erwinia* spp. populations, such as bacteriophages (8,9), serology (3,27,28), protein profiles (26), FAME analysis (4), random amplified polymorphic DNA-PCR (14), ribotyping (18), and restriction fragment length polymorphism (1,4,11,19). Most of these techniques have shown that *Erwinia* spp., especially *E. carotovora*, are made up of large heterogenic populations. Some of the techniques used in these studies have been useful for distinguishing populations geographically and by host of origin. In this study, rep-PCR was utilized to fingerprint isolates. Using rep-PCR to characterize *Erwinia* populations is not unique. McManus and Jones (16) successfully used rep-PCR to distinguish between populations of *E. amylovora* infecting tree-fruit crops and those infecting *Rubus* spp.

In this study, using rep-PCR, *Erwinia* strains were grouped into two large clusters containing the two distinct species. Within the clusters, distinct subclusters were evident that contained high percentages of strains isolated from water. The *Erwinia* strains from plants were collected over a 17-year period; therefore, it is not clear if strains isolated from water are genetically different due to the sources or whether these genetic differences represent evolutionary changes in the population over the sampling period.

A number of studies have been conducted on variations in virulence and host range of *Erwinia* strains (1,7,24,25). Virulence of the *Erwinia* strains were not measured in this study. However, 99% (76/77) of the strains from water were HR-positive on tobacco and pathogenic on dieffenbachia. A similar percentage of strains (98%) of those from plant sources also were pathogenic on dieffenbachia. Tropical ornamentals, especially from the *Araceae* family, are known to be susceptible to *Erwinia* soft rots. This is evident from the distribution of isolates collected from ornamental plants (Table 3). With the high susceptibility of tropical ornamentals to *Erwinia* soft rot, it would seem inadvis-

able to use untreated water from lakes, recycled irrigation, or stormwater runoff. Also, it appears that pathogenic *Erwinia* populations increase within water recycled from nursery retention ponds. More research is needed to identify a direct link between the use of contaminated irrigation water and an increased incidence of soft rot in nurseries.

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