Survival of *Xanthomonas fragariae* on Strawberry in Summer Nurseries in Florida Detected by Specific Primers and Nested Polymerase Chain Reaction

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ABSTRACT

Roberts, P. D., Jones, J. B., Chandler, C. K., Stall, R. E., and Berger, R. D. 1996. Survival of *Xanthomonas fragariae* on strawberry in summer nurseries in Florida detected by specific primers and nested polymerase chain reaction. Plant Dis. 80:1283-1288.

Genomic DNA from strain XF1425 of *Xanthomonas fragariae* was amplified with primers RST2 and RST3 from the *hrp*-gene region of *Xanthomonas campestris* pv. *vesicatoria.* The polymerase chain reaction (PCR) product was sequenced. Four primers were selected at sites unique to *X. fragariae,* which were identified by comparison with the sequences of PCR products amplified by the same primers from DNA of three strains of *X. campestris* pv. *vesicatoria.* Three primers were specific for amplification of DNA from *X. fragariae* but not from strains of 16 pathovars of *X. campestris* or nonpathogenic xanthomonads from strawberry. Bacteria were detected at approximately 10^4 to 10^5 CFU/ml by a single round of PCR. A nested PCR technique enabled detection to approximately 18 cells. Restriction endonuclease digestion patterns of the PCR product were unique to *X. fragariae* and confirmed amplification of DNA from the target organism. Bacteria were detected from symptomatic and asymptomatic plant tissue by the nested technique. From strawberry plants inoculated with a rifampicin-resistant strain of *X. fragariae* and planted in the field in Florida, bacteria were detected by nested PCR and by recovery onto media at 2-week intervals for 92 days after planting. Daughter plants of the inoculated plants were positive for *X. fragariae* by nested PCR amplification, indicating that *X. fragariae* survived on plants in summer nurseries in Florida and was disseminated to daughter plants.

Additional keywords: angular leaf spot of strawberry, *Fragaria,* restriction endonuclease analysis

Strawberry (*Fragaria* × *ananassa* Duchesne) angular leaf spot, caused by the bacterium *Xanthomonas fragariae,* was first reported in Minnesota in 1959 and now occurs in many areas of strawberry production throughout the world (8,15). The disease caused the loss of 75% of fruit in Wisconsin (3). Angular leaf spot was first found in Florida in 1968 (6). In Florida, strawberry plants that arrive from northern nurseries for transplanting in the fall frequently have angular leaf spot.

The epidemiology of *X. fragariae* is mostly unknown in strawberry fields in Florida, where production is from annual crops. Howard (6) was unable to determine the inoculum source for infected plants from nurseries in Florida or other states. Bacteria may survive in the soil on infested leaves (9), but plants are usually treated with the herbicide paraquat at the end of the season in Florida and removed. The bacterium does not survive freely in the soil (9). Cool temperatures (≈24°C) are optimal for disease symptom expression, and high temperatures (>28°C), such as those that occur in Florida during the summer months, are unfavorable for symptom development (8). In surveys conducted in 1968, 1970, 1993, and 1994, nursery plants with symptoms of angular leaf spot in the spring did not have symptoms of the disease the following August. In 1969, mild infections on one variety were observed in the nursery in mid-August, but plants of this variety did not develop angular leaf spot in production fields (6; P. D. Roberts, *unpublished*). Neither the survival of the bacterium on plants in summer nurseries in Florida nor inoculum sources other than infected transplants has been established.

Identification of plants infected with *X. fragariae* is a priority because of the ease of transmission via infected but asymptomatic plants (16). International movement of infected plants is blamed for the introduction of angular leaf spot into Greece and New Zealand (2,19). Nursery plant producers are pressured to provide disease-free plants by foreign countries and by farmers who refuse to buy infected transplants. The European Plant Protection Organization (EPPO) lists *X. fragariae* as a quarantined pest and has prescribed phytosanitary procedures. In the future, regulatory issues may be of greater concern. The production of pathogen-free plants is essential for control of angular leaf spot. Therefore, accurate identification of infected plants is imperative. Available detection techniques are limited in their usefulness and accuracy in detecting the low populations of the bacterium that may exist in asymptomatic tissue. *X. fragariae* may be identified in the early stages of leaf infection by the diagnostic translucent, water-soaked lesions viewed with transmitted light; however, older lesions may be confused with symptoms caused by fungal pathogens (9). Diagnosis based on symptoms is very difficult and not applicable for asymptomatic plants. Detection based on isolation and characterization of the causal agent may also be difficult because *X. fragariae* grows slowly and may be masked by faster growing organisms (5). Expression of water-soaked lesions takes 6 days or longer after inoculation. Thus, tests to confirm pathogenicity are difficult and time-consuming.

Assays have been developed with improved sensitivity and specificity for the detection of plant pathogens in plant tissue. A specific indirect enzyme-linked immunosorbent assay (ELISA) was developed to detect *X. fragariae* from symptomatic plant tissue (22). The polyclonal antisera did not react with bacterial strains of 16 pathovars of *Xanthomonas campestris* or with nonpathogenic bacteria from strawberry leaves. A single cross-reaction occurred to a strain of *X. campestris* isolated from *Nerium oleander.* The assay detected bacteria directly from a visible lesion on a strawberry leaf at approximately 10^4 CFU/ml. However, bacteria on asymptomatic plants may not be detected by this ELISA.

Polymerase chain reaction (PCR) amplification of specific DNA sequences has been used to detect and identify many plant pathogens, including some members of the genus *Xanthomonas* (4,14). Primers specific to regions of the *hrp* gene cluster

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Florida Agricultural Experiment Station Journal Series R-05324.

Accepted for publication 19 August 1996.

Publication no. D-1996-0916-07R
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from *X. campestris* pv. *vesicatoria* detected only pathogenic xanthomonads, since saprophytic and nonpathogenic xanthomonads lack these genes, which confer hypersensitivity and pathogenicity (12,13,24,27). Differentiation of *X. campestris* pathovars was made by restriction endonuclease analysis (REA) patterns generated by digestion of the PCR products with frequent cutting enzymes (11,12). The primers were used to detect *X. campestris* pv. *vesicatoria* DNA in seed lots of naturally infected pepper and tomato (11). The limit of detection by PCR is generally 10^3 to 10^2 CFU/ml (4,11,18). In nested PCR, the limit of detection is decreased by using PCR products from an amplification as target DNA in a second round of amplification by a second set of primers internal to the first (23). McManus and Jones (17) reported the nested PCR detected 1,000 times fewer *Erwinia amylovora* cells than the minimum number detected in a single round of PCR amplification.

Our objective was to develop a sensitive and specific technique for detection of *X. fragariae*. Our approach was to design primers specific to the region of genomic DNA from *X. fragariae* related to the *hrp* genes of *X. campestris* pv. *vesicatoria*. The survival of *X. fragariae* on nursery strawberry plants in the field at two locations in Florida and dissemination to daughter plants was examined to understand the disease cycle of angular leaf spot in Florida.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Strains of *X. fragariae* and nonpathogenic xanthomonads isolated from strawberry were maintained at 24°C on Wilbrink's (WB) medium (10). *X. fragariae* colonies on this medium were pale yellow and mucoid, and appeared 2 to 3 days after plating. Pathovars of *X. campestris* were cultured on nutrient agar (Difco Laboratories, Detroit, MI) and incubated at 28°C. Long-term storage was at –70°C in 15% glycerol. Bacteria for plant inoculations and DNA extractions were grown in 5 ml of nutrient broth on a rotary shaker at 200 rpm at 24°C for 16 h. A rifampicin-resistant mutant of strain XF1425 was selected on Wilbrink's medium supplemented with rifampicin at 100 µg/ml by the gradient plate technique (25). Strain information and sources for *X. fragariae* and nonpathogenic strains of xanthomonads isolated from strawberry are listed in Table 1. Additional strains tested were ATCC type strains of *X. campestris* pathovars *begoniae* ATCC 11726, *campestris* ATCC 33913, *carotae* ATCC 10547, *celebensis* ATCC 19045, *glycines* ATCC 43911, *incanae* ATCC 49073, *manihotis* ATCC 49073, *musacearum* ATCC 49084, *papavericola* ATCC 14179, *pelargonii* ATCC 8721, *phaseoli* ATCC 9563, *poinsettiaecola* ATCC 11643, *raphani* ATCC 49079, *taraxaci* ATCC 19318, *vignicola* ATCC 11648, and *vitians* ATCC 19320.

Pathogenicity tests. Bacteria from overnight cultures in nutrient broth were centrifuged and washed three times with sterile water. The concentration of cells was adjusted in either 10 mM $MSO_4 \cdot 7H_20$ or sterile water to 2×10^8 CFU/ml, determined by the dilution plate method (26) converted to a spectrometric reading of $OD_{600} = 0.1$, and diluted to a final concentration of 10^5 CFU/ml. Inoculum was sprayed to runoff on cv. Sweet Charlie strawberry plants placed under mist 24 to 48 h prior to inoculation. Inoculated plants were maintained under mist or put into growth chambers (Percival, Boone, IA) at 24°C with a 12-h photoperiod. The tips of runners from Sweet Charlie plants were rooted under mist in sterile soil, transplanted to 10-cm-diameter pots, and inoculated 6 to 8 weeks after transplanting. Two plants per strain were inoculated, and inoculations were repeated twice if symptoms did not develop.

Sequencing and primer design. Primers RST2 and RST3 from the *hrp* gene cluster of *X. campestris* pv. *vesicatoria* (12) amplified the genomic DNA of 49 strains of *X. fragariae*. The PCR product from *X. fragariae* strain XF1425 was isolated from agarose gel, cleaned by the Promega Wizard Kit (Promega, Madison, WI), and sequenced at the ICBR DNA Sequencing Facility, University of Florida, Gainesville. The nucleotide sequence was compared to sequences of the PCR products amplified by the same primers from three *X. campestris* pv. *vesicatoria* strains representing tomato races 1, 2, and 3 using the Seqaid II computer program (21). Four primers were selected from the sequence of *X. fragariae* based on unique DNA sequences and low homology compared with the DNA sequences from *X. campestris* pv. *vesicatoria*. The four primers synthesized were: XF9 (5′-TGGGCCATGCCGGTGGAACTGTGTGG-3′); XF10 (5′-TGGAACTGTGTGGCGAGCCAG-3′); XF11 (5′-TACCCAGCCGTCGCAGACGACCGG-3′); and XF12 (5′-TCCCAGCAACCCAGATCCG-3′). Primers XF10 and XF12 were located internal to the other two primers. Oligonucleotide primers were synthesized with a model 394 DNA synthesizer (Applied Biosystems, Foster City, CA) at the ICBR Facility.

PCR amplification and nested amplification. Total genomic DNA was extracted by the method described by Ausubel et al. (1). PCR amplification was in a DNA Thermal Controller PT-100 (MJ Research, Watertown, MA). The 50-µl reaction volume contained 1× amplification buffer with $MgCl_2$ (Promega, Madison, WI), 100 µM each dNTP (Promega, Madison, WI), 50 µM each primer, 1.25 U *Taq* DNA polymerase, and 100 ng of purified genomic DNA in 3 µl of TE (10 mM Tris and 1 mM EDTA, pH 8.0) buffer. Each reaction was overlaid with 50 µl of sterilized mineral oil (Sigma Chemical Co., St. Louis, MO) for a total volume of 100 µl in sterile 0.6-ml microcentrifuge tubes. In later experiments, the thermocycler was equipped with a heated lid controller (The Hot Bonnet, MJ Research), which eliminated the need for the mineral oil overlay. Amplification of the DNA proceeded after template DNA was denatured at 95°C for 2 min followed by 30 amplification cycles and a final extension step at 72°C for 5 min. For primer set XF9 and XF11, each amplification cycle consisted of denaturation at 95°C for 30 s, annealing at 65°C for 30 s, and extension at 72°C for 45 s. For primer XF12 with either primer XF9 or XF10, the program was identical except that the annealing temperature was 58°C.

For nested PCR, first-round amplification was as described for primers XF9 and XF11. In the second round of amplification, 3 µl of sample of the first amplification mixture was added to primers XF9 and XF12 and reaction components at the concentrations described above. In all PCR runs, including the nested assays, a water

Table 1. Geographic origin, year, and source of *Xanthomonas fragariae* and nonpathogenic xanthomonads used in this study

Strain designations	Geographic origin	Year	Source
X. fragariae			
1238, 1240, 1243, 1245, 1249, 1250	CA	1990	ARC[a]
1290, 1291, 1293, 1295, 1298	CA	1989	ARC
1425, 1428, 1426,1427, 1429, 1431	FL	1992	ARC
1514, 1516	CA	1993	ARC
1517, 1518, 1526	NC	1993	ARC
1520, 1523, 1524,1525	CA	1993	ARC
1533, 1534	WI	1993	ARC
100, 101, 103, 104, 105, 106, 107, 113, 114, 115	FL	1993	PDR
116, 117, 119, 124, 125, 126, 127, 138, 146, 153	CANADA	1993	PDR
ATCC 33239	MN	1960	ATCC
Nonpathogenic xanthomonads isolated from strawberry plants			
1142, 1225, 1226, 1226, 1239	CA	1990	ARC
109, 110, 111, 112	FL	1993	PDR

[a] A. R. Chase, Apopka Research and Education Center, University of Florida, Apopka; P. D. Roberts, this study; ATCC = American Type Culture Collection.

sample was included as a negative control. An 8-µl PCR sample was loaded into the wells of a 1% Seakem GTG agarose gel (FMC BioProducts, Rockland, ME) containing ethidium bromide at 0.5 µg/ml in TAE buffer at 8 V/cm.

Restriction endonuclease analysis (REA) of PCR products. PCR samples with the overlay of mineral oil were cleaned by the method of Minsavage et al. (18). Samples without the mineral oil were restricted directly. An 8-µl sample of the PCR product was digested with restriction endonuclease *Sau*3AI, *Hae*III, or *Cfo*I under conditions specified by the manufacturer (Promega). Restricted products were separated in 4% agarose gel (3% NuSieve, 1% SeaKem GTG, FMC BioProducts) containing ethidium bromide at 0.5 µg/ml in TAE buffer at 8 V/cm, as described by Leite et al. (12). DNA molecular weight marker XI (Boehringer Mannheim, Indianapolis, IN) was used for standard weight markers. Gels were photographed over a UV transilluminator with type 55 Polaroid film.

Detection of bacteria from infected plant tissue. The limit of detection of the assay in the presence of plant tissue was determined by adding 200 µl of 10-fold dilutions of a bacterial suspension adjusted to 10^8 CFU/ml to plant samples. A 9-mm-diameter disk of plant tissue was macerated with mortar and pestle in 200 µl of phosphate buffer (pH 7.0) containing 5% polyvinylpolypyrrolidone (PVPP) (PVP40, Sigma) and 0.02 M sodium ascorbate (PPA). The mixture was incubated at room temperature for a minimum of 1 h. The volume of plant sample and bacterial cells was adjusted to 582 µl with TE, and DNA extraction proceeded as described. A minimum of three experiments with three replicates of each treatment for each primer set and nested reaction was performed. REA was performed on the final PCR products.

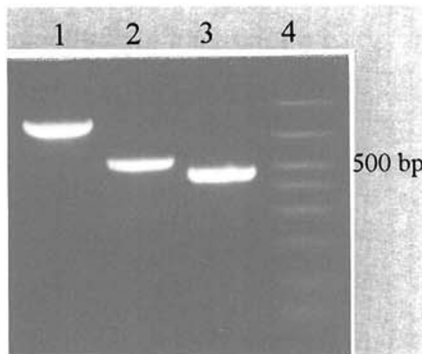

Fig. 1. Agarose gel of the polymerase chain reaction (PCR) products generated by amplification of genomic DNA from a strain of *Xanthomonas fragariae* with the *hrp*-primers RST2 and RST3 (lane 1) and the XF-specific primers XF9 paired with XF11 (lane 2) and with XF12 (lane 3), and molecular weight marker XI (Boehringer Mannheim, Indianapolis, IN; lane 4).

Strawberry leaves with lesions either from field infections or spray inoculation were assayed by removing a 9-mm-diameter disk of tissue surrounding a lesion and processing as above. Tomato leaf tissue was used as a negative control. For confirmation, a sample of the ground tissue suspension was plated on Wilbrink's medium amended with 0.05% Bravo 720 (chlorothalonil; ISK Biosciences, Mentor, OH). Plates were incubated at 24°C, and pale yellow, mucoid colonies appearing after 2 to 3 days were tested by nested PCR and REA.

Field experiments to examine survival of bacteria on summer nursery plants. Six-week-old rooted transplants of Sweet Charlie were spray-inoculated with *X. fragariae* strain XF1425^{rif} 2 weeks prior to planting. Plants with lesions of angular leaf spot were transplanted into the field on 27 June 1995 at Gulf Coast Research and Education Center, Dover, FL, and on 29 June 1995 at Gulf Coast Research and Education Center, Bradenton, FL. Plants were sampled over a 14-week period through 27 September 1995. A set of inoculated plants was placed into Percival growth chambers at a 12-h photoperiod and 24°C and maintained for the duration of the experiment. Noninoculated, asymptomatic plants were placed in a greenhouse for the negative control. At 2-week intervals, five plants from the field, one plant from the growth chamber, and one plant from the greenhouse were sampled. Two samples of three daughter plants of the inoculated plants were sampled 132 days after planting from both locations.

Individual plants were assayed as follows. All the leaves from a single plant were placed in a flask with 200 ml of phosphate buffer containing 0.02% Tween-20 and shaken either on a wrist action (Burrell Corp., Pittsburgh, PA) or a rocker platform (Bellco Biotechnology, Vineland, NJ) shaker for 2 to 16 h. A 200-µl sample was plated onto Wilbrink's medium plus rifampicin at 100 µg/ml plus 0.5% Bravo 720 and incubated at 24°C for 72 h. Bacteria characteristic of *X. fragariae* were examined by nested PCR with the XF-specific primers followed by REA of the 458-bp fragment. The remainder of the phosphate buffer sample was concentrated by vacuum filtration onto a 0.45-µm membrane disk (Millipore Corp., Bedford, MA). The disk was washed in 1.5 ml of TE, and the suspension was centrifuged 5 min at 14,000 × *g*. The pellet was resuspended in 582 µl of TEPA (TE buffer containing 5% PVP40 + 0.02 M sodium ascorbate) and incubated at room temperature for 1 h. DNA was extracted as described.

The crown of the plant was sectioned and macerated by mortar and pestle in 10 ml of PPA. The plant tissue debris and PVPP were collected by centrifugation at 1,000 × *g* for 1 min. The supernatant with the bacterial cells was removed to a clean centrifuge tube and centrifuged at 14,000 × *g* for 5 min. The pellet was resuspended in 582 µl of TE, and DNA extraction proceeded as described above. A sample was plated onto Wilbrink's agar plus rifampicin at 100 µg/ml and 0.5% Bravo and incubated at 24°C for 72 h. The nested PCR reaction and REA were performed as described.

RESULTS

Pathogenicity assays. All strains identified as *X. fragariae* caused disease symptoms typical of angular leaf spot on strawberry plants. Other xanthomonads isolated from strawberry tissue were nonpathogenic on strawberry plants.

Specificity of primers. For all strains of *X. fragariae*, the PCR products amplified by primers RST2 and RST3 were approximately 840 bp (Fig. 1). The REA profile that resulted from restriction of the PCR products with *Cfo*I or *Hae*III was identical for the 49 strains of *X. fragariae* (data not shown). Genomic DNA was not amplified by the primers from nonpathogenic strains of *Xanthomonas* isolated from strawberry.

Primer XF9 paired with XF11 or XF12 delineated a 537-bp or 458-bp fragment, respectively (Fig. 1). Primers XF10 and XF12 delineated a 448-bp fragment. PCR products were the estimated sizes based on sequence data. All 49 strains of *X. fragariae* were amplified with primer sets XF9 and XF11, XF9 and XF12, and XF10 and XF12. DNA from strains other than *X.*

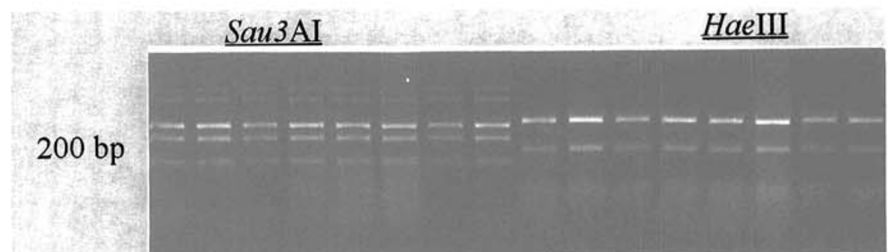

Fig. 2. NuSieve 3:1 agarose gel showing the restriction patterns generated after digestion of the polymerase chain reaction (PCR) product amplified with the primers XF9 and XF12 from the genomic DNA of strains of *Xanthomonas fragariae*. The PCR product was digested by restriction endonucleases *Sau*3AI (lanes 1 to 8) and *Hae*III (lanes 9 to 16).

fragariae was not amplified by primer XF9 paired with XF11 or XF12. Except for *X. campestris* pv. *pelargonii,* genomic DNA from these strains could not be amplified with primer set XF10 and XF12. Due to the nonspecific amplification of DNA from *X. campestris* pv. *pelargonii* by primer XF10 paired with XF12, primer XF10 was not used in subsequent experiments.

REA of PCR products. PCR products from DNA of *X. fragariae* amplified with primer set XF9 and XF12 restricted with *Cfo*I, *Sau*3AI, or *Hae*III produced distinct banding patterns for each enzyme (Fig. 2). Five bands resulted from *Cfo*I restriction (125, 108, 90, 75, and 60 bp; data not shown) and three bands each from the *Hae*III restriction (258, 150, and 50 bp) and *Sau*3AI restriction (250, 125, and 83 bp). Polymorphisms were not observed among strains of *X. fragariae* in the REA of the restricted PCR products for the enzymes tested.

Limit of detection of *X. fragariae* in plant tissue by PCR. *X. fragariae* was detected in the presence of plant tissue by amplification with primer XF9 and either XF11 or XF12, and by nested PCR of the two primer sets. The limit of detection of bacterial cells by amplification with one round of PCR was approximately 10^5 to 10^4 CFU/ml. Nested PCR detected 1,000-fold fewer cells (Fig. 3) and enabled detection of approximately 18 cells. PCR was used to detect bacteria directly from infected plant tissue both from inoculated strawberry and from plant samples collected in the field. The PCR product was generated from a single lesion on plant material ground and processed representing a bacterial population of 10^4 CFU/ml or above as determined by dilution plating. A single-round PCR was sufficient to detect bacteria from a lesion. In all experiments, identification of *X. fragariae* was confirmed by REA of PCR products.

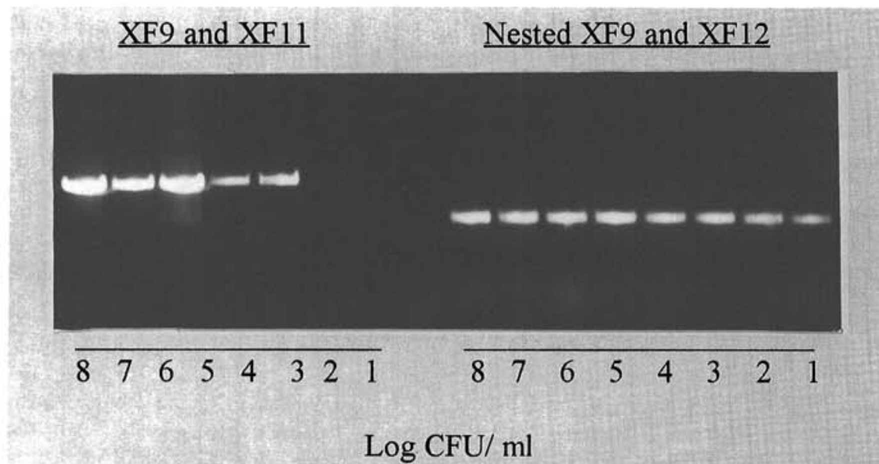

Fig. 3. Agarose gel showing the number of cells detected by polymerase chain reaction (PCR) and nested PCR in the presence of plant tissue. The genomic DNA of *Xanthomonas fragariae* was extracted and amplified by primers XF9 and XF11 and used in a second round of nested amplification with primers XF9 and XF12.

Detection of bacteria on plants in field experiments. All plants exhibited visible symptoms of angular leaf spot at planting. However, by 34 days after planting, symptoms of angular leaf spot were not visible (Table 2). Bacteria were recovered on WB supplemented with rifampicin and Bravo 720, and colonies of *X. fragariae* were identified by morphology, resistance to rifampicin, DNA amplification by the XF-specific primers, and REA. Bacteria were recovered on medium with antibiotic from a few samples throughout the experiment, including the final date, indicating that bacteria were viable on plants throughout the summer.

X. fragariae was detected from leaf samples by nested PCR on each sample date. The percentage of leaf samples that were positive by the nested PCR assay ranged from 20 to 100% for the sample dates. From the leaves, all samples were initially positive, but by 51 days after planting, the number of positive samples declined to 20%. By 92 days after planting, all samples were again positive. A single round of amplification was occasionally sufficient to identify positive samples. Bacteria from the crowns of plants were detected by PCR amplification on all sample dates except one. REA analysis of the PCR products confirmed banding patterns of *X. fragariae.* Plants inoculated and placed in the growth chamber at the favorable temperatures for disease development were positive at each sampling date except one. Noninoculated plants from the greenhouse were positive by this technique in approximately 20% of the samples.

Table 2. Percentage of strawberry plants with *Xanthomonas fragariae* detected by polymerase chain reaction (PCR) and a recovery onto medium in summer nurseries from June through September at two locations in Florida

	Leaves						Crown					
Location	DAP[a]	NonI[b]	Inoc[c]	XF911[d]	Nested[e]	Medium[f]	Symptoms	NonI	Inoc	XF911	Nested	Medium
Dover	0	–	+	60	100	TC	+	–	+	20	40	TC
	11	–	+	0	40	TC	+	–	+	0	40	TC
	31	–	+	0	20	0	+	–	+	0	40	0
	40	+	+	0	20	NS	–	–	+	0	0	NS
	66	–	–	0	60	0	–	–	+	0	40	0
	85	–	+	20	100	60	–	–	+	20	100	20
	92	–	+	40	100	40	–	–	+	0	80	40
Bradenton	0	–	+	60	100	TC	+	–	+	0	40	TC
	20	–	+	20	20	20	+	+	+	0	60	0
	34	+	+	0	20	NS	–	–	+	0	40	NS
	40	–	+	0	20	20	–	–	+	0	100	20
	54	–	+	0	40	0	–	–	+	0	40	0
	68	+	+	20	100	60	–	+	+	20	80	20
	82	–	+	20	80	40	–	+	+	20	80	40

[a] Days after planting.
[b] Negative control of noninoculated plants in greenhouse: – = negative by PCR amplification, + = positive by PCR amplification.
[c] Inoculated strawberry plants in growth chamber at 22°C: – = negative by PCR amplification, + = positive by PCR amplification.
[d] Percentage of samples amplified by first round PCR with primer set XF9 and XF11.
[e] Percentage of samples amplified by nested PCR with primers XF9 and XF12.
[f] Wilbrink's medium plus rifampicin at 100 mg/ml and 0.05% Bravo; TC = too contaminated to distinguish individual colonies; NS = not assayed; 0 to 100 = percentage of plates with colonies of *X. fragariae*.

DISCUSSION

Leite et al. (12) used the sequence variation within the *hrpB* operon among plant-pathogenic xanthomonads to select primers with different specificities, and this approach was successful in our studies to identify primers specific to *X. fragariae*. The *hrp*-primers RST2 and RST3 amplified DNA with identical restriction patterns from all strains of *X. fragariae*. The homology within this amplified region presented a site to select primers universal to *X. fragariae*. Primers XF9, XF11, and XF12 were specific for amplification of DNA only from strains of *X. fragariae*. The primer XF10 was responsible for the nonspecific amplification of a strain of *X. campestris* pv. *pelargonii* in preliminary tests and therefore was not included in later experiments.

The level of detection techniques for specific bacteria (20,23) is comparable to the level achieved with a single round of PCR amplification. PCR was reported to be more sensitive than ELISA (11), and with our primers, a single round of PCR is 10 times more sensitive than the ELISA for *X. fragariae* developed by Rowhani et al. (22). In addition, their antibodies cross-reacted to another xanthomonad, while we were able to eliminate the nonspecific reaction by discontinuing the use of primer XF10. The *X. fragariae* primers were suitable for use in the nested technique, and the limit of detection of the PCR reaction decreased by 1,000-fold. This level of detection was achieved in assays to detect the bacteria in the presence of plant tissue and is well below the number of cells needed to cause visible lesions. Therefore, the nested technique is applicable to detecting bacteria associated with asymptomatic tissue.

Cross-contamination among samples and PCR reagents is a problem associated with the nested technique (17). We experienced false positives in some initial experiments and finally determined that the mineral oil was contaminated. Although we eliminated the need for mineral oil overlay by using a hot bonnet, extreme care is needed to avoid contamination and subsequent false positives. In our experiments, negative control samples were always included for each amplification round of the nested assay to check for aerosol or reagent contamination. In the field experiments, a problem encountered with the nested technique was amplification of bacterial DNA from the negative control plants. Although these plants were physically isolated from a contamination source during the experiments, they were initially obtained from fields at GCREC-Dover, where plants infected with angular leaf spot were located, and the plants may have been infested with the bacterium. Cross-contamination of samples may have occurred during preparation of plant samples from handling infected material or using nonsterile instruments. Aerosols may also have been a source of contamination of PCRs (7). However, system contamination should be detected by amplification in the negative controls. Negative controls from every PCR run were consistently negative; therefore, contamination of PCR reagents or aerosols was unlikely.

Confirmation that the PCR product is from amplification of the target DNA is possible by REA. The REA was used to identify PCR products amplified by the *hrp*-primers from cells of *X. campestris* pv. *vesicatoria* added to seed washing of tomato and pepper (11). Similarly, we applied REA to identify PCR products amplified by the XF-primers. The profiles of the restricted PCR products were distinct for each enzyme. The profiles from REA should be different for unrelated organisms, since it is highly unlikely that the same restriction sites would exist for two heterologous pieces of DNA (11).

The nested PCR technique was useful to detect bacteria on strawberry plants in nurseries in Florida. Symptoms and recovery of the rifampicin-marked strain on the medium were not useful to identify bacteria when populations were extremely low. However, recovery of the rifampicin-marked strain from some samples indicated that the bacteria were viable throughout the summer. Visible symptoms on plants disappeared soon after plants were placed in the field, and recovery of bacteria on media was difficult due to the slow-growing nature of *X. fragariae* and overgrowth by contaminants. While bacterial populations were not enumerated, the levels were deduced to be less than 10^3 CFU/ml, since bacteria were detected in the nested PCR but not by a single round of amplification. This level of detection would be useful to screen asymptomatic nursery plants to detect plants contaminated with *X. fragariae*, a concern of nursery-plant producers and regulatory agencies of plant shipment (16).

The field studies have implications regarding the disease cycle of angular leaf spot in Florida. The decline and subsequent increase in the number of positive samples through the summer indicates that populations of the bacteria declined throughout the summer and increased when more favorable conditions, especially cooler weather, occurred. The bacterial populations did not completely die. Therefore, to eliminate disease, the production of plants in nurseries in Florida must begin with plants free of the bacterium.

Researchers reported the systemic movement of *X. fragariae* in plants (5). In our research, inoculation of strawberry plants resulted in survival of bacteria on leaves and crown for extended periods under conditions not optimum for growth of the bacteria. In addition, bacteria were detected by PCR technique on daughter plants in the field. Dissemination to the daughter plants could have been due to either systemic movement through the vascular system of the runner or dispersal of bacterium by mechanical means.

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