New Disease on Ornamental Asparagus Caused by *Xanthomonas campestris* in Florida

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ABSTRACT

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From dark, water-soaked lesions on stems of asparagus tree fern (*Asparagus virgatus*) in commercial nurseries in Florida, 33 xanthomonad strains were isolated. Strains formed large, round, butyrus, bright yellow colonies on yeast dextrose calcium carbonate medium, and were gram negative, oxidase negative, catalase positive, motile, strictly aerobic, and did not hydrolyze starch. Strains were further characterized by carbon substrate utilization patterns (Biolog), and by fatty acid methyl esters (FAME) analyses. The metabolic fingerprints of most strains were similar to *Xanthomonas campestris* pv. *vitians*, and *X. campestris* pv. *dieffenbachiae* from *Xanthosoma* or *Synгонium*. Representative strains from *A. virgatus* were not pathogenic on *Dieffenbachia*. *X. campestris* pv. *dieffenbachiae* strains that did not hydrolyze starch produced scattered lesions on *A. virgatus* stems. However, starch-hydrolyzing strains of *X. campestris* pv. *dieffenbachiae* did not produce symptoms when inoculated onto *A. virgatus*. FAME analysis indicated the strains were *X. campestris* pv. *vitians* or *X. campestris* pv. *translucens*; however, low similarity indices ($\bar{x} = 0.461$) indicated that the asparagus strains were not represented in the MIDI library database. FAME analysis profiles were also compared to the University of Florida database, which contains 1,048 *X. campestris* strains of which 200 are *X. campestris* pv. *dieffenbachiae*. Similarity indices were again low with 15 strains matched to *X. campestris* pv. *secalis* ($\bar{x} = 0.412$), seven strains to *X. fragariae* ($\bar{x} = 0.224$), six strains to *X. campestris* pv. *translucens* ($\bar{x} = 0.437$), and five strains matched < 0.20 to other pathovars. Five representative strains were tested on six *Asparagus* species or cultivars: *A. virgatus*, *A. setaceus*, *A. macowanii*, *A. densiflorus* 'Sprengeri', *A. densiflorus* 'Myers', and *A. officinalis*. All five strains were pathogenic on *A. virgatus* but were less virulent on *A. setaceus* and *A. densiflorus* 'Sprengeri'.

The delicate, dark-green foliage of asparagus fern has made it one of the most popular cut foliage crops. The ornamental asparagus industry in Florida began in the late 1800s with the production of *Asparagus setaceus* (Plumosa fern) (12). Shipments of cut asparagus fern were sent by train to the northeastern United States for use in flower arrangements. By 1956, 2,000 acres of asparagus fern were in production in Florida (20). By that time, primary production had shifted from growing *A. setaceus* to *A. virgatus* (tree fern). Currently, over 3,700 acres of asparagus ferns are grown, with 2,000 acres being planted in *A. virgatus* and the rest with *A. setaceus*, *A. macowanii*, and *A. densiflorus* (21). Asparagus fern grows well in the sandy soil found in Florida. Plants are cultivated

mainly under natural oak hammocks but also to a limited extent in shadehouses.

Fungal pathogens such as *Fusarium* and *Alternaria* are common on commercially produced edible asparagus (*A. officinalis* L.) (4,6,19,23). No major bacterial diseases have been recorded for edible asparagus or the closely related ornamental asparagus. This is the first report of bacterial disease of ornamental *A. virgatus* plants. Diseased plants have water-soaked lesions on stems, and the lesions turn dark brown to black with age, making infected fronds unmarketable as a cut foliage green for floral arrangements.

MATERIAL AND METHODS

Pathogen isolations. In April of 1995, dark green to brown water-soaked lesions were observed on stems of *A. virgatus* in Volusia County, Florida. Lesions varied in length from a few millimeters to 1 cm. Samples were surface sterilized for 30 s in 0.25% sodium hypochlorite solution, ground in 300 μ l of sterile distilled water, and dilution-streaked onto modified Kelman's (14) triphenyltetrazolium chloride medium (TZC). Plates were incubated at $27 \pm 1^\circ\text{C}$ for 48 h. Single colonies were

streaked onto TZC media to assure purity. Colonies were smooth, round, entire, butyrus, and brick red in color. A total of 33 strains were collected during an 8-month period from Volusia and Putnam counties in Florida. All strains were further characterized on yeast dextrose calcium carbonate medium (YDC) (29) for colony color and morphology and on cellobiose starch (CS) medium for starch hydrolysis (14). Bacteria grown 48 h on TZC plates were suspended in sterile saline (NaCl, 8.5 g/L) to a concentration of 1×10^8 CFU/ml using techniques of Kolmer et al. (7). Growth characteristics were determined on YDC and CS after incubation at $27 \pm 1^\circ\text{C}$ for 4 days. Each strain was also tested for gram reaction, oxidase, catalase, oxidation-fermentation of glucose, and motility following protocols of Suslow et al. (24) and MacFaddin (11).

Pathogenicity and host range. All 33 strains isolated were tested for pathogenicity by mist-inoculating onto *A. virgatus* plants. Inoculum was prepared by growing strains for 24 h at $27 \pm 1^\circ\text{C}$ on Bacto nutrient agar amended with 5% sucrose. Bacterial growth was subsequently suspended in sterile saline, spectrophotometrically adjusted (A_{600}) to 1×10^8 CFU/ml, and suspensions were applied to two plants with hand sprayers until run-off. Inoculated plants were placed in clear polyethylene bags in the glasshouse for 24 h, and then bags were removed. During the following weeks, plants were misted with water every 30 min for 15 s during daylight hours. Temperatures and lighting in the glasshouse were maintained between 18.3 and 32°C and at $266 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, respectively. Plants were examined after 2 weeks for symptom development. If symptoms were present, samples were taken and bacteria were reisolated following the previously described protocol. Reisolated bacterial strains were characterized on YDC and CS media and tested for gram, oxidase, and catalase reactions. For host range testing, five strains that had been isolated from three separate farms over a two county area were selected. Two plants of each of the following six *Asparagus* spp. or cultivars were used following previously mentioned cultural and inoculation techniques: *A. virgatus* (Asparagus tree

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fern); *A. setaceus* (Plumosa fern); *A. macowanii* (Ming fern); *A. densiflorus* 'Sprengerii' (Sprengerii fern); *A. densiflorus* 'Myers' (Foxtail fern); and *A. officinalis* (edible asparagus). The host range experiment was repeated three times.

Utilization of carbon sources and fatty acid profile comparison. The 33 strains were tested for their ability to utilize 95 carbon sources using the Biolog GN System, version 3.5 (Biolog, Hayward, CA). Strains were grown on Difco tryptic soy agar for 48 h at $27 \pm 1^\circ\text{C}$. Bacterial growth was subsequently suspended in sterile saline and spectrophotometrically adjusted at A_{600} to 0.190–0.210 OD. Individual wells of 96-well test plates were filled with 150 μl of the bacterial suspension and plates were incubated at $27 \pm 1^\circ\text{C}$. After 48 h, optical densities were recorded and compared to the Biolog database and the percentage of strains utilizing each carbon source was calculated. *X. campestris* pv. *begoniae* (strain X1271) was used as a control.

Fatty acid methyl esters (FAME) analyses were also carried out on the 33 bacterial strains using the MIDI Microbial Identification System, software version TSBA 3.90 (Microbial ID, Inc., Newark, DE). Strains were grown for 24 h at $27 \pm 1^\circ\text{C}$ on Trypticase soy broth amended with Bacto (Difco) agar (15 g/L). After 24 h of growth, approximately 40 mg of bacterial cells (wet weight) were placed in 13 $\times$ 100 mm glass tubes fitted with Teflon-lined screw caps. Cellular fatty acids were extracted and derivatized to their FAMES using procedures described by Miller (13); FAME profiles were statistically compared to the MIDI TSBA 3.90 library (17,18). Three controls were utilized in each fatty

acid extraction: 1) reagent control; 2) *Stenotrophomonas maltophilia* (strain X1128, used because of multiple peaks as an evaluation of the extraction process); and 3) *X. campestris* pv. *begoniae* (strain X1271 used as a check to confirm specificity of analysis to pathovar level). To further compare FAME profiles, data were compared to the University of Florida's database (ver. 3.90), which contains 1,048 *X. campestris* strains of various pathovars.

Pathogenicity on *Dieffenbachia*. Using the Biolog assay, 85% of the 33 strains tested had high similarity values ($\bar{x}$ similarity index [s.i.] > 0.7) to *X. campestris* pv. *dieffenbachiae*. To determine if strains isolated from *A. virgatus* are *X. campestris* pv. *dieffenbachiae*, a substantially different variant of *X. campestris* pv. *dieffenbachiae*, or a new pathovar, the five prementioned strains along with six *X. campestris* pv. *dieffenbachiae* strains were tested for pathogenicity on *D. maculata* 'Camille' and *A. virgatus* plants. Strains of *X. campestris* pv. *dieffenbachiae* were selected from the two biotypes present in the population, those that can hydrolyze starch (strains X175, X186, and X195) and those that cannot (strains X454, X758, and X868). Strains X454 and X758 were isolated from *Anthurium andraeanum* in Hawaii and Florida, respectively, while the other four strains were isolated from *Dieffenbachia* in Florida. These bacterial strains were cultured, inoculated by mist inoculation onto two plants of each species and reisolations were done after 4 weeks as previously described and experiment was repeated at least once.

To further determine if xanthomonads isolated from *A. virgatus* might be capable of causing infections on *Dieffenbachia*, the

five strains from *A. virgatus* and the six *X. campestris* pv. *dieffenbachiae* strains were grown, adjusted spectrophotometrically in saline to 1×10^4 CFU/ml, and injected (5 cm^2 infiltration zones) with a 25-gauge hypodermic needle into two leaves of a *D. maculata* 'Camille' plant. Two controls were utilized—sterile saline and *Erwinia herbicola* (strain E101). Symptom development was monitored over a 4-week period.

RESULTS

Pathogen isolations. All strains of *Xanthomonas* isolated from asparagus fern grew readily on YDC medium and formed large round, butyrus, bright yellow colonies within 48 h. However, growth on CS medium was slow and colonies developed only after 4 days. No starch hydrolysis zones were evident around colonies of any of the strains tested. Additionally, all strains tested in this study were gram negative, oxidase negative, catalase positive, motile, and strictly aerobic.

Pathogenicity and host range. All 33 strains were pathogenic on *A. virgatus*, forming light green water-soaked lesions on stems within 2 weeks. Over the next 2 weeks, margins of these lesions turned brown and eventually lesions became completely necrotic. Number and size of lesions varied greatly among plants; some formed large discrete water-soaked lesions approximately 1 cm in length, while others formed hundreds of small lesions < 0.3 cm. The five strains utilized in the host range testing produced lesions on *A. virgatus* and additionally formed scattered lesions on new growth of *A. macowanii* and *A. densiflorus* 'Sprengerii' (Fig. 1). However, extent and severity of symptoms varied with indi-

Fig. 1. Symptoms caused by *Xanthomonas campestris* sp. on stems of (A) *Asparagus virgatus* (asparagus tree fern) and similar symptoms produced when inoculated onto (B) *A. macowanii* (plumosa fern) and (C) *A. densiflorus* (sprengerii fern).

vidual strains tested (Table 1). *Xanthomonas* was reisolated and identified using previously outlined techniques from all plants exhibiting lesion development.

Utilization of carbon sources and fatty acid profile comparison. Using the 95 carbon source Biolog System and accompanying software (ver. 3.5), the 33 strains were grouped into four classification groups based on their metabolic fingerprints: 1) *X. campestris* pv. *vitians* (4 strains, s.i. = 0.604); 2) *X. campestris* pv. *dieffenbachiae* from *Xanthosoma* (21 strains, $\bar{x}$ s.i.=0.777); 3) *X. c.* pv. *dieffenbachiae* from *Syngonium* (7 strains, $\bar{x}$ s.i.=0.750); and 4) *X. oryzae* (1 strain, s.i. = 0.447). Similarity indexes ≥ 0.50 are considered matches with 1.0 considered a perfect match. Percentages of the 33 strains utilizing each carbon source can be seen in Figure 2. The *X. campestris* pv. *begonia* strain utilized as a control was identified as *X. campestris*. However, no match was made to a specific pathovar.

FAME analysis using the MIDI data base divided the 33 strains into five groups as follows: 1) *X. campestris* pv. *vitians* (13 strains, $\bar{x}$ s.i. = 0.469); 2) *X. campestris*

pv. *translucens* (11 strains, $\bar{x}$ s.i. = 0.529); 3) *X. campestris* pv. *begoniae* (7 strains, $\bar{x}$ s.i.=0.388); 4) *X. campestris* pv. *pelargonii* (1 strain, s.i. = 0.206); and 5) *X. campestris* pv. *phaseoli* (1 strain, s.i. = 0.397). Using the Biolog and FAME analysis techniques, only two strains were classified by both systems as *X. campestris* pv. *vitians*. The two control strains, *X. campestris* pv. *begoniae* (strain X1271, s.i. = 0.859) and *Stenotrophomonas maltophilia* (X1128, s.i.=0.903), were identified to the pathovar and species level, respectively. Using the University of Florida's FAME database, similarity indices were again low with 15 strains being matched to *X. campestris* pv. *secalis* ($\bar{x}$ = 0.412), 7 strains to *X. fragariae* ($\bar{x}$ = 0.224), and 6 strains to *X. campestris* pv. *translucens* ($\bar{x}$ = 0.437), with the other 5 strains having matches < 0.20 to other pathovars. Percentages of the five most prevalent fatty acids found in the FAME profiles of the 33 *X. campestris* strains isolated from *A. virgatus* are shown in Table 2.

Pathogenicity on *Dieffenbachia*. All five xanthomonads from *A. virgatus* formed lesions within 2 weeks on *A. vir-*

gatus plants. However, no symptoms were observed on *Dieffenbachia* plants over a 4-week period. The three *X. campestris* pv. *dieffenbachiae* strains that were starch hydrolysis negative formed scattered dark brown necrotic lesions on *A. virgatus* stems within 2 weeks. No symptoms were produced on *A. virgatus* plants by the *X. campestris* pv. *dieffenbachiae* strains that were capable of utilizing starch. All six *X. campestris* pv. *dieffenbachiae* strains were pathogenic on *Dieffenbachia* forming water-soaked lesions on leaves within 4 weeks. *Xanthomonas* strains were reisolated from lesions of test plants.

Leaves of all dieffenbachia infiltrated with *X. campestris* pv. *dieffenbachiae* strains became water-soaked within four days, necrotic within 2 weeks and disease

Table 1. Host range of five xanthomonad strains isolated from *Asparagus virgatus*, on five of the most commonly cultivated *Asparagus* species^a

Genus/Species	Common Name	Symptoms ^b
<i>Asparagus virgatus</i>	Tree fern	+ ^c
<i>A. setaceus</i>	Plumosa fern	+/- ^d
<i>A. macowanii</i>	Ming fern	- ^e
<i>A. densiflorus</i> 'Sprengeri'	Sprengeri fern	+/- ^f
<i>A. densiflorus</i> 'Myers'	Foxtail fern	-
<i>A. officinalis</i> ^g	Edible asparagus	-
Control (saline)		-

^a Experiment repeated three times with two replicate plants of each cultivar.

^b A suspension containing *Xanthomonas* at 1×10^8 CFU/ml was misted onto surface of plants.

^c Water-soaked lesions on stem of asparagus plants.

^d Two of the five strains produced a few (1 to 4), scattered, water-soaked lesions.

^e No symptoms observed four weeks after inoculation.

^f All five strains produced lesions but only on half of the plants.

^g Jersey Knight Hybrid, Park Seed, Greenwood, SC.

	1	2	3	4	5	6	7	8	9	10	11	12
A	CONTROL		9		100	97		21		15		97
B		100	6		9	100				3		94
C			82				61	94	33		100	
D												
E												
F	15			6			18		6	78		39
G									48			
H												

Fig. 2. Percentages of the 33 xanthomonad strains utilizing each carbon compound, using the 95 carbon compound GN Biolog assay. Carbon compounds are defined by letters (rows) and numbers (columns) as follows: A2, α -cyclodextrin; A3, dextrin; A4, glycogen; A5, Tween 40; A6, Tween 80; A7, *N*-acetyl-D-galactosamine; A8, *N*-acetyl-D-glucosamine; A9, adonitol; A10, L-arabinose; A11, D-arabitol; A12, cellobiose; B1, *meso*-erythritol; B2, D-fructose; B3, L-fucose; B4, D-galactose; B5, gentiobiose; B6, α -D-glucose; B7, *meso*-inositol; B8, α -D-lactose; B9, lactulose; B10, maltose; B11, D-mannitol; B12, D-mannose; C1, D-melibiose; C2, β -methyl-D-glucoside; C3, D-psicose; C4, D-raffinose; C5, L-rhamnose; C6, D-sorbitol; C7, sucrose; C8, D-trehalose; C9, turanose; C10, xylitol; C11, methylpyruvate; C12, mono-methylsuccinate; D1, acetic acid; D2, *cis*-aconitic acid; D3, citric acid; D4, formic acid; D5, D-galactonic acid lactone; D6, D-galacturonic acid; D7, D-gluconic acid; D8, D-glucosaminic acid; D9, D-glucuronic acid; D10, α -hydroxybutyric acid; D11, β -hydroxybutyric acid; D12, γ -hydroxybutyric acid; E1, *p*-hydroxyphenylacetic acid; E2, Itaconic acid; E3, α -ketobutyric acid; E4, α -ketoglutaric acid; E5, α -ketovaleric acid; E6, DL-lactic acid; E7, malonic acid; E8, propionic acid; E9, quinic acid; E10, D-saccharic acid; E11, sebacic acid; E12, succinic acid; F1, bromosuccinic acid; F2, succinamic acid; F3, glucuronamide; F4, alaninamide; F5, D-alanine; F6, L-alanine; F7, L-alanyl-glycine; F8, L-asparagine; F9, L-aspartic acid; F10, L-glutamic acid; F11, glycyl-L-aspartic acid; F12, glycyl-L-glutamic acid; G1, L-histidine; G2, hydroxy-L-proline; G3, L-leucine; G4, L-ornithine; G5, L-phenylalanine; G6, L-proline; G7, L-pyrroglutamic acid; G8, D-serine; G9, L-serine; G10 L-threonine; G 11, DL-carnitine; G12, γ -aminobutyric acid; H1, urocanic acid; H2, inosine; H3, uridine; H4, thymidine; H5, phenyl ethylamine; H6, putrescine; H7, 2-aminoethanol; H8, 2,3-butanediol; H9, glycerol; H10, DL α -glycerolphosphate; H11, glucose 1-phosphate; H12, glucose 6-phosphate.

Table 2. Percentages of five fatty acids^a in the FAME profile of 33 *Xanthomonas campestris* strains isolated from *Asparagus virgatus*

Fatty Acid	Mean	SD ^b	Std/Mean
11:0 ISO	5.63	0.41	0.07
14:0	2.01	0.14	0.07
15:0 ISO	28.29	2.62	0.09
15:0 anteiso ^c	7.26	1.22	0.17
NRD ^d	24.97	2.04	0.08

^a These five fatty acids comprised approximately 68% of the total profile of the strains tested.

^b Standard deviation.

^c A structural isomer of 15:0 ISO, note higher variability in production relative to mean.

^d NRD = not reliably differentiated by MIDI includes 16:1 ω 7cis and 15:0 iso 2OH.

spread within the plant became evident by week four. However, no symptoms were observed on *Dieffenbachia* plants in which xanthomonad strains from *A. virgatus*, saline, or *E. herbicola* controls had been infiltrated.

DISCUSSION

Host range studies on asparagus species and on *dieffenbachia* indicate that the xanthomonads isolated from *A. virgatus* have a very limited host range. Only *A. virgatus* was infected, and scattered lesions were also produced on stems of *A. macowanii* and *A. densiflorus* 'Sprengeri'. In the field, diseased *A. virgatus* plantings within a few feet of plantings of *A. macowanii* or *A. densiflorus* 'Sprengeri' produced no infections on these species. In infested production fields, outbreaks were always observed in *A. virgatus* plantings, which either had a very thick growth of asparagus ferns or an abundance of weeds. This suggests that the high humidity produced in the thick growth of plants may be necessary for infection. Weeds in infested fields were not infected by xanthomonads. In laboratory inoculations, infection occurred only on young asparagus stems that were still growing, never on old hardened-off fronds. New infections in production fields were observed in the spring and in the fall. This may be related to flushes of new growth or to more moderate temperatures.

It is difficult to discriminate among *X. campestris* pathovars without extensive pathogenicity testing. However, different techniques have been successfully employed such as monoclonal antibodies (1,9), SDS polyacrylamide gel electrophoresis (26,27), DNA hybridization (5,25), restriction fragment-length polymorphism (8), and genomic DNA fingerprinting (3,10,15). However, widespread use of some of these methods is limited due to expense, time, spectrum of use, or ability to differentiate strains at the pathovar level. Both FAME analysis and metabolic fingerprints (Biolog) have been successfully used to distinguish pathovars of *X. campestris* (2,16,22,27,28). Controls utilized in the FAME analysis had high similarity values with existing databases. However, low similarity values were obtained for the 33 strains tested from asparagus. These low values were not produced in a single FAME extraction but were consistent even when new extractions were made. The low values obtained when using either the MIDI or University of Florida database indicate that these xanthomonad strains are probably a distinct *X. campestris* pathovar or a unique variant that is metabolically similar to *X. campestris* pv. *dieffenbachiae* strains that do not hydrolyze starch. *X. campestris* pv. *dieffenbachiae* strains isolated from *Syngonium* and *Xanthosoma* are usually unable to hydrolyze starch, which

may substantiate relatedness of these strains to those from asparagus. It appears from this study that the *X. campestris* strains from asparagus may in fact be a variant of *X. campestris* pv. *dieffenbachiae* and that the ability to hydrolyze starch may be directly related to host range of members of this very heterogeneous pathovar. To answer these questions, additional research involving genetic comparisons and extensive host range testing would be needed.

The xanthomonads infecting *A. virgatus* plantings are currently found only on a few farms in two counties in Florida but have the potential, under appropriate environmental conditions, to become a major pathogen of *A. virgatus* plantings.

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