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Ecological and genetic analysis of streptomycin-resistant *Erwinia amylovora* in Michigan and epidemiology of fire blight in an apple nursery

McManus, Patricia S., Ph.D.

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U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

ECOLOGICAL AND GENETIC ANALYSIS OF STREPTOMYCIN-RESISTANT *ERWINIA AMYLOVORA* IN MICHIGAN AND EPIDEMIOLOGY OF FIRE BLIGHT IN AN APPLE NURSERY

By

Patricia S. McManus

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ABSTRACT

ECOLOGICAL AND GENETIC ANALYSIS OF STREPTOMYCIN-RESISTANT *ERWINIA AMYLOVORA* IN MICHIGAN AND EPIDEMIOLOGY OF FIRE BLIGHT IN AN APPLE NURSERY

By

Patricia S. McManus

Fire blight, caused by *Erwinia amylovora* (Burrill) Winslow *et al.*, is the most serious bacterial disease affecting commercial production of apple (*Malus* × *domestica* Borkh.). Streptomycin-resistant strains of *E. amylovora* were isolated from 14 of 63 Michigan apple orchards surveyed during 1991 through 1993. The genes encoding streptomycin resistance, *strA* and *strB*, were usually located on DNA homologous to transposon Tn*5393* and to the self-transmissible plasmid pEa34. Ten percent of the resistant strains from one orchard in Van Buren County contained DNA homologous to Tn*5393* on the chromosome or a resident plasmid, pEA29. Plasmids homologous to pEa34 but lacking Tn*5393* were found in only three orchards. A streptomycin resistance mechanism unrelated to *strA-strB* was detected in all resistant isolates from two adjacent orchards, in 30% of the resistant isolates from a distant orchard, but not in resistant isolates from other orchards. Strains with the alternate mechanism were resistant to higher concentrations of streptomycin than strains with *strA-strB*. Streptomycin was superior to oxytetracycline in controlling bacterial populations and blight on blossoms inoculated with a streptomycin-sensitive strain, but oxytetracycline was superior to streptomycin in controlling bacterial populations and blight on blossoms inoculated with a streptomycin-resistant strain.

The roles of wind-driven rain, rain-generated aerosols, and *E. amylovora*-contaminated budwood in the incidence and spatial pattern of fire blight in a Michigan apple nursery and a simulated nursery planting at Michigan State University (MSU) were investigated. The MARYBLYT disease prediction model related several major outbreaks of fire blight to previous storms, but often erroneously predicted or failed to predict blight. Spatial lag autocorrelation analysis showed that disease was clustered either within rows or around infection foci early in the season. Following storms containing wind-driven rain, significant autocorrelations were across rows, often at high-order, noncontiguous spatial lags. Ordinary runs analysis indicated strong within-row aggregation of fire blight in the MSU plots throughout the season, whereas significant across-row aggregations were apparent following storms. All air samples collected during rain contained *E. amylovora* whereas samples collected during dry periods contained zero or very few colony forming units of the pathogen. Using traditional plating techniques, *E. amylovora* was not detected in 115 10-bud samples collected from a budwood orchard. *E. amylovora* was detected in 6 and 16% of 106 samples of washings from budsticks and leaves by plating and a polymerase chain reaction technique, respectively.

To my family, who, about three years ago, kindly quit asking,

"So, will you be finished soon?"

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INTRODUCTION, LITERATURE REVIEW AND OBJECTIVES

INTRODUCTION

Fire blight, caused by the bacterium *Erwinia amylovora*, is an extremely destructive disease affecting apple, pear and several other rosaceous plants. The pathogen can thrive in any part of a susceptible host. Afflicted plant organs show oozing of the pathogen, and eventually appear brown or black, as though charred by fire. While blossom and fruit infections reduce yield in the current season, root and stem infections are persistent and can kill a tree within a few years.

Pear trees are so susceptible to fire blight that large-scale pear production in North America is largely confined to dry regions in the West. Many popular apple cultivars are also highly susceptible, and growers in Michigan sustained losses estimated at $3.8 million following an epidemic in 1991. Despite the losses, apple production is a growing industry in Michigan. In 1992, 1,080 million pounds of apples were produced, ranking Michigan behind Washington (4,800 million pounds) and New York (1,170 million pounds) (38). Also, a large fruit tree nursery in Michigan, specializing in apple cultivars, supplies growers throughout North America. In recent years, fire blight in the nursery has resulted in significant losses financially and threatens the reputation of the business. Thus, research applicable to the prevention and control of fire blight is a high priority of apple producers in Michigan.

LITERATURE REVIEW

Fire blight was first observed in the Hudson River Valley of New York in 1780 (W. Denning, cited in 51, 66). Throughout the 1800s numerous theories arose regarding the cause of blight, including sun scald (W. Coxe, cited in 66), insects (37), and frozen sap (11, 22). In the late 1800s Burrill presented evidence (15), and Arthur proved (5) that fire blight was caused by a bacterium, now known as *E. amylovora*.

The means by which *E. amylovora* is disseminated have been debated for over a century and are still not fully understood. The most frequently cited means of dispersal supported by experimental evidence involve rain, wind, and insects. Migratory birds and international commerce involving fruit and propagation wood may also have facilitated dispersal of *E. amylovora*, especially over long distances, but experimental data substantiating such reports are lacking. The use of contaminated pruning tools is the most widely documented means by which humans spread the fire blight pathogen from tree to tree (66).

Gossard and Walton (27) provided the earliest data demonstrating the role of rain in disseminating the fire blight pathogen, supporting previous observations that blight was more severe following rain (57, 69). The role of wind was first investigated by Stevens *et al.* (56). Subsequent studies (7, 8, 9, 13, 25, 40, 47, 65) confirmed that rain, especially when accompanied by strong winds or hail, was highly effective in distributing *E. amylovora* over short distances, such as within an orchard or nursery planting. Wind-borne bacterial strands have been proposed as a means of long-distance transport of *E. amylovora* in dry conditions (8, 29, 33). Southey and Harper (55) determined that exposure to the open air was deleterious to aerosol-borne *E. amylovora*, but a fraction of

cells, deemed epidemiologically significant, remained viable after two hours. However, the role of meteorological factors in the long-distance dispersal of airborne *E. amylovora* remains conjectural.

Seventy-seven genera of insects have reportedly been involved in the spread of fire blight (66), although most were cited in single studies and are probably not of general epidemiological significance. Several studies (28, 34, 40, 47, 48) have established that honey bees are critical in moving *E. amylovora* among blossoms within orchards and perhaps over distances up to 5 km (10). The role of piercing and sucking insects such as aphids and leafhoppers in inoculating succulent terminals is controversial with compelling arguments in favor of (13, 14, 58, 59) and opposing (40, 49, 56) their involvement. Any insect that comes into contact with an oozing fire blight infection and then flies or crawls to susceptible tissue can spread *E. amylovora*, and wounds inflicted by insects serve as portals of entry for bacteria. However, insect-mediated dissemination is probably less efficient than wind-driven rain.

The relative importance of weather, insects, and cultural practices in spreading *E. amylovora* within pome fruit nurseries was considered by Stewart (57, 58) in New York and later by Bauske (7, 9) in Iowa. Stewart assigned the greatest importance to insects, especially aphids and tarnished plant bugs. An increase in blight following rain was attributed to the increase in succulent growth of terminals which attracted insects. Although he believed that *E. amylovora* had likely been introduced on contaminated budwood, he did not consider spread of the pathogen during grafting significant in the development of epiphytotics in the nursery. In contrast to Stewart, Bauske cited wind and rain as principal factors contributing to fire blight epidemics in Iowa pear nurseries (7).

The severity of disease was directly related to the degree of exposure of trees to prevailing southerly winds, and spread of blight was curtailed by the use of wind breaks. Winds of 3-6 m/s moved water-borne *E. amylovora* at least 1 m, the distance between rows of trees in nurseries. Wind also damaged foliage thereby facilitating infection, although immature tissue became infected even when protected from injury. A lesser role was ascribed to insects since chemical control of insects did not decrease the incidence of fire blight.

The sudden development of fire blight epidemics following severe storms, even in the absence of visible sources of inoculum, suggested that *E. amylovora* existed as an epiphyte on the surface of symptomless flowers and leaves of pear (41), or internally as a "resident" bacterium in stems of apple and pear (32). Subsequent studies confirmed that populations as great as 10^5-10^7 cells of *E. amylovora* per flower occurred without causing infection in pear (63, 64). Thomson (62) proposed that the moist surfaces of stigmata on apple and pear allow multiplication of *E. amylovora* even when relative humidities are low, and infection results when rain or heavy dew transports the pathogen to the hypanthium. Whether *E. amylovora* exists as an epiphyte on leaves is unclear. *E. amylovora* has been isolated from the surfaces of apparently healthy leaves, but usually only after symptoms were observed in the orchard (41, 60, 63).

Controlling *E. amylovora* has been the goal of growers and researchers since fire blight was first described two centuries ago. Early attempts to control fire blight involved pruning out infections (11, 68). Burrill (16) recommended applying linseed oil or limewater-saturated phenol to wounds to prevent infection and suggested disinfecting pruning tools. Other early topical remedies and disinfectants included corrosive sublimate

(mercuric chloride) (69), mercuric cyanide (50), zinc chloride (21) and cadmium salts (47). The first sprays used against *E. amylovora*, lead arsenate and lime sulfur (53) and later Bordeaux mixture (39), controlled fire blight but reduced tree vigor, caused russeting of fruit, and were hazardous to the applicator and to the environment.

Chemical control of fire blight was revolutionized during the 1950s with the introduction of antibiotics for use against phytobacteria. In 1951, Murneek (43) reported 58% fewer fire blight infections in Jonathan apple trees sprayed with streptomycin during bloom compared to untreated controls. Several other field studies conducted during the 1950s proved the efficacy of streptomycin (2, 35, 71), and tested the effects of timing and methods of application and the addition of adjuvants. An additive to streptomycin sprays was oxytetracycline, but the combination was not significantly more effective than streptomycin alone (3, 23, 26). Thus, oxytetracycline was eventually eliminated from the formulation. By the 1960s, streptomycin was used in commercial apple and pear orchards throughout the United States.

Besides conferring unprecedented control of fire blight, streptomycin was favored because it did not russet fruit, and residues were relatively short-lived (6). Thus, in the western United States, where fire blight was most serious on pear, 10-18 applications of streptomycin at 120-240 µg/ml were made during the long bloom period (up to 12 weeks) (42, 63) and continued until 30 days before harvest (6, 51). By contrast, in the central and eastern states, apple was the predominant fire blight host grown, and fewer applications of streptomycin were made due to the relatively short bloom period (1-2 weeks).

In 1971, streptomycin failed to control fire blight in some California pear orchards

even when the application rate and frequency were doubled (42). Strains of *E. amylovora* resistant to streptomycin at 200 μg/ml, the highest level tested, were isolated from these orchards (41). By 1973, streptomycin-resistant strains of *E. amylovora* were recovered from areas encompassing half of California's pear-growing acreage (42). Resistance was presumed to be caused by a single-step chromosomal mutation, since strains tolerated very high levels of streptomycin (>1000 μg/ml) (52).

A few years after the discovery of resistance in California, streptomycin-resistant strains of *E. amylovora* were recovered from 35 pear orchards in Washington and Oregon (20). Use of oxytetracycline and copper to control fire blight was resumed in the Western states during the 1970s in orchards harboring streptomycin-resistant *E. amylovora*. A recent survey of pear orchards in Washington showed that while streptomycin-resistant strains of *E. amylovora* were ubiquitous, none of the isolates was resistant to oxytetracycline or copper (36). Surveys of apple orchards in Michigan (60) and apple and pear orchards in New York (12) during the mid-1970s revealed no resistant strains of *E. amylovora*, but in the early 1980s resistant strains were discovered in Missouri (54).

Severe fire blight, despite timely application of streptomycin in a large commercial orchard in Van Buren county in 1990, prompted a reassessment of the resistance situation in Michigan. Streptomycin-resistant isolates were recovered from this orchard (17). Subsequent studies showed that the resistance genes, *strA* and *strB*, were located on a transposon, Tn*5393*, which was inserted on the self-transmissible plasmid pEa34 (18). The resistance genes encode two streptomycin phosphotransferases (19), enzymes that inactivate streptomycin by phosphorylation. In 1991, resistant isolates of *E. amylovora* were detected in orchards surrounding the original site of resistance in Van Buren county

and in two adjacent orchards in Kent county 94 km north northeast of the original site (A. L. Jones, unpublished data). The mechanism of resistance in the isolates from Kent county was apparently unrelated to *strA-strB* based on DNA homology tests.

In 1991, oxytetracycline was registered for use on apples and pears in Michigan in areas where the presence of streptomycin-resistant *E. amylovora* was confirmed. The efficacy of oxytetracycline against streptomycin-resistant strains of *E. amylovora* is not known; however, oxytetracycline is less effective than streptomycin in controlling blight incited by streptomycin-sensitive strains (26, 46, 71).

Control of fire blight through the use of resistant cultivars and rootstocks has been advocated (1, 31, 61), and may become crucial with the emergence of bactericide resistance and as the perceived health and environmental risks associated with the use of chemical bactericides increase. While conventional breeding methods established in the early 1900s are still commonly used (44), recently apple was transformed with insect genes that encode anti-bacterial lytic enzymes (45). In greenhouse trials, the transgenic apple plants were significantly more resistant to fire blight than their nontransformed counterparts.

Today fire blight control is best achieved by integrating sanitation, the use of resistant cultivars, and the judicious and timely application of bactericides. Epiphytic microorganisms antagonistic to *E. amylovora* have long been recognized (4, 24, 47) and are being exploited as potential means of biological control (30, 67, 70). Though some bacterial formulations are available commercially, biological control of fire blight is not widely practiced, in part because the organisms have not been as effective as chemical bactericides in combatting *E. amylovora*. However, with the emergence of streptomycin-

resistant strains and the limited arsenal of chemical bactericides available to growers, the pursuit of alternate means of control will likely occupy researchers seeking a solution to fire blight during the next several years.

OBJECTIVES

The objectives of the research presented in this dissertation were to: (1) identify Michigan apple orchards containing streptomycin-resistant *E. amylovora*; (2) identify the genetic determinants of streptomycin resistance in strains from these orchards; (3) compare the efficacy of streptomycin and oxytetracycline in restricting multiplication of *E. amylovora* on stigmata of apple blossoms and in controlling blossom blight; and (4) identify factors involved in the introduction and spread of fire blight at an apple nursery in Michigan.

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PART I

ECOLOGICAL AND GENETIC ANALYSIS OF STREPTOMYCIN-RESISTANT *ERWINIA AMYLOVORA* AND EVALUATION OF OXYTETRACYCLINE FOR CONTROL

INTRODUCTION

Fire blight, caused by *Erwinia amylovora*, is the most devastating bacterial disease affecting apple and pear trees. Streptomycin was first used to control phytopathogenic bacteria, including *E. amylovora*, in the late 1950s. After nearly two decades of intense use, strains of *E. amylovora* resistant to streptomycin were discovered in pear orchards in California, Oregon, and Washington in the early 1970s (6, 18), and in apple orchards in Missouri in 1983 (27). Surveys conducted in New York and Michigan in the 1970s did not reveal streptomycin-resistant *E. amylovora* (1, 31), but in 1990, streptomycin-resistant strains of *E. amylovora* were detected at one orchard in southwestern Michigan (4). Resistance at this orchard was mediated by aminoglycoside phosphotransferase enzymes encoded by *strA* and *strB*, genes carried by transposon Tn*5393* which was inserted on the conjugative plasmid pEa34 (3, 4, 5). The genetic determinants of streptomycin resistance in strains from the western United States are apparently unrelated to *strA* and *strB* (4, 19, 25).

Streptomycin is the preferred bactericide for controlling fire blight, except in areas where streptomycin-resistant strains have emerged. Oxytetracycline has been used since the late 1970s to control fire blight in pear orchards in the western United States (20), and was recently approved by the Environmental Protection Agency (EPA) for use in Michigan in counties or townships where streptomycin resistance has been confirmed. Antibiotic sprays are recommended during bloom since, under favorable environmental conditions, *E. amylovora* multiplies rapidly on stigmata before infecting flowers (32). Although restricting multiplication of *E. amylovora* during bloom is pivotal in preventing blossom blight, the relative effectiveness of streptomycin and oxytetracycline for the

control of blossom blight incited by streptomycin-resistant strains has not been demonstrated.

In this report, Michigan apple orchards containing streptomycin-resistant strains of *E. amylovora,* and the genetic determinants of streptomycin resistance, are identified. In addition, the efficacy of streptomycin and oxytetracycline in restricting multiplication of *E. amylovora* on stigmata of apple blossoms, and in controlling blossom blight, is compared.

MATERIALS AND METHODS

Bacterial strains. Streptomycin-resistant strain Ea88 of *E. amylovora*, isolated from a pear orchard in Washington, was provided by R. G. Roberts, Tree Fruit Research Laboratory, Wenatchee, WA. Resistant strain CA11 was isolated from a crabapple orchard in Van Buren county, MI (4). Resistance in CAll is mediated by streptomycin resistance genes *strA* and *strB* carried by transposon Tn*5393* inserted on the self-transmissible plasmid pEa34 (4, 5). All other strains of *E. amylovora* and streptomycin-resistant strain BC9 of *E. herbicola* were isolated from apple orchards in Michigan.

Orchard survey. Sixty-three orchards in 12 counties throughout the major apple-producing regions of Michigan were sampled for streptomycin-resistant *E. amylovora* during June and July of 1991-1993 (Figure 1); however, not all orchards were sampled every year. Only orchards which had been sprayed with streptomycin were sampled. If resistant strains were detected in an orchard, then neighboring orchards were sampled. A blighted shoot, spur or fruit comprised one sample, and approximately 25 samples were tested per orchard. Samples were transported to the laboratory in a cooler and processed within 24 h.

Isolation and identification of *E. amylovora*. Bacterial ooze or small pieces of tissue from infected shoots, spurs and fruits were streaked in parallel onto King's medium B (KB) (12) supplemented with cycloheximide at 50 µg/ml (KBc) and KBc supplemented with streptomycin sulfate at 100 µg/ml (KBsc). Plates were incubated at 28 C for 2-3 days. Non-fluorescent, cream-colored colonies typical of *E. amylovora* were transferred to either CG or CCT, the differential media of Crosse and Goodman (7) and Ishimaru and Klos (9), respectively. Single colonies characteristic of *E. amylovora* on the differential media were transferred to KBc and KBsc to confirm their phenotype. Pathogenicity was determined by inoculating immature pear fruit. The identity of each isolate collected in 1992 and 1993 was confirmed with an *E. amylovora*-specific DNA probe (8) (see Colony hybridizations, below).

Bacteria for population and blossom blight studies. A spontaneous mutant of streptomycin-sensitive strain G11 of *E. amylovora* resistant to nalidixic acid at 100 µg/ml (G11nal^r) was mated with streptomycin-resistant strain CA11 as described previously (4). Transconjugant strain G11nal^r(pEa34), which was genetically identical to G11nal^r except for the presence of pEa34, was used as the streptomycin-resistant strain in laboratory studies. Inoculum was prepared by suspending in sterile distilled water 48-h-old bacterial colonies from plates of KB amended with the appropriate antibiotics. The suspensions were adjusted to an optical density of 0.14 at 640 nm which corresponded to 2-4 $\times$ 10^8 colony forming units (cfu) per milliliter.

Effect of antibiotics on populations of *E. amylovora* on stigmata and blossom blight incidence. In the population study, newly-opened blossoms were cut at the peduncle from 2-yr-old potted apple trees (cultivar Gala, highly susceptible to fire blight)

in the greenhouse. The peduncle of each flower was inserted through a hole in the cap of a sterile, disposable 5-ml plastic culture tube filled with sterile distilled water. The tubes with blossoms were placed in test-tube racks. A micropipette was used to deliver 3 µl of a suspension of G11nal^r to the stigmata of 90 flowers; 90 flowers were similarly inoculated with a suspension of G11nal^r(pEa34). Thirty flowers from each inoculation group were then sprayed until run-off with streptomycin at 100 µg/ml (Agrimycin at 0.6 g/L), oxytetracycline at 200 µg/ml (Mycoshield at 1.2g/L), or water. These concentrations were the rates recommended on product labels for field application. Immediately after spraying and at 12-h intervals up to 60 h, the petals were removed from five replicate flowers of each treatment, and the remaining flower parts were macerated in 1 ml sterile distilled water in a microcentrifuge tube. The homogenates were serially diluted, and 0.1 ml aliquots of the appropriate dilutions were plated onto KBc supplemented with nalidixic acid at 25 µg/ml or KBsc supplemented with nalidixic acid for flowers inoculated with G11nal^r or G11nal^r(pEa34), respectively. Plates were incubated at 28 C for 2-3 days and colonies were counted. Immediately after the initial sampling, the blossoms were enclosed in clear, plastic bags to maintain high humidities and were incubated at room temperature. This experiment was performed twice. To determine whether *E. amylovora* or other bacteria existed on uninoculated flowers from trees in the greenhouse, 10 fresh blossoms were macerated, and the homogenates diluted and plated onto CGc or KBc.

In the blossom blight study, fruit spurs with newly-opened blossoms were collected from apple trees (cultivar Jonathan, highly susceptible to fire blight) at the Botany and Plant Pathology Farm of Michigan State University at East Lansing. In the laboratory, the stems of the spurs were submerged in water in 120-ml baby-food jars so

that each jar contained 16-22 blossoms. The blossoms were sprayed until run-off with 0.6 g iprodione plus 0.6 g benomyl per liter of water to inhibit fungi. After the blossoms dried, they were inoculated with either G11nal^r or G11nal^r(pEa34), or mock-inoculated with water by dipping a cotton swab into a suspension of the appropriate bacterial strain or water and touching the swab to the stigmata. Immediately after inoculation, blossoms in five jars from each of the three inoculation groups were sprayed until run-off with streptomycin (100 µg/ml), oxytetracycline (200 µg/ml), or water. The jars were randomized and incubated in a dew chamber at 28 C with 12 h of light per day provided by fluorescent bulbs. After 7 days, flowers were observed for symptoms of blossom blight. This experiment was performed twice.

Colony hybridizations. Putative isolates of *E. amylovora* collected in 1992 and 1993 were spotted onto nylon membranes (Amersham, Arlington Heights, IL) which had incubated for 20 h on the surface of KBc. After incubation at 28 C for 20-30 h, colonies were lysed and the membranes neutralized (10). DNA was fixed to the membranes by baking at 80 C for 2 h. A 5-kb *Sal*I fragment (probe 5SUP) of pEA29, the 29-kb plasmid ubiquitous in and unique to *E. amylovora* (8, 15), was purified by electrophoresis onto DEAE-cellulose paper (26) and labeled with digoxigenin-11-dUTP by the randomized oligonucleotide labeling method using the Genius DNA Labeling and Detection kit (Boehriger Mannheim Corp., Indianapolis, IN). Prehybridization, hybridization, and colorimetric or chemiluminescent detection of the probe were performed according to the manufacturer's instructions. A digoxigenin-labeled 0.5-kb *Bam*HI-*Ava*I internal fragment of Tn*5393*, containing a portion of *strA* (6) and designated probe SMP3 (21), was used to identify strains with *strA-strB*. The presence of pEa34, either with or without Tn*5393*,

was detected with a digoxigenin-labeled 26-kb *Sma*I fragment of pEa34 (probe 26-Ea34). Plasmid and total genomic DNA from streptomycin-resistant isolates with DNA homologous to SMP3 but not to 26-Ea34 in colony hybridization was subjected to Southern analyses.

Isolation and Southern analysis of DNA. Indigenous plasmids of *E. amylovora* were isolated using the Magic Minipreps DNA Purification System (Promega, Madison, WI) and resolved on agarose gels (0.5% w/v) run at 5 V/cm in TAE electrophoresis buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). Total genomic DNA was recovered by a miniprep procedure (35). Plasmid DNA for restriction analysis was purified by cesium chloride centrifugation (17). Southern analyses of intact plasmid DNA, restriction enzyme-digested plasmid DNA, and *Ava*I-digested total genomic DNA were done by standard procedures (17). Probe SMP3 was used to identify the 2.7-kb internal *Ava*I fragment indicative of Tn*5393* in *Ava*I-digested plasmid and total genomic DNA (4, 5). Membranes used for multiple probings were stripped by gentle shaking in boiling 0.5% sodium dodecyl sulfate (SDS) until the SDS reached room temperature and then rinsing briefly in distilled water. The insertion site of Tn*5393* into pEA29 was mapped by digesting pEA29::Tn*5393* with *Bgl*II, *Cla*I, *Hin*dIII, *Kpn*I, *Sal*I, and *Pst*I, probing Southern blots with SMP3, and comparing the results to published restriction maps of pEA29 (8, 15).

Determination of minimum inhibitory concentration (MIC). MICs were determined by the agar dilution method (14). Bacteria were cultured overnight in LB broth (17) at 25 C with shaking and diluted 100-fold in sterile distilled water to ~10^7 cfu/ml. Three microliters of the diluted bacterial suspensions were spotted onto duplicate

plates of KB amended with streptomycin sulfate at 0, 10, 50, 100, 500, 750, 1000, or 2000 µg/ml. The MIC was the lowest concentration of streptomycin sulfate that inhibited bacterial growth in the spots after 24 h incubation at 28 C.

RESULTS

Orchard survey. In 1991, resistant strains of *E. amylovora* were recovered from orchard V-A, the site where resistance was originally discovered in Michigan in 1990 (4), and from orchards V-B, V-C, and V-D, all within 2 km of V-A in Van Buren county (Figure 1, Table 1). Also in 1991, resistance was detected at two adjacent orchards, K-A and K-B, in Kent county at a site 94 km north northeast of V-A. In 1992, streptomycin-resistant *E. amylovora* were found in six Van Buren county orchards, V-B, V-C, V-D, V-E, V-F, and V-G, but not in V-A, the original site of resistance. Orchards K-A and K-B and two Newaygo county orchards, N-A and N-B, 132 km north of V-A, also harbored resistant strains in 1992. By 1993, streptomycin-resistant isolates were detected at orchards V-A, V-B, V-C, V-D, V-E, V-F, V-G, V-H, and V-I in Van Buren county; K-A and K-B in Kent county; and N-A, N-B, and N-C in Newaygo county. The frequency of streptomycin resistance (number of resistant samples/total number of samples × 100) ranged from 0 to 100% (Table 1). Resistant isolates of *E. amylovora* were detected at 14 of the 63 orchards surveyed (Figure 1A).

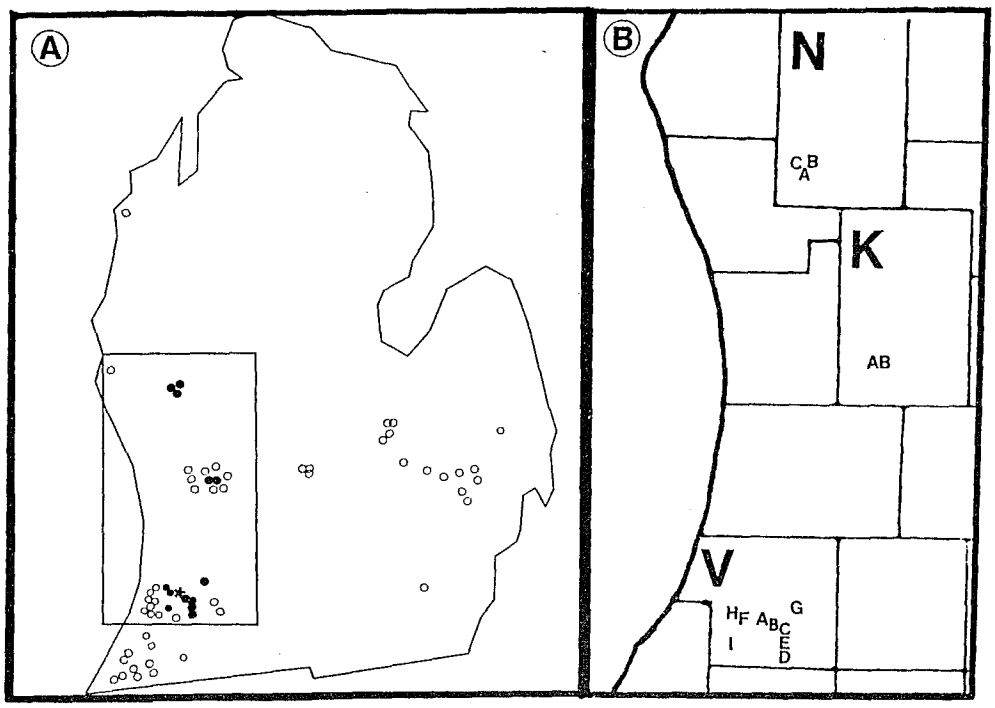

Figure 1. Distribution of Michigan apple orchards surveyed for streptomycin-resistant *Erwinia amylovora*, 1991-1993. **A,** Orchards from which only sensitive strains were isolated (o), orchards harboring resistant strains (•), and orchard where resistance was first discovered in 1990 (*)(4); boxed region is projected in **B.** **B,** orchards harboring resistant strains in Kent (K), Newaygo (N) and Van Buren (V) counties. Orchard V-A is the original site of resistance.

Table 1. Distribution and frequency of streptomycin-resistant (Sm^r) *Erwinia amylovora* in Michigan apple orchards relative to antibiotic spray history[a]

		Frequency of Sm^r samples[b] (Total no.samples) Spray history[c]		
County	Orchard	1991	1992	1993
Kent	K-A[d]	>0 (≥6)[e]	13 (48) M, C	4(49) M, C
	K-B[d]	>0 (≥6)[e] S:7	17 (18) M:4	0 (1)[f] M:2
Newaygo	N-A	---[g] S:5, C:1	100 (24) S:2, C:1	48 (29) M:3, C:1
	N-B	--- S:5, C:1	21 (28) S:2, C:1	0 (21) M:3, C:1
	N-C	---	---	43 (23) M
Van Buren	V-A	9 (11)	0 (28) M	9 (47) M
	V-B	64 (11)	44 (27) M	82 (62) S:4
	V-C	36 (11)	27 (37) M	68 (22) S:3
	V-D	50 (4)	50 (8) S:3	38 (16)
	V-E	---	41 (22) M:3	62 (13)
	V-F	---	100 (7) S	85 (39)
	V-G[d]	--- S:8	18 (45) S:3, M:4	6 (32) M:3
	V-H	---	---	84 (25) S
	V-I	---	---	92 (24) S

Table 1 (cont'd)

[a]Sixty-three orchards in 12 counties were sampled for streptomycin-resistant *E. amylovora*. Resistance was detected in three counties; data only for orchards where resistance was detected are presented. Streptomycin-resistant isolates from each orchard contained DNA homologous to probe SMP3 unless otherwise noted.

[b]Frequency (%) = number of streptomycin-resistant samples/total number of samples × 100.

[c]Spray history data were based on personal communication with growers. S = streptomycin, M = oxytetracycline, C = copper; if blank then no information regarding bactericide use was available. Numbers after a bactericide indicate the number of applications; no number indicates that the number of applications was unknown.

[d]Probe SMP3 hybridized to DNA from 0% of the streptomycin-resistant isolates from orchards K-A and K-B, and to 70% of the resistant isolates from orchard V-G.

[e]Six streptomycin-resistant samples were isolated; the total number of samples is unknown.

[f]Trees yielding streptomycin-resistant isolates were removed the previous year.

[g]---indicates that the orchard was not sampled during that year.

Effect of antibiotics on populations of *E. amylovora* on stigmata and blossom blight incidence. Initial populations of streptomycin-sensitive G11nal^r and streptomycin-resistant G11nal^r(pEa34) were ~5.0 × 10^5 cfu/flower for all treatments (Figure 2). After 12 h, populations of G11nal^r and G11nal^r(pEa34) on blossoms sprayed with water, and of G11nal^r(pEa34) on blossoms sprayed with streptomycin, increased to 3.2 × 10^6 - 2.1 × 10^7 cfu/flower; populations on these blossoms peaked at ~6.0 × 10^7 cfu/flower by 36 h (Figure 2). Populations of G11nal^r on blossoms sprayed with streptomycin decreased significantly by 48 h and continued to decline thereafter. Populations of G11nal^r and G11nal^r(pEa34) on blossoms sprayed with oxytetracycline were similar to each other and did not fluctuate significantly throughout the experiment. However, 12 h after inoculation, bacterial populations on oxytetracycline-treated blossoms were significantly lower than populations on water-treated controls. Similar results were obtained in a replicate experiment (data not shown). No *E. amylovora* were isolated from uninoculated blossoms from the greenhouse, and the number of unidentified bacteria ranged from 0-7 cfu/ml.

One-way analysis of variance of the blossom blight data indicated that the coefficients of variablility for the two experiments were low and similar. Also, the F test between the error mean squares of the two runs showed that the error variances of the two runs were not significantly different ($P > 0.05$), indicating that the data from the two experiments could be pooled for further statistical analysis. Blossom blight incidence was >90% when stigmata were inoculated with streptomycin-sensitive G11nal^r or streptomycin-resistant G11nal^r(pEa34) and then sprayed with water (Figure 3). The incidence of blight on blossoms inoculated with G11nal^r and then sprayed with

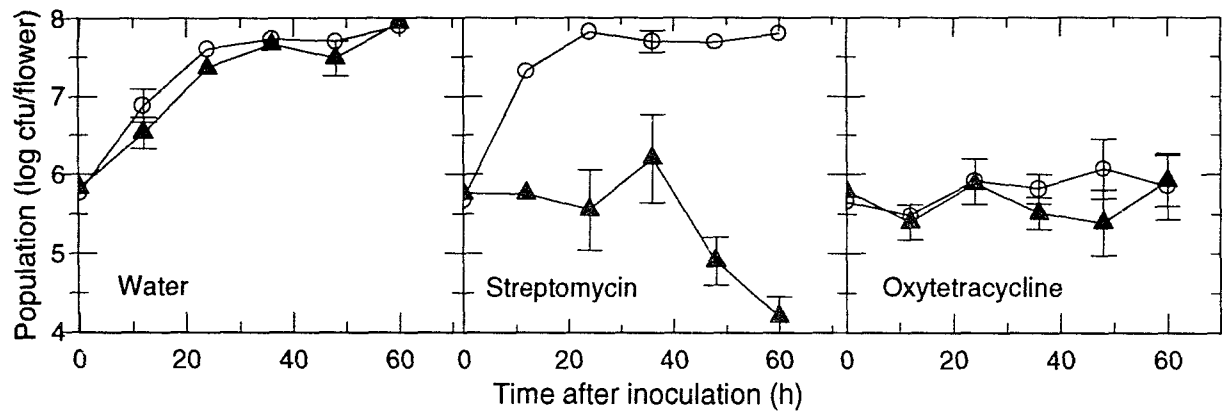

Figure 2. Colonization of detached flowers of apple by streptomycin-sensitive *Erwinia amylovora* strain G11nal^r (▲) or streptomycin-resistant *E. amylovora* strain G11nal^r(pEa34) (○), after inoculation of stigmata and spraying with water, streptomycin (100 µg/ml), or oxytetracycline (200 µg/ml). Bars represent two standard errors of the mean.

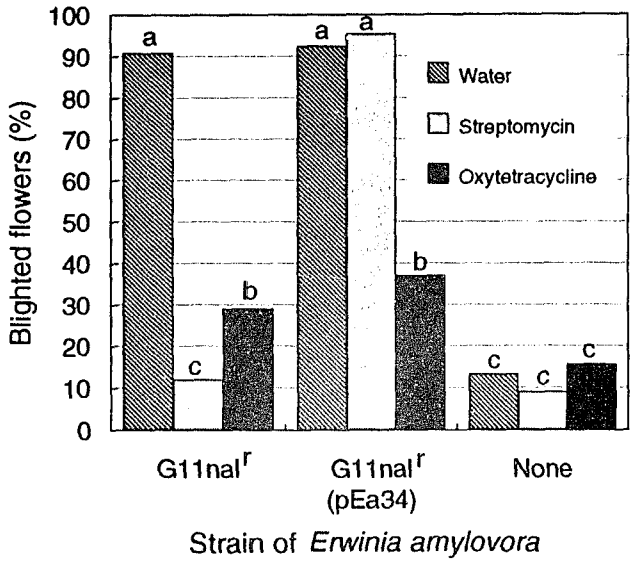

Figure 3. Incidence of blossom blight in apple flowers 7 days after inoculation with strain G11nal^r (streptomycin-sensitive) or G11nal^r(pEa34) (streptomycin-resistant) of *Erwinia amylovora*, or mock-inoculation with water, and spraying with water, streptomycin (100 µg/ml), or oxytetracycline (200 µg/ml). Letters above the bars denote significant differences (P = 0.05) between the means by Least Significant Difference Analysis, LSD = 8.9.

streptomycin was 12%; this level of blight did not differ significantly from that of uninoculated blossoms. Blight incidence was 96% on streptomycin-sprayed blossoms and 93% on water-sprayed blossoms inoculated with G11nal^r(pEa34). The incidence of blight on oxytetracycline-sprayed blossoms inoculated with G11nal^r or G11nal^r(pEa34) was similar (29 and 37%, respectively), but significantly higher than the incidence of blight on uninoculated control blossoms, and significantly lower than the incidence on water-sprayed inoculated blossoms. None of 20 blighted blossoms from the uninoculated control group yielded nalidixic acid-resistant strains of *E. amylovora*, indicating that the blight resulted from epiphytic *E. amylovora* acquired in the field.

Colony hybridizations, plasmid profiles and Southern analyses. Probe SMP3 hybridized to DNA from 0, 100, and 98.6% and probe 26-Ea34 hybridized to DNA from 0, 100, and 95.3% of the streptomycin-resistant strains of *E. amylovora* collected in 1992 and 1993 from orchards in Kent, Newaygo, and Van Buren counties, respectively. Ten percent of the resistant strains from orchard V-B contained DNA homologous to SMP3 but not to 26-Ea34, accounting for 3.3% of all resistant strains from Van Buren county. Thirty percent of the resistant strains from orchard V-G contained DNA that was not homologous to SMP3, accounting for 1.4% of all resistant strains from Van Buren county. None of the streptomycin-sensitive isolates contained DNA that hybridized to probe SMP3, while 11, 39, and 91% of the streptomycin-sensitive strains from three orchards in central Michigan, but none from the remaining 60 orchards, contained strains with DNA that hybridized to probe 26-Ea34.

Southern analysis of plasmid preparations of streptomycin-resistant and -sensitive strains of *E. amylovora* from various locations showed that each strain

contained a plasmid homologous to probe 5SUP, while the plasmid of streptomycin-resistant strain BC9 of *E. herbicola* did not hybridize to 5SUP (Figure 4). Streptomycin-resistant *E. amylovora* strains CA11, H6b, and M3a, and *E. herbicola* strain BC9 from Van Buren county, and streptomycin-resistant *E. amylovora* strain RN2b from Newaygo county, each had a 34-kb plasmid that hybridized to probes SMP3 and 26-Ea34. Streptomycin-resistant strains BCN12, BCN16, BCN20, and BCN87, which showed DNA homology to probe SMP3 but not to probe 26-Ea34 in colony hybridizations, each lacked a 29- and 34-kb plasmid but contained a 36-kb plasmid homologous to both SMP3 and 5SUP. Streptomycin-resistant strains BCN74, BCN75, and BCN77 each contained a 29-kb plasmid which hybridized only to probe 5SUP, although in colony hybridization, DNA from these strains showed homology to probe SMP3. Plasmid DNA of streptomycin-resistant strains BB8 and Ea88 from Kent county and Washington, respectively, and of streptomycin-sensitive strains BCN27, PW23, and G11 did not hybridize to either SMP3 or 26-Ea34.

Probe SMP3 hybridized to a 2.7-kb fragment in *Ava*I-digested plasmid and total genomic DNA from strains of *E. amylovora* or *E. herbicola* which contained a plasmid homologous to probe SMP3 (Figure 5, Table 2). A weak hybridization signal was detected in *Ava*I-digested total genomic but not in plasmid DNA from *E. amylovora* strains BCN74, BCN75, and BCN77, indicating that Tn*5393* was inserted on chromosomal DNA in these strains. Probe SMP3 did not hybridize to *Ava*I-digested DNA from streptomycin-sensitive strain G11. Probe SMP3 hybridized to a 2.7-kb *Ava*I fragment of the 36-kb plasmid in strain BCN87 (Figure 5, Table 2) and strains BCN12, BCN16, and BCN20 (Table 2), demonstrating that pEA29 had acquired Tn*5393* to form

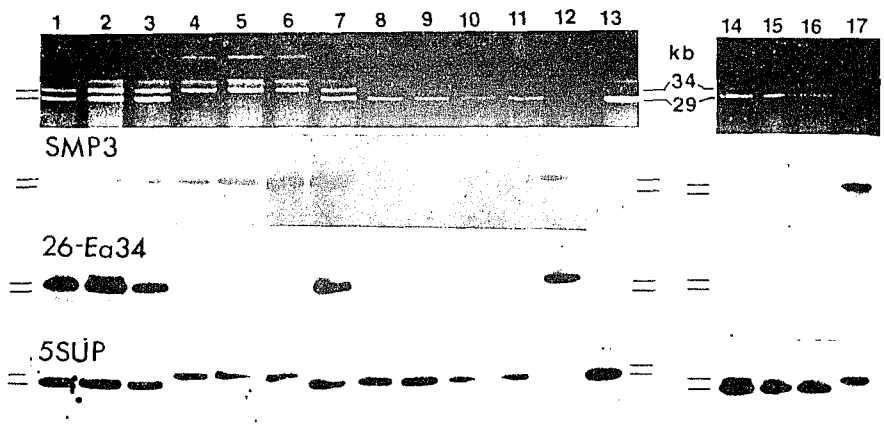

Figure 4. Plasmid profiles and Southern analyses of streptomycin-resistant (Sm^r) and streptomycin-sensitive (Sm^s) strains of *Erwinia amylovora* (all lanes except lane 12) and a Sm^r strain of *E. herbicola* (lane 12). Lane 1, Sm^r CA11; lane 2, Sm^r H6b; lane 3, Sm^r M3a; lane 4, Sm^r BCN12; lane 5, Sm^r BCN16; lane 6, Sm^r BCN20; lane 7, Sm^r RN2b; lane 8, Sm^r BB8; lane 9, Sm^s BCN27; lane 10, Sm^s PW23; lane 11, Sm^s G11; lane 12, Sm^r BC9; lane 13, Sm^r Ea88; lane 14, Sm^r BCN74; lane 15, Sm^r BCN75; lane 16, Sm^r BCN77; and lane 17, Sm^r BCN87. The gel with lanes 14-17 included plasmids of strains CA11 and G11 as controls. The gels were blotted to nylon membranes and sequentially hybridized with digoxigenin-11-dUTP-labeled probes SMP3, 26-Ea34 and 5SUP. The positions of 29-kb and 34-kb plasmids are marked in all panels.

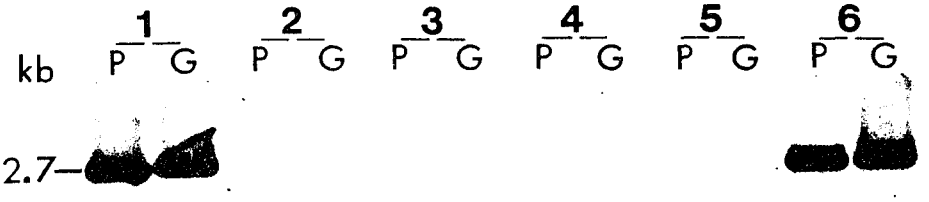

Figure 5. Hybridization of *Ava*I-digested plasmid (P) and total genomic (G) DNA of streptomycin-resistant (Sm^r) and streptomycin-sensitive (Sm^s) strains of *Erwinia amylovora*. 1, Sm^r CA11; 2, Sm^s G11; 3, Sm^r BCN74; 4, Sm^r BCN75; 5, Sm^r BCN77; and 6, Sm^r BCN87. The 2.7-kb internal *Ava*I restriction fragment of Tn*5393* (4, 5) is marked.

Table 2. Miniumum inhibitory concentrations of streptomycin (MIC)[a] and detection of Tn*5393*[b]

Species Strain	MIC (µg/ml)	Orchard of origin[c]	Location of Tn5393[b] Plasmid DNA	Total DNA
Erwinia amylovora				
CAll	750	V-A	+	+
H6b	750	V-F	+	+
M3a	750	V-D	+	+
RN2b	750	N-B	+	+
BCN12	750	V-B	+	+
BCN16	750	V-B	+	+
BCN20	750	V-B	+	+
BCN87	750	V-B	+	+
BCN74	500	V-B	-	+
BCN75	500	V-B	-	+
BCN77	500	V-B	-	+
BB8	>2000	K-B	-	-
Ea88	>2000	--[d]	-	-
BCN27	10	V-B	-	-
PW23	10	--[d]	-	-
G11	10	--[d]	-	-
Erwinia herbicola				
BC9	750	V-B	+	+

[a]MICs were determined by spotting 3 µl of an ~10^7 cfu/ml suspension of each strain onto KB medium amended with 0-2000 µg/ml streptomycin sulfate.

[b]Hybridization of probe SMP3 to a single 2.7-kb internal *Ava*I restriction fragment was indicative of Tn*5393* (4, 5).

[c]All orchards contained streptomycin-resistant *E. amylovora* and were located in Kent, Newaygo or Van Buren counties (Figure 1) unless otherwise noted.

[d]Ea88 was from Washington state; PW23 was from an orchard in southeastern Michigan lacking streptomycin-resistant *E. amylovora*; G11 was from an orchard in Van Buren county lacking streptomycin-resistant *E. amylovora*.

the 36-kb plasmid pEA29::Tn*5393*. Restriction digests and Southern analysis of pEA29::Tn*5393* from strains BCN12, BCN16, BCN20, and BCN87 showed that the transposon was inserted into a region overlapped by 1.4-kb *Bgl*II and 1.6-kb *Hin*dIII fragments at approximately 27 kb from the previously designated *Bam*HI origin of pEA29 (8, 15) (data not shown).

MICs. Streptomycin-resistant strains of *E. amylovora* lacking DNA homologous to probe SMP3 had MICs >2000 µg/ml streptomycin (Table 2). Bacteria containing plasmid DNA which hybridized to SMP3 had MICs of 750 µg/ml streptomycin, whereas strains with total genomic but not plasmid DNA homologous to SMP3 had MICs of 500 µg/ml streptomycin. Sensitive strains of *E. amylovora* were inhibited by 10 µg/ml streptomycin.

DISCUSSION

Streptomycin-resistant strains of *E. amylovora* were isolated from 22% of 63 Michigan apple orchards surveyed during 1991-1993; the majority of orchards contained only streptomycin-sensitive strains (Figure 1A). By contrast, Loper *et al.* (16) reported streptomycin-resistant strains in 86% of the orchards throughout the major pear-growing regions in Washington. It is likely that streptomycin resistance emerged earlier and is more widespread in pear orchards in the West than in apple orchards in Michigan because of more intense use of streptomycin in the West (24, 33).

Beginning in 1991, EPA regulations permitted growers in Van Buren county to use oxytetracycline for fire blight control. Growers used oxytetracycline in some orchards where resistance was confirmed, but were reluctant to use it rather than streptomycin in nearby orchards where resistance had not been confirmed. Streptomycin-resistant *E.*

amylovora continued to increase and spread in the county as the number of orchards with resistant strains increased from one (out of 20 orchards sampled) in 1990 (4) to nine in 1993. Moreover, the frequency of resistance was high at several Van Buren county orchards where streptomycin was applied in the presence of resistant strains (Table 1). In particular, at orchards V-B and V-C the frequencies of resistance declined from 1991 to 1992 when oxytetracycline rather than streptomycin was used, but populations of streptomycin-resistant *E. amylovora* quickly rebounded in 1993 when streptomycin use was resumed. Previously, Stall and Thayer (28) reported a rapid resurgence by streptomycin-resistant *Xanthomonas campestris* pv. *vesicatoria* following repeated application of the antibiotic in field studies on tomato in Florida. Thus, our data and those of Stall and Thayer underscore the importance of avoiding streptomycin as the primary bactericide at sites where streptomycin resistance has been confirmed. The proliferation of streptomycin-resistant *E. amylovora* in Van Buren county illustrates the impossibility of containing resistance in areas where growers are unwilling to adopt alternate control strategies.

Streptomycin was superior to oxytetracycline in controlling colonization of stigmata by a streptomycin-sensitive strain of *E. amylovora*, but oxytetracycline was superior in controlling colonization by a streptomycin-resistant strain (Figure 2). These data are consistent with the conclusion that the tetracycline derivatives are bacteriostatic, whereas streptomycin is bacteriocidal (17). The bacteriocidal activity of streptomycin versus the bacteriostatic activity of oxytetracycline may explain why streptomycin was more effective than oxytetracycline in reducing blossom blight incited by a sensitive strain in our laboratory experiments (Figure 3), and in field studies in New York (22). The

failure of streptomycin and success of oxytetracycline in inhibiting multiplication of and infection by G11nal^r(pEa34) illustrate the futility of attempting to combat streptomycin-resistant *E. amylovora* with streptomycin, and the value of oxytetracycline for controlling resistant strains.

The clustering of orchards harboring streptomycin-resistant *E. amylovora* within each county, the finding that strains within a county shared a common resistance mechanism, and the geographic separation of the three regions with resistant strains, indicate that resistance probably arose independently in each county. Certainly this was the case in Kent county, where a streptomycin resistance mechanism unrelated to *strA-strB* was detected. The alternate resistance mechanism detected in Michigan may be similar to the resistance mechanism in strains from the western United States, since strains from both locations had MICs >2000 µg/ml (Table 2). The possibility of intercounty dispersal of *E. amylovora* with *strA-strB* on pEa34 mediated by humans, or introduction of the pathogen from a common source such as a nursery, cannot be eliminated. However, these scenarios are unlikely since orchards in Newaygo county were managed independently of orchards in Van Buren county, and resistance would be ubiquitous in Michigan if nursery stock were contaminated with *E. amylovora* containing *strA-strB*.

Several lines of evidence suggest that plasmid-mediated streptomycin resistance originated from the transfer of pEa34 to *E. amylovora*, probably from the ecologically associated bacterium *E. herbicola*. Some streptomycin-resistant strains of *E. herbicola* contain pEa34 (Figures 4, reference 5), and conjugal transfer of pEa34 from *E. herbicola* to *E. amylovora* and *E. coli* has been demonstrated in laboratory mating studies (5).

However, pEa34 is not ubiquitous among bacteria associated with apple trees since DNA from 142 epiphytic gram-negative bacteria with *strA-strB* did not hybridize to probe 26-Ea34 (5). It is likely that pEa34 and not just Tn*5393* was transferred to *E. amylovora*, because streptomycin-sensitive strains of *E. amylovora* containing a plasmid homologous to probe 26-Ea34 are rare; they were found only in three adjacent orchards in central Michigan and have not been reported in populations of *E. amylovora* originating outside Michigan (29). Thus, pEa34 moved into *E. amylovora* relatively recently, and our data pertain to streptomycin resistance during its early stages in Michigan.

While pEa34 is rare in gram-negative bacteria from apple orchards, Tn*5393* is widespread among streptomycin-resistant gram-negative bacteria in Michigan (4). In New York, DNA from streptomycin-resistant *Pseudomonas syringae* pv. *papulans* and strains of *Pseudomonas* spp. and unidentified yellow bacteria associated with fire blight infections hybridized to probe SMP3, but no resistant *E. amylovora* strains were detected (2, 21). Minsavage *et al.* (19) identified plasmid-borne streptomycin-resistance genes in *X. c. vesicatoria* that were related to those of *P. s. papulans* strain Psp36 and speculated that a transposon was involved in the insertion of these genes on the chromosome of *X. c. vesicatoria* strain 87-77 from Ohio. Sundin and Bender (30) proposed that transposition might account for the presence of *strA-strB* homologs on plasmids of various sizes in *P. s. syringae* from Oklahoma. Thus, *strA-strB*, and possibly Tn*5393*, are widely dispersed among phytobacteria in the United States.

Strains containing pEA29::Tn*5393* or containing Tn*5393* inserted on the chromosome lacked pEa34, implying that after transposition of Tn*5393*, pEa34 was lost in subsequent generations. Alternatively, a plasmid other than pEa34 might have been

transferred to *E. amylovora* and delivered Tn*5393*, but was unable to survive in its new host. Although transposition of Tn*5393* from pEa34 to pEA29 or the chromosome was not demonstrated, the occurrence of Tn*5393* on genetic elements common to all *E. amylovora* is evidence that the streptomycin-resistance transposon can reside on genetic elements that are stable in the species and is not associated exclusively with a conjugative plasmid.

Tn*5393* is a Tn*3*-type transposon (5), and transposition of Tn*3* and related Tn*1721* to the chromosome in *E. coli* occurs at a frequency 10^3-10^4 times lower than transposition to plasmids (13, 34). If Tn*5393* transposes to the chromosome at such a low frequency in *E. amylovora*, then it is surprising that strains were found with Tn*5393* on the chromosome. Perhaps such strains became more prevalent in the population because they were favored by the insertion of Tn*5393*, other than becoming streptomycin-resistant. For example, Kearney *et al.* (11) reported that the transposable element IS476 inactivated $avrBs_1$ of *X. c. vesicatoria* allowing the pathogen to evade the host defense response normally triggered by this avirulence gene.

Strains carrying Tn*5393* on the chromosome probably have fewer copies of *strA-strB* than strains with pEa34 or pEA29::Tn*5393*, both medium-copy-number plasmids. This could explain the weaker hybridization signal in Southern blots of *Ava*I-digested genomic DNA probed with SMP3 (Figure 5). Low copy number of *strA-strB* might also account for lower MICs among strains with Tn*5393* inserted on the chromosome compared to strains with plasmid-borne Tn*5393* (Table 2).

Tn*5393* was inserted at the same site on pEA29 in all four strains containing pEA29::Tn*5393*, possibly because several clones arose following one insertion event.

Previous laboratory studies showed that Tn*5393* inserted at different sites on plasmids in *E. coli* (5). Other than a preference for AT-rich regions, Tn*3*-type transposons show little target-site specificity (23); thus, Tn*5393* probably inserts into other sites on pEA29. Plasmid pEA29 is not required for pathogenicity, but its genes are believed to modulate the development of fire blight symptoms (8, 15). Strains harboring pEA29::Tn*5393* were similar to strains with plasmids pEA29 and pEa34 in MIC (Table 2), pathogenicity, and growth rate in liquid culture (data not shown), indicating that the insertion had not disrupted genes involved in these functions.

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PART II

THE ROLE OF WIND-DRIVEN RAIN, AEROSOLS, AND CONTAMINATED BUDWOOD IN INCIDENCE AND SPATIAL PATTERN OF FIRE BLIGHT AT AN APPLE NURSERY

INTRODUCTION

Fire blight, caused by *Erwinia amylovora*, is the most destructive bacterial disease affecting apple, pear, and rosaceous ornamentals. *E. amylovora* is difficult to control and nearly impossible to eradicate, because once established in its host, low populations persist and overwinter in symptomless tissue (5, 7, 24, 31, 34, 42). Despite intense efforts to prevent its introduction, *E. amylovora* is often present in pome tree nurseries (3, 39, 45). Nursery trees are at high risk for fire blight since regular fertilization and irrigation promote succulent growth which is especially vulnerable to the pathogen (3, 8, 12, 26, 39, 45). Contaminated propagation wood and nursery stock are potential means of long-distance dispersal of *E. amylovora* (2, 9, 34, 39, 43). Maintaining uncontaminated nursery stock is crucial in preventing the introduction of *E. amylovora* into areas where blight is not known, and in limiting the introduction of new genotypes of the pathogen to areas where blight is already established.

Few studies have attempted to determine how *E. amylovora* enters nurseries or how it survives and is disseminated after introduction. Pear trees in a nursery in New York succumbed to blight after *E. amylovora* was apparently introduced on budwood, then spread on budding knives (39). Internally-infested scionwood was implicated as the source of *E. amylovora* when three of 600 pear rootstock seedlings became infected after being grafted with surface-sterilized, symptomless buds (44). Pruning tools have also been reported to transmit blight (39, 41, 45). Bauske (3) reported that the severity of fire blight epidemics in pear nurseries in Iowa was directly related to the degree of exposure of trees to prevailing southerly winds, and spread of blight was reduced by the use of windbreaks. Moreover, winds of 3-6 m/s moved water-borne *E. amylovora* at least 1 m,

the distance between nursery rows (3, 4). While the pattern of blighted trees in the nursery seemed to be affected by the weather, the data were not analyzed statistically. Also, no attempt was made to identify sources that introduced *E. amylovora* into the nursery.

Spatial lag autocorrelation analysis has been used to test whether disease incidence at one location in a field plot is dependent on values of the variable at neighboring locations (10, 11, 16, 17, 19, 20, 33). Spatial pattern analyses, including lag autocorrelation and ordinary runs, have been used to relate incidence and aggregation of bacterial diseases in citrus nurseries in Florida and Argentina to biological and environmental factors as well as cultural practices (16, 18, 19, 21). In the citrus nursery studies, factors affecting spatial patterns of disease included host susceptibility, aggressiveness of the pathogen strain, defoliation and regrowth of the host, introduction of the pathogen on contaminated rootstock, bactericide application, mechanical spread during routine maintenance operations, and wind-driven rain. Within-row aggregation of disease was generally attributed to cultural and mechanical factors, whereas across-row aggregation of disease was related to previous severe weather.

The purpose of this study was to identify factors involved in the introduction and spread of fire blight at an apple nursery by analyzing spatial patterns of disease and relating these patterns to weather phenomena and cultural practices. Additionally, the possibility that *E. amylovora* entered the nursery on contaminated budwood was investigated.

MATERIALS AND METHODS

In 1992, three plots were established at an apple nursery in Michigan in a block of various fire blight-susceptible cultivars on M.26 rootstock, also susceptible to fire blight. Plots were 30 m × 10, 11, or 13 rows running north-south. Trees were spaced about 25 cm apart within rows with 1.5 m between rows. Thus, a plot of 10, 11, or 13 rows contained approximately 1,200, 1,320, or 1,560 trees, respectively. In 1993, four plots were established in a nursery block 1.6 km from the 1992 site. Plots contained various fire blight-susceptible cultivars on M.9 or Mark rootstocks, both susceptible to fire blight. Plots were 30 m × 15 rows running east-west, and spacing of trees and rows was the same as for the 1992 plots. Thus, each plot contained approximately 1,800 trees. The trees were trickle-irrigated, and fertilizer and pesticides, including copper bactericides and streptomycin, were applied as needed to maintain vigorous growth. Plots were monitored for fire blight at least once per week from June through August of each year. On each census date, the position of each blighted tree was mapped within the plot and then removed from the nursery.

A simulated nursery planting of four plots, each consisting of five rows of 22 1-year-old apple trees of various fire blight-susceptible cultivars on susceptible rootstocks, was established at the Botany and Plant Pathology Farm of Michigan State University (MSU) at East Lansing on 3 June 1992. Trees were planted 30 cm apart within rows with 1.5 m between rows which ran north-south. In 1993, trees were cut back to 5-7 cm above the graft union, and the scion trained to a single shoot. The trees were trickle-irrigated, and fertilizer and pesticides (no bactericides) were applied as needed until late August. A nalidixic acid-resistant mutant of *E. amylovora* was selected from plates

inoculated with 0.1 ml of a suspension of *E. amylovora* containing ~10^9 colony forming units (cfu) per milliliter onto a plate of King's Medium B (25) supplemented with 100 µg/ml nalidixic acid. Two trees in the center of each plot were inoculated with the nalidixic acid-resistant strain on 15 June 1993 by wounding succulent apical tissue with a dissecting needle and smearing bacteria into the wounds. After symptoms developed on inoculated trees, the plots were monitored 2-3 times per week for fire blight symptoms, and the locations of diseased trees were recorded. Bacterial ooze or water-soaked tissue was streaked onto CCT, the differential medium of Ishimaru and Klos (23), supplemented with 25 µg/ml nalidixic acid and 100 µg/ml cycloheximide (CCTnc) to verify that the marked strain was present in the blighted trees.

Relation of meteorological events to fire blight incidence. In 1992, temperature and precipitation at the nursery were measured with a wet/dry bulb thermometer and tipping bucket rain gauge, respectively, and recorded with a RSS 411 apple scab predictor (Reuter-Stokes, Inc., Cleveland, OH). Wind speed was measured with a three-cup totalizing anemometer and was averaged hourly with a CR10 micrologger (Campbell Scientific, Inc., Logan, UT) at a station 15 km from the nursery. In 1993, temperature, precipitation, wind speed and wind direction were monitored at the nursery every minute with a thermistor probe, tipping bucket rain gauge, three-cup totalizing anemometer and wind vane, respectively, and averaged hourly with a 21× micrologger (Campbell Scientific, Inc.). Temperature and precipitation at MSU were measured with a thermistor probe and a tipping bucket rain gauge, respectively, and recorded with an EnviroCaster (Neogen Corp., Lansing, MI). Wind speed and direction data were obtained from a National Weather Service station located 12 km from the MSU plots. The MARYBLYT

computer program (27, 37, 38) was used to predict dates of fire blight outbreaks. "Trauma blight" (38) refers to fire blight that develops after plant tissue is damaged by wind, hail, or other sudden, injurious stress. For both 1992 and 1993, a trauma event was entered into the program if rain fell during an hour when the mean wind speed was ≥ 6.5 m/s. In 1993, the fastest 1-min mean wind speed for each day, as well as hourly mean wind speeds, were recorded. Therefore, a trauma event was also entered if rain fell during an hour in which a 1-min mean wind speed was ≥ 7.7 m/s and hourly mean wind speed was ≥ 4.0 m/s. The goal was not to compare disease incidence among plots, but rather to evaluate disease incidence on each assessment date so that the MARYBLYT model could be tested. Thus, disease incidence was standardized by defining it as the number of new infections in a plot expressed as a percentage of the seasonal total number of blighted trees in that plot.

Spatial pattern analyses. After disease incidence and mapping data had been collected, nursery plots were partitioned into 1.5 m square quadrats, each containing approximately six trees within one row. Disease incidence within a quadrat was expressed as the fraction of trees with fire blight. Disease assessment dates were grouped according to the observed clumping of fire blight outbreaks and infection periods predicted by the MARYBLYT model (38). Spatial lag autocorrelation analysis was performed using the LCOR2 computer program (20) to assess autocorrelation among disease incidence values of quadrats. Autocorrelation matrices were generated in which each quadrat value was compared to values in all proximal quadrats. Autocorrelation matrices and their associated two-dimensional proximity patterns were interpreted as described previously (11, 16, 17, 19, 20, 33). Because the MSU plots were relatively

small, quadrat-based analysis was not appropriate. However, trees were arranged in a lattice of rows and across-row "columns", and the status (*i.e.*, diseased or healthy) of every tree was known, permitting the use of ordinary runs (28) to analyze the spatial pattern of disease. Disease assessment dates were grouped as described above. In each plot, within-row runs were calculated by treating the five rows of trees as a single contiguous row by counting runs up one row and down the next (10, 29). Similarly, across-row runs were calculated by treating the 22 columns of trees as a single column. A continuity correction (0.5) was added to the observed number of runs prior to calculating Z-statistics (15). A nonrandom distribution of diseased trees was indicated if the probability of the Z value was ≤ 0.050.

Air sampling. An Andersen six-stage microbial impaction sampler (1) (Andersen 2000 Inc., Atlanta, GA) was used in 1993 to sample air in an MSU apple orchard with fire blight and, later in the season, in the simulated nursery plantings at MSU. Glass petri dishes designed for use with the air sampler were filled with 27 ml CCT supplemented with 100 µg/ml cycloheximide (CCTc) for sampling in the orchard, or CCTnc for sampling in the plots. The sampler was supported on a horizontal platform and placed among foliage with conspicuous bacterial ooze. A battery-powered vacuum pump drew air through the sampler for 20 min at a flow rate of 0.028 m^3/min. Eleven samples were collected during rain, and 10 samples were collected during dry weather, although in some cases, leaves were wet from dew or previous rain. During rain, the sampler was shielded with an umbrella to insure that only aerosol-sized particles and not splash droplets would reach the sampler orifice. Counts for each stage were summed to determine total counts per air sample, and expressed as cfu/m^3.

Screening of budwood for *E. amylovora*. Budwood from various cultivars of apple in the main budwood orchard for the nursery was collected during late July through August of 1992 and 1993 when nursery workers were collecting budwood for grafting. Though the trees were symptomless at the time of collection, fire blight infections were pruned from trees in this orchard during June and July of both years. In 1992, 10-bud samples from each of 115 trees were ground in 5 ml 0.01 M potassium phosphate buffer pH 7 (PPB), enriched with 5 ml CCT prepared without agar, and shaken for 12-16 h at room temperature. One hundred-microliter aliquots of 10^0, 10^{-1}, and 10^{-2} dilutions of each sample were spread onto plates of CCTc. In 1993, 106 budsticks ~50-cm in length, were defoliated and cut into several pieces. Leaves and stems were shaken in 100 ml PPB for 1-2 h. Samples were filtered (Whatman No. 1, Whatman Ltd., Maidstone, UK) to remove debris, concentrated by centrifugation for 15 min at 6,000 rpm, and resuspended in 210 µl water. One hundred-microliter aliquots of 10^0, 10^{-1}, and 10^{-2} dilutions of the suspension were spread onto plates of CCTc. Ten microliter aliquots of the concentrated sample were used in the polymerase chain reaction (PCR) to identify *E. amylovora* (6). Primers were 17-mer oligonucleotides from the borders of a 0.9 kb *Pst*I fragment of a plasmid common in all strains of *E. amylovora* (6, 14), and were synthesized at the Macromolecular Facility Laboratory at MSU with an automatic 380B DNA Synthesizer (Applied Biosystems, Foster City, CA). PCR was carried out in a total volume of 50 µl containing (final concentrations) 25 pmol of each primer, 2 U of *Taq* DNA polymerase (Gibco, BRL, Gaithersburg, MD), 0.2 mM each dATP, dCTP, dGTP, and dTTP (Promega Corp., Madison, WI), 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 8 µg bovine serum albumin, 5% dimethyl sulfoxide, and 1% Tween 20.

Ten microliters of sterile water and 10 µl of a suspension containing ~10^4 cfu/ml of *E. amylovora* were run as negative and positive controls, respectively. A dilution series of *E. amylovora* and a series put through the centrifugation, filtering and resuspension steps were run to determine the sensitivity of the assay. Samples were overlaid with a drop of light mineral oil, and PCR was performed in a Coy TempCycler (Coy Corp., Grass Lake, MI). Denaturation was at 93 C (in first cycle for 2 min and in subsequent cycles for 1 min), annealing was at 52 C for 2 min, and polymerization was at 72 C for 2 min. After 37 cycles, the PCR products were separated on 1.0% agarose gels, stained with ethidium bromide and photographed on a UV transilluminator. The presence of a 0.9-kb DNA fragment was indicative of *E. amylovora* (6).

Pathogen identification. Representative colonies characteristic of *E. amylovora* on CCTc or CCTnc were tested with an *E. amylovora*-specific DNA probe (30). Pathogenicity of putative isolates of *E. amylovora* was tested by inoculating immature pear fruit.

RESULTS

Relation of meteorological events to fire blight incidence. Disease sufficient for analysis developed at the nursery in three plots, N92-1, N92-2, and N92-3, in 1992, and in one plot, N93-3, in 1993. Since diseased trees were removed immediately after detection of fire blight, data for each assessment date represent new infections only and not cumulative counts of blighted trees. At MSU, disease sufficient for analysis developed in two plots, MSU2 and MSU3.

Although the MARYBLYT model predicted the dates of some major outbreaks of fire blight in the nursery plots, it also erroneously predicted, or failed to predict, disease

on other dates (Figures 1-3, Table 1). In 1992, MARYBLYT predicted shoot blight symptoms (not trauma related; see ref. 38) on 13 June, and shoot blight was first detected in plot N92-1 on 16 June (Figure 1). Three severe storms occurred at the nursery during mid-June through mid-July, and trauma blight symptoms were predicted for 1, 12, and 20 July. In plot N92-1, the incidence of blighted trees, based on a seasonal total of 121 blighted trees, was 22 and 32% on 1 July, and the combined dates of 20 and 24 July, respectively (Figure 1, Table 1). In plot N92-2, fire blight was detected as predicted by the model on 1 July (8% of the seasonal total of 197 blighted trees) and on the combined dates of 20 and 24 July (73%) (Figure 1, Table 1). In plot N92-3, fire blight was detected as predicted by the model on 1 July (14% of the seasonal total of 155 blighted trees) and on the combined dates of 20 and 24 July (57%) (Figure 1, Table 1). However, little blight was found in any of the plots on 15 July, although MARYBLYT had predicted symptoms for 12 July (Figure 1, Table 1).

In 1993, trauma blight symptoms were predicted at the nursery on 25 June, 4 July, 5 July, and 16 July (Figure 2, Table 1). Blight was first observed in plot N93-3 on 6 July, and 24% of the seasonal total of 97 blighted trees was recorded on 19 July. Symptoms were not predicted in late July, but 19% of the seasonal total of blighted trees was recorded on 28 July (Figure 2).

At MSU, symptoms on foci were detected on 19 June. Trauma blight symptoms were predicted on 29 June, 16 July, 13 August, and 29 August (Figure 3, Table 1). Symptoms on non-focus trees were first noted on 29 June in plots MSU2 and MSU3. In plot MSU2, the incidence of blighted trees, based on a seasonal total of 51 blighted trees,

Table 1. Weather data in relation to dates of predicted and observed outbreaks of fire blight in nursery trees[a]

Location / Year	Date of trauma[b]	Rain (mm)[c]	Wind speed (m/s)[d] and direction[e]	Date symptoms Predicted	Date symptoms Observed[f]	Plot:Incidence (%)[g]
Nursery						
1992	17 June	32.5	10.8 n.a.	1 July	1 July[h]	N92-1: 22 N92-2: 8 N92-3: 14
1992	4 July	9.7	6.5 n.a.	12 July	15 July	N92-1: 3 N92-2: 1 N92-3: 1
1992	13 July	29.5	6.5 n.a.	20 July	20 and 24 July	N92-1: 32 N92-2: 73 N92-3: 57
1993	18 June	9.4	4.5; 8.6 SW	25 June	28 June	N93-3: 0
1993	27 June	15.2	4.5; 9.0 SW	4 July	6 July[i]	N93-3: 5
1993	30 June	7.1	5.0; 8.1 SE	5 July	6 July[i]	N93-3: 5
1993	9 July	0.8	5.4; 8.6 SW	16 July	19 July	N93-3: 24
Michigan State University						
1993	19 June	19.3	7.4; 7.7 SW	29 June	30 June[j]	MSU2: 4 MSU3: 4

Table 1 (cont'd)

Location Year	Date of trauma[b]	Rain (mm)[c]	Wind speed (m/s)[d] and direction[e]	Date symptoms Predicted	Observed[f]	Plot:Incidence (%)[g]
Michigan State University						
1993	9 July	5.1	6.1; 9.8 SW	16 July	19 July	MSU2: 8 MSU3: 15
1993	3 Aug.	2.0	4.0; 8.8 SW	13 Aug.	16 Aug.	MSU2: 14 MSU3: 15
1993	24 Aug.	4.1	5.9; 6.2 S	29 Aug.	29 Aug.	MSU2: 22 MSU3: 13

[a]The trauma blight feature of the MARYBLYT model (38) was used to predict dates of fire blight outbreaks.

[b]Trauma refers to sudden, injurious stress to trees. In 1992 and 1993, trauma was recorded if rain fell during an hour when mean wind speeds were ≥ 6.5 m/s; in 1993, trauma was also recorded if rain fell during an hour in which a 1-min mean wind speed was ≥ 7.7 m/s and hourly mean wind speed was ≥ 4.0 m/s.

[c]Total rainfall on date of trauma.

[d]Maximum mean hourly wind speed (m/s) recorded during rain; fastest 1-min mean wind speed recorded during rain.

[e]S = south; SW = southwest; SE = southeast; n.a. = not available.

[f]Disease assessment dates within 4 days following the date that symptoms were predicted.

[g]The number of new infections expressed as a percentage of the seasonal total number of blighted trees.

[h]Date on which fire blight was first detected in plots N92-2 and N92-3.

[i]Date on which fire blight was first detected in plot N93-3.

[j]Date on which fire blight was first detected on non-focus trees in plots MSU2 and MSU3.

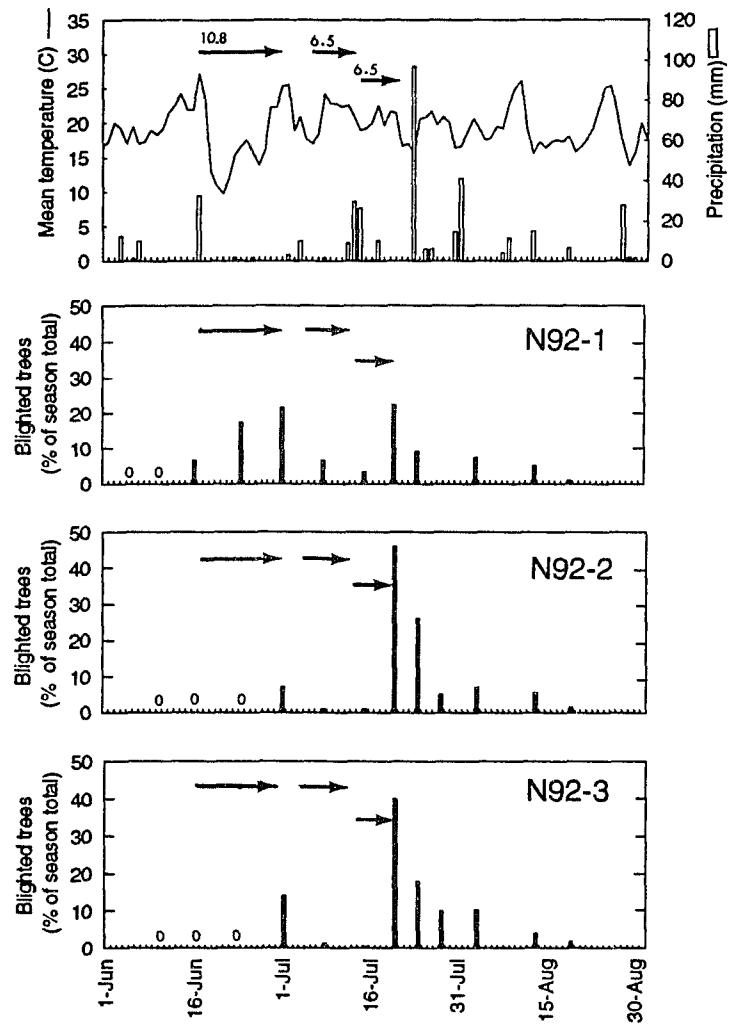

Figure 1. Relation of weather to the incidence of fire blight at an apple nursery in Michigan in 1992. Arrows in each graph indicate dates and duration of trauma blight infection periods as predicted by the MARYBLYT model (38). Numbers above arrows in top graph are mean hourly wind speeds (m/s) recorded during the storms. Blighted trees in plots N92-1, N92-2, and N92-3 are new infections detected on each assessment date. A zero indicates that no blighted trees were detected. Disease was not assessed on other dates.

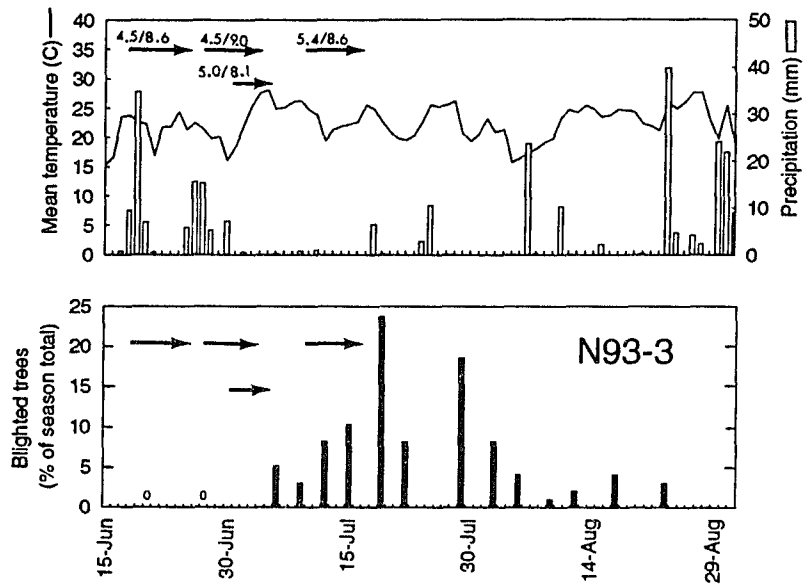

Figure 2. Relation of weather to the incidence of fire blight at an apple nursery in Michigan in 1993. Arrows in both graphs indicate dates and duration of trauma blight infection periods as predicted by the MARYBLYT model (38). Numbers above arrows in top graph are mean hourly wind speeds followed by maximum 1-min mean wind speeds (m/s) recorded during the storms. Blighted trees in plot N93-3 are new infections detected on each assessment date. A zero indicates that no blighted trees were detected. Disease was not assessed on other dates.

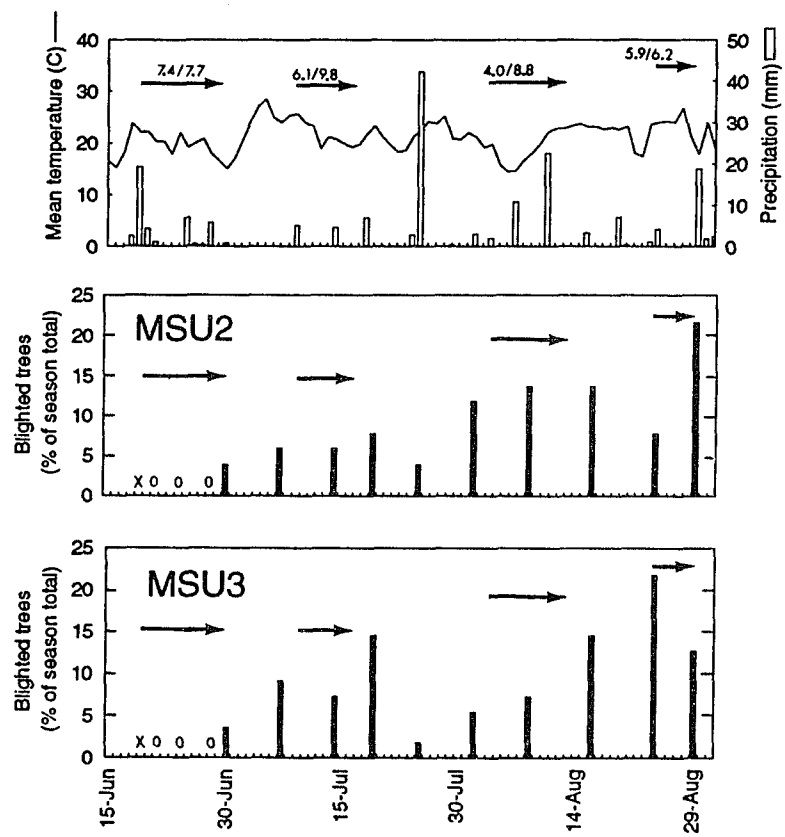

Figure 3. Relation of weather to incidence of fire blight in simulated nursery plots at Michigan State University in 1993. Arrows in each graph indicate dates and duration of trauma blight infection periods as predicted by the MARYBLYT model (38). Numbers above arrows in top graph are mean hourly wind speeds followed by maximum 1-min mean wind speeds (m/s) recorded during the storms. The date on which focus trees first showed symptoms is indicated by ×. Blighted trees in plots MSU-2 and MSU-3 are new infections detected on each assessment date. A zero indicates that no blighted trees were detected. Disease was not assessed on other dates.

was relatively constant through mid-July, and increased during August, with 22% of the seasonal total recorded on 29 August. A similar trend was detected in plot MSU3 with 22% of the seasonal total of 55 diseased trees recorded on 24 August.

Spatial pattern analyses. No consistent pattern of significantly autocorrelated lags was noted early in the season, with two plots (N92-1 and N92-2) showing strong within-row patterns, while the other two plots (N92-3 and N93-3) showed tightly clustered patterns (Figure 4). On later dates, proximity patterns showed a few significant across-row autocorrelations, often noncontiguous and at high-order spatial lags. Examination of the autocorrelation matrices corresponding to diagonal directions revealed significant high-order autocorrelations by mid-July in all plots (not shown). Significant negative autocorrelations were not detected. For plot N92-1, there was extensive significant autocorrelation among spatial lags within field rows (north-south), and no autocorrelation across rows (east-west) when data from early assessment dates were combined. Throughout July, within-row autocorrelations decreased, and across-row autocorrelations were detected. Later in the season, matrices for the diagonal directions indicated significant autocorrelations, especially at high-order lags. For plot N92-2, extensive within-row (north-south) autocorrelations, and first-order across-row (east-west) autocorrelations among spatial lags were detected on 1 July. On later dates, the matrix corresponding to an alignment parallel with the plot (Figure 4) and matrices for diagonal directions indicated significant autocorrelations at high-order, noncontiguous lags. For plot N92-3, a large tight cluster of significantly autocorrelated spatial lags noted on 1 July, disintegrated by mid-July despite extensive disease in the plot. However, matrices for the diagonal directions indicated several significant autocorrelations at high-order

Figure 4. Proximity patterns of significant spatial lag autocorrelations of fire blight in an apple nursery in Michigan. Black squares denote significant positive autocorrelations ($P \leq 0.050$) at the indicated spatial lag positions. For plots N92-1, N92-2, and N92-3, lags north-south (N-S) are within field rows; for plot N93-3, lags east-west (E-W) are within field rows. Plots N92-1, N92-2, N92-3, and N93-3 contained 11, 13, 10, and 15 field rows, respectively.

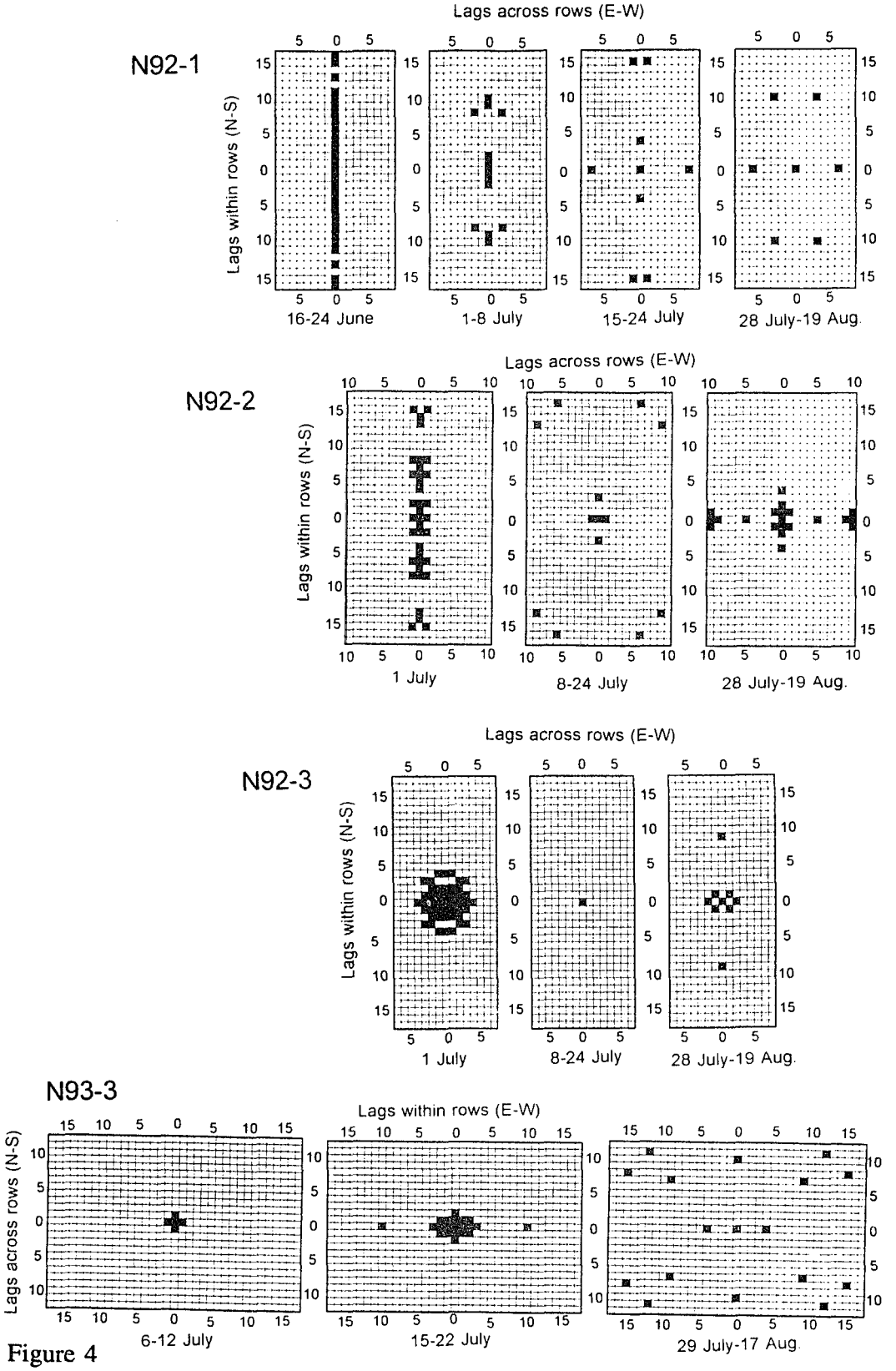
Lags across rows (E-W)
N92-1
Lags within rows (N-S)
16-24 June
1-8 July
15-24 July
28 July-19 Aug.
Lags across rows (E-W)
N92-2
Lags within rows (N-S)
1 July
8-24 July
28 July-19 Aug.
Lags across rows (E-W)
N92-3
Lags within rows (N-S)
1 July
8-24 July
28 July-19 Aug.
N93-3
Lags within rows (E-W)
Lags across rows (N-S)
6-12 July
15-22 July
29 July-17 Aug.

Figure 4

spatial lags. Late-season infections resulted in first-order autocorrelation in a diagonal direction, second-order autocorrelation across rows (east-west), and at the ninth spatial lag within rows (north-south). For plot N93-3, first-order autocorrelations were initially significant within (east-west) and across (north-south) rows. The cluster of quadrats with significant autocorrelations expanded during July. However, later in the season the pattern of new infections resulted in significant autocorrelations at high-order, noncontiguous spatial lags. Matrices for the diagonal directions indicated significant autocorrelations at high-order spatial lags.

Ordinary runs analysis of data from plots MSU2 and MSU3 showed significant within-row (north-south) aggregation throughout the season (Table 2). There was no evidence of across-row aggregation until late July through early August. The Z-values calculated from across-row runs were much less negative than Z-values calculated from within-row runs.

Air sampling. All 11 air samples collected during rain contained *E. amylovora*; the mean was 103 cfu/m^3 with a standard deviation of 106. Three of 10 air samples collected during dry weather contained *E. amylovora*; the mean was 2.4 cfu/m^3 with a standard deviation of 1.0. Eighty-two percent of aerosols containing *E. amylovora* were deposited on stages 1-3 and were therefore >2.1 μm in diameter, while 18% of aerosols containing *E. amylovora* were deposited on stages 4-6 and were therefore < 2.1 μm in diameter. Representative colonies were identified as *E. amylovora*.

Table 2. Ordinary runs analysis of fire blight on apple trees in simulated nursery plots at Michigan State University (MSU)

Plot	Z-values[a] (*P*)[b]	
Disease assessment dates	Within row (north-south)	Across row (east-west)
MSU2		
25 June-14 July	-6.80 (<0.001)	0.03 (0.382)
16 July-8 August	-5.07 (<0.001)	-1.93 (0.027)
11 August-29 August	-5.49 (<0.001)	-1.06 (0.145)
MSU3		
25 June-14 July	-5.81 (<0.001)	-0.23 (0.409)
16 July-8 August	-4.33 (<0.001)	-1.65 (0.050)
11 August-29 August	-6.30 (<0.001)	-1.64 (0.051)

[a]Standardized variable; large negative values indicate a nonrandom, aggregated distribution of disease.

[b]Significance level; levels of $P \leq 0.050$ indicate a nonrandom distribution of disease.

Screening of budwood for *E. amylovora*. *E. amylovora* was not detected in any of 115 10-bud samples collected in 1992. In 1993, *E. amylovora* was detected in 6% of 106 samples using traditional plating and identification methods and in 16% of the samples using PCR. All samples testing positive on plates also tested positive by PCR. Bacterial populations of the plate-positive samples ranged from 2×10^4 - 2×10^5 cfu/ml corresponding to 2×10^2 - 2×10^3 cfu per PCR reaction, assuming a plating efficiency of 100%. The lower detection limit of the PCR assay was 1×10^2 cfu/ml when *E. amylovora* was added directly to the reaction buffer and 5×10^2 cfu/ml when bacteria were put through the manipulations that field samples underwent.

DISCUSSION

The data indicate that wind-driven rain was the most important factor involved in spreading *E. amylovora* after the pathogen had entered the nursery. Severe outbreaks of fire blight could sometimes be traced back to a storm containing wind-driven rain. When blight was initially detected in the nursery each season, significant autocorrelations among spatial lags were generally within-row or formed tight clusters (Figure 4). However, after severe weather, autocorrelation matrices for directions parallel with (Figure 4) and at a diagonal to the plots often showed significant values at high-order, noncontiguous spatial lags. Such a pattern suggests that strong winds capable of blowing inoculum several meters impacted the nursery plots at an oblique angle. For example, proximity patterns for N92-1 and N92-2 indicated high-order spatial lag autocorrelations in the east-west direction after summer storms, which in Michigan generally come from the southwest.

Although the MARYBLYT model predicted the dates on which fire blight was first detected in the six plots analyzed, and dates of some other major outbreaks, it was

not always accurate. The trauma blight feature of MARYBLYT is somewhat subjective, since the user defines a trauma event. A trauma event was entered when rain and strong winds occurred during the same hour. However, the importance of foliage-damaging winds that occurred before or after a storm, or wind gusts, was not considered. Extreme values of wind speed, as opposed to averages, are more important in the liberation of fungal spores from host tissue (35), and might be similarly critical in dispersal of bacterial pathogens. This could explain outbreaks of fire blight that were not predicted by MARYBLYT.

The role of weather in relation to the incidence and spatial pattern of fire blight at the nursery was more easily discerned early in the summer when disease was first detected and following the first major storm of the season. By late July and throughout August, interpretation of data was complicated by the formation of secondary foci. In our study, secondary foci did not expand because diseased trees were continually removed by nursery personnel. This is in contrast to studies of *Xanthomonas campestris* pv. *citri* in citrus nurseries where extensive secondary foci coalesced by later assessment dates (19, 21). It was suggested that spatial lag distances between primary and secondary foci might provide an estimate of the distance that inoculum is splash-disseminated (19). Secondary foci increased the amount of bacteria-laden ooze available to initiate new infections and might have been associated with an increase in epiphytic *E. amylovora* (13, 32, 40, 42). As ooze became more abundant, dispersal of the pathogen to healthy trees probably occurred even in the absence of severe storms. It was also possible for disease to occur throughout the plot with no significant autocorrelations among spatial lags (Figure 4, N92-3). This might be expected if disease were randomly dispersed among quadrats.

The MSU plots were established to evaluate the development and spread of fire blight when diseased trees were not removed. A build-up of bacterial ooze and epiphytic *E. amylovora* was probably the reason that new infections as a percentage of the seasonal total steadily increased in the MSU plots. Ordinary runs analysis showed strong within-row aggregation of disease throughout the season (Table 2). The distance between trees within rows was much less than that across rows, allowing more frequent contact and transfer of inoculum among trees in the same row. Across-row aggregation of disease was significant only later in the season, possibly because of a storm on 9 July. Though total precipitation from the storm was not great (5.1 mm), it fell in a short period of time (~20 min), and was accompanied by strong gusts of wind from the southwest. Alternatively, across-row aggregation may have been due to the simultaneous spread of disease within adjacent rows.

The importance of rain in the release and dispersal of *E. amylovora* was further supported by the air sampling data. Air samples collected during rain always contained *E. amylovora*, whereas samples collected during dry periods contained zero or very few cfu of the pathogen. Southey and Harper (36) reported that *E. amylovora* remained viable up to 2 h in aerosol particles exposed to the open air. Aerosols containing soft rot *Erwinia* spp. remained suspended for at least 1 h (22). Thus, wind might result in the dispersal of viable *E. amylovora* even after rain has stopped. Presumably, a cfu represented at least one bacterium. However, since cells of *E. amylovora* are ~0.7 × 1.0 μm in size (45), and most colonies arose from aerosol particles >2.1 μm in diameter, it would be possible for a single particle to carry more than one cell. Under favorable environmental conditions, bacteria would multiply rapidly if aerosols landed on highly

susceptible wind-damaged shoot tips (12, 26). Infection was frequently associated with wind-damaged leaves. Thus, aerosols could be an epidemiologically significant source of *E. amylovora* even if the number of cells per particle were low.

Contaminated budwood has been implicated as a means by which *E. amylovora* enters pome tree nurseries (2, 9, 39, 44, 45). Populations of *E. amylovora* are apparently very low in buds from symptomless shoots of apple and pear since numerous attempts to recover the pathogen from such tissue have yielded few positive results (7, 9, 32, 42). Though not a model epiphyte (32), *E. amylovora* has commonly been isolated from the surfaces of apparently healthy shoots of apple and pear, but only after symptoms were observed in the orchard (32, 40, 42). Although *E. amylovora* was not isolated from buds alone, the pathogen was detected in samples of budsticks and leaves by plating and PCR. Dueck and Morand (13) reported that in Ontario, populations of *E. amylovora* on apple leaves were highest during mid-July through August. If conditions were similar in Michigan, then high populations of *E. amylovora* would coincide with the collecting and grafting of buds. In the budwood orchard, bundles of budsticks with leaves are wrapped in wet cheese cloth and stored for up to three days before buds are grafted to rootstocks. Thus, *E. amylovora* might have entered the nursery externally on budwood, and invaded the rootstocks after grafting with contaminated buds.

Several lines of circumstantial evidence support the premise that budwood was a source of *E. amylovora* in the nursery. Rootstocks in plots N92-1 and N92-2 were budded in 1991, and there was strong within-row aggregation of disease on the earliest dates that blight was detected during the 1992 season. In plot N92-1, a few rootstocks within one row had active cankers by mid-May. At the other nursery location, several

rootstocks which had been budded with the same cultivar exhibited fire blight cankers at the graft union 2-4 weeks after budding in 1992. Infected trees were not randomly dispersed down the row, but appeared to be aggregated, indicating that they might have been budded from the same budstick or bundle of budsticks. Removal of infected trees in the autumn of 1992 eliminated potential sources of inoculum in the spring of 1993 and may have contributed to the lower incidence of fire blight in 1993.

Due to the large number of buds required by nurseries and the limitations in detection of *E. amylovora* in buds, it would be impractical to screen budwood prior to grafting. Furthermore, budwood sources are finite, and it may be impossible to maintain a mature budwood orchard completely free of the pathogen, especially in apple-growing regions. Thus, the introduction of *E. amylovora* on budwood into nursery plantings may be inevitable. However, our study has demonstrated the urgency of preventing the overwintering of inoculum in rootstocks, because once established in the nursery, *E. amylovora* is readily disseminated by wind-driven rain and becomes aerosol-borne during rain with or without wind.

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APPENDIX A

DETECTION OF *ERWINIA AMYLOVORA* IN A MICHIGAN APPLE NURSERY

DETECTION OF *ERWINIA AMYLOVORA* IN A MICHIGAN APPLE NURSERY

Isolation of *Erwinia amylovora*, the fire blight bacterium, was attempted from several potential sources at an apple nursery in Michigan. The purpose was to determine how the pathogen might have entered the nursery, where it resided and overwintered, and how it was spread within the nursery.

The nursery was monitored at least once per week during May through September of 1992 and 1993. Samples were collected from four farms at the nursery (Table 1). Identification of *E. amylovora* was based on colony morphology on a differential medium, pathogenicity on immature pear fruit, hybridization with a DNA probe and a polymerase chain reaction (PCR) technique (see Parts I and II of this dissertation for more detail).

Isolates from the nursery were identified and were tested for streptomycin resistance by attempting to grow them on a medium supplemented with the drug (Table 2). All isolates were sensitive to streptomycin at 100 μg/ml.

Table 1. Farms at a Michigan apple nursery sampled for *Erwinia amylovora*

Farm	Description
A	Rootstock planted in spring 1991; budded in summer 1991; sampled in 1992
B	Rootstock planted in spring 1992; budded in summer 1992; sampled in 1992 and 1993
C	Rootstock planted in spring 1993; budded in summer 1993; sampled in 1993.
D	Budwood orchard; sampled in 1992 and 1993

Table 2. Detection of *Erwinia amylovora* from potential sources at a Michigan apple nursery

Source	Farm[a]	Date	Identification method[b]	*E. amylovora* detected?	Comments
Stem cankers	A	May 1992	M, P, DNA	Yes	12 rootstocks (M.26); active cankers
	B	Sept. 1992	M, P, DNA	Yes	22 rootstocks (M.9); cankers at graft union
	C	Sept. 1993	M, P, DNA	Yes	3 rootstocks (M.9); cankers at graft union
Blighted shoots	A	June-Sept. 1992	M, P, DNA	Yes	Several rootstocks and cultivars
	B	Aug.-Sept. 1992; June-Sept. 1993	M, P, DNA	Yes	Several rootstocks and cultivars
	C	Aug.-Sept. 1993	M, P, DNA	Yes	Several rootstocks and cultivars
	D	July 1992 July 1993	M, P, DNA	Yes	cv. Rome in 1992; Empire in 1993
Blossoms[c]	A	May 1992	M, P, DNA	Yes	cvs. DS-165, Fulford Gala, Imperial Gala, Lawspur Rome, Manchurian, Sweet 16

Table 2 (cont'd)

Source	Farm[a]	Date	Identification method[b]	*E. amylovora* detected?	Comments
Blossoms (cont'd)	B	May 1993	M, P, DNA	Yes	cv. Braeburn
				No	cvs. Red Harolson, Redmax, Spuree Rome, William's Pride
				No	cvs. Redmax, Empire
	D	May 1992	M	No	cvs. Fulford Gala, Imperial Gala, Redcort, Sweet 16, Jonnee, Mutsu
Symptomless buds[d]	D	July-Aug. 1992	M	No	10 buds × 115 trees; see Part II for details
Symptomless shoots[d]	D	July-Aug. 1993	M, DNA	Yes	6 of 106 samples; see Part II for details
		July-Aug. 1993	PCR	Yes	16 of 106 samples; see Part II for details
Liquid from containers with budsticks[e]	C	Aug. 1993	PCR	No	18 samples taken during budding

Table 2 (cont'd)

Source	Farm[a]	Date	Identification method[b]	*E. amylovora* detected?	Comments
Pruning tools[f]	A	July 1992	M, P, DNA	Yes	3 of 5 samples; disinfectant not used
			M	No	10 samples; disinfectant used
Disinfectant[g]	A	July 1992	M	No	12 samples collected from buckets in the field
Worker's hands[f]	A	July 1992	M, P, DNA	Yes	2 of 5 samples
Bags containing trees[f]	A	July 1992	M, P, DNA	Yes	4 of 5 samples; blighted trees tore holes in some bags
Insects[h]	A	July 1992	M, P	Yes	17 of 22 10-aphid samples; 2 samples of 3 and 5 leafhoppers
			M	No	Washed, ground aphid samples

[a]See Table 1 for farm descriptions.

[b]*E. amylovora* was identified by colony morphology on differential media (M), pathogenicity on immature pear fruit (P), DNA homology with an *E. amylovora*-specific probe (DNA), or the polymerase chain reaction using *E. amylovora*-specific primers (PCR). See Parts I and II of this dissertation for details.

[c]Blossoms were shaken vigorously for 30-60 s in 0.01 M potassium phosphate buffer pH 7 (0.5 ml buffer per flower).

Table 2 (cont'd)

[d]See Part II of this dissertation for details on isolation from symptomless tissue.

[e]Samples of 100-200 ml were filtered to remove debris and then concentrated by centrifugation.

[f]Sources were rinsed in 100-200 ml water and then concentrated by centrifugation.

[g]Disinfectant used was Greenshield™ (n-alkyl dimethyl benzyl ammonium chloride 10%, n-alkyl dimethyl ethylbenzyl ammonium chloride 10%). Samples of 10-15 ml were concentrated by centrifugation.

[h]Samples of 10 aphids were rinsed in 1 ml 0.01 M potassium phosphate buffer pH 7, and 0.1 ml was plated. Samples were then washed three times, ground, and replated.

Stem cankers occurring at the graft (bud) union are potentially significant overwintering sites for *E. amylovora* in the nursery. They can become active during the spring and release inoculum which is disseminated by rain. Cankers at the graft union imply bud-mediated entry of *E. amylovora*. While *E. amylovora* was not detected in symptomless buds, the pathogen was isolated from symptomless shoots collected from the budwood orchard. However, the pathogen was not detected in samples taken from the containers in which the budsticks were stored prior to grafting. Nevertheless, removing leaves of budsticks before storing for transport to the nursery might prevent entry of the pathogen on externally contaminated buds.

The importance of wind-driven rain in disseminating *E. amylovora* from blighted shoots in nursery plantings was illustrated in Part II of this dissertation. Blighted shoots in the budwood orchard are also a serious problem. Even if infections are removed, inoculum persists on symptomless tissue. Thus, under favorable weather conditions, the stage is set for an epiphytotic.

Nursery trees grafted with vegetative buds will not produce bloom. However, if flower buds are inadvertently grafted and environmental conditions are favorable, populations of *E. amylovora* could multiply rapidly on stigmata. Rain or heavy dew transports the pathogen *en masse* to the hypanthium where infection can occur. Also, pollinating insects can move bacteria among blossoms. Thus, blossoms can be an important source of inoculum in the nursery. In 1993, nursery workers removed blossoms as they appeared. While excessive contact with and unnecessary manipulations of the young trees should be avoided, removing bloom is probably a wise practice as long as sanitation is practiced diligently.

The disinfectant used in the nursery was effective against *E. amylovora*, but it was not used consistently. The pathogen was isolated from workers' hands and tools when they were removing lateral shoots. The pathogen was not isolated from tools after they were quickly immersed in the disinfectant.

The outer surfaces of large plastic bags used to carry blighted trees from the nursery were contaminated with *E. amylovora*. In many cases the bags were full, and diseased trees punctured the bags thereby exposing inoculum. Care should be taken to prevent this from happening.

Insect populations were usually low at the nursery due to consistent use of insecticides, thereby preventing extensive insect sampling. *E. amylovora* was isolated from the outer surfaces of aphids and leaf hoppers but not from samples after washing and grinding. Insects could probably spread the pathogen after coming into contact with oozing shoot termini, but apparently *E. amylovora* is not persistent internally. Additional insecticide use would probably not be worth the cost economically or the risk of insects developing resistance to the chemicals.

APPENDIX B

SURVIVAL OF *ERWINIA AMYLOVORA* IN RAINWATER

SURVIVAL OF *ERWINIA AMYLOVORA* IN RAINWATER

In Part II of this dissertation, wind-driven rain was cited as the major factor in spreading *E. amylovora* at an apple nursery. *E. amylovora* probably invades its host quickly during storms, since wind not only moves water-borne inoculum, but also damages host tissue thereby facilitating entry of the pathogen. It is not known how long *E. amylovora* remains viable in rainwater following a storm. Following heavy downpours, flooding occurred in the nursery during 1992 and 1993. In some cases, water remained stagnant for several days. If *E. amylovora* could survive for long periods of time in rainwater, then its dissemination would be enhanced during routine nursery operations and subsequent storms which cause splashing. This study was conducted to determine how long *E. amylovora* survives in rainwater.

Approximately 10^6 colony forming units (cfu) of the streptomycin-resistant *E. amylovora* strain CA11 were added to 25 ml of sterile rainwater, distilled water, or 0.01 M potassium phosphate buffer in an erlenmeyer flask. The flasks were incubated on a lab bench (room temperature with fluorescent and natural light). At various intervals, 0.1 ml aliquots from each flask were diluted and plated onto King's B medium supplemented with streptomycin at 100 µg/ml and cycloheximide at 100 µg/ml. Colonies were counted after two days.

The data indicate that *E. amylovora* survives approximately 1 day in rainwater but

for several days in distilled water or buffer. Thus, the long-term survival of *E. amylovora* in rainwater may not be of epidemiological significance in the nursery. The goal of a preliminary test was to monitor the survival of *E. amylovora* in water collected from stagnant puddles in the field, and in liquid collected from the containers in which budsticks were stored prior to grafting. The liquids were not sterilized, however, and contaminants obscured the detection of *E. amylovora* on the plates. More repetitions of these experiments would be required to ascertain the importance of biotic (*e.g.*, competing microorgansims) and abiotic (*e.g.*, pH, ultraviolet light) factors on the survival of *E. amylovora* in fresh rainwater or stagnant water in the field.

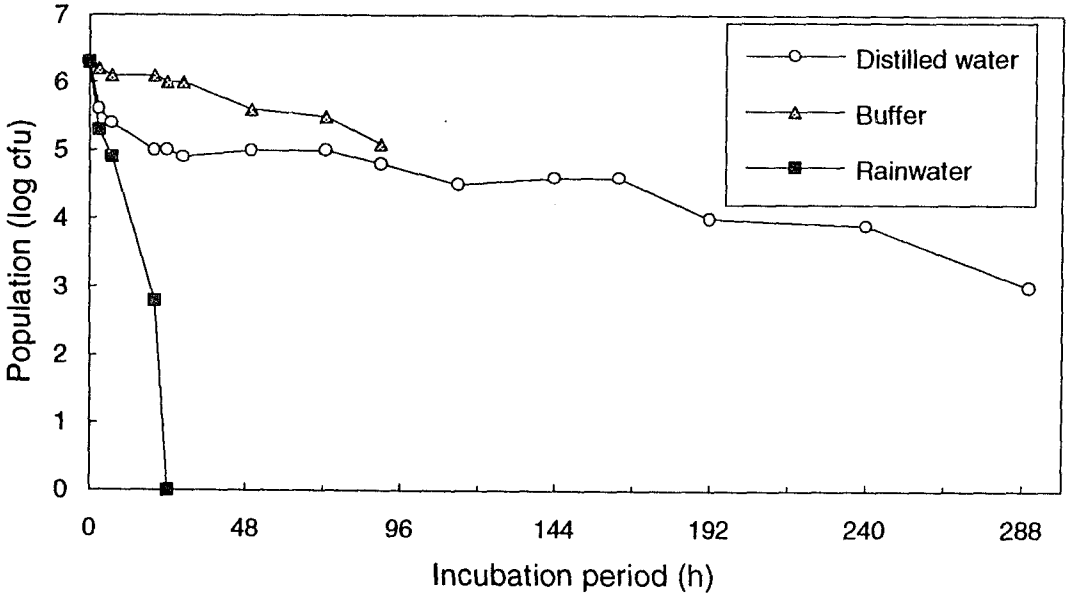

Figure 1. Population of *E. amylovora* in distilled water, 0.01 M potassium phosphate buffer, or rainwater after incubation at room temperature. After 90 h, the buffer became contaminated; therefore buffer sampling was discontinued.

APPENDIX C

POTENTIAL USE OF DNA FINGERPRINTING IN ECOLOGICAL STUDIES OF *ERWINIA AMYLOVORA*

POTENTIAL USE OF DNA FINGERPRINTING IN ECOLOGICAL STUDIES OF *ERWINIA AMYLOVORA*

DNA-based strain differentiation has been accomplished by analysis of restriction fragment length polymorphisms (RFLPs), and more recently, polymerase chain reaction-based techniques such as randomly amplified polymorphic DNAs (RAPDs). A relatively new technique uses repetitive extragenic palindromic (REP) and enterobacterial repetitive intergenic consensus (ERIC) sequences as primers in the PCR (13). PCR products are separated on agarose gels, and strains that are closely related produce similar banding patterns, whereas unrelated strains produce distinct patterns. REP- and ERIC-PCR technology is rapid, sensitive and widely applicable, especially among genera of enteric bacteria (6, 9, 13), making *E. amylovora* a suitable candidate.

Parts I and II of this dissertation raise two important questions related to the issue of long-distance dispersal which remain unanswered:

1. Did *strA-strB*-containing strains of *E. amylovora* arise independently in Newaygo and Van Buren counties, or do the strains share a common origin?

2. What was the original source of *E. amylovora* in the nursery?

Although hypotheses were proposed based on observations and circumstantial evidence, the questions remain unanswered. At the root of these questions specific to the dissertation research is a more general quandary:

To what extent has the long-distance spread of *E. amylovora* been mediated by commercial trade versus natural means such as birds, insects, and meteorological factors?

For the purposes of this discussion, long-distance dispersal refers to the spread of *E. amylovora* between counties, states, countries, or continents as opposed to short-distance dispersal which refers to spread within an orchard or nursery planting. Intercontinental, and in many cases, international spread of *E. amylovora* has presumably been mediated by the importation of contaminated fruit and propagation material (4, 5, 12). Circumstantial evidence suggested that migratory starlings carried *E. amylovora* from England to the European continent (2). However, when a small number of bacteria are introduced by inefficient means (*eg*: on fruit crates or birds' feet), outbreaks of fire blight probably occur long after the pathogen is first introduced to a new location. Thus, it is virtually impossible to pinpoint origins of the pathogen by the time fire blight is discovered at a new site. Consequently, implementation of quarantines with profound economic and political impact has been justified by fearful speculation based on circumstantial evidence at best, and not experimental proof.

DNA fingerprinting techniques might enable researchers to identify the origins of *E. amylovora* long after the pathogen has been introduced. The premise is that strains of *E. amylovora* emanating from a common source would be more similar genetically than strains emanating from diverse sources. Determining the genetic structure of populations of *E. amylovora* from around the world would establish the relatedness of strains from distinct locations and might reveal patterns of long-distance spread. For example, Momol and Zeller (10) hypothesized that fire blight outbreaks in Turkey in 1990 and 1991 were part of an epidemic originating in Egypt in the mid-1980s that expanded into the eastern

Mediterranean and Balkan regions. However, no explanation regarding the means of dispersal were provided. If the strains from the Mediterranean and Balkan regions did emanate from Egypt in just five years, then they would likely be genetically very similar to each other and to strains from Egypt. However, if strains were introduced from a distant location, such as the United States, then they would be more genetically similar to strains from the United States than from Egypt.

The discussions in Parts I and II of this dissertation proposed answers to the two questions that opened this section. Briefly, *strA-strB*-containing strains arose independently in Newaygo and Van Buren counties, and *E. amylovora* entered the nursery on budwood. Orchards situated between Newaygo and Van Buren counties lacked *strA-strB*-containing strains of *E. amylovora*. Such discontinuity would not be expected if meteorological factors or insects were responsible for the dispersal of resistant strains between counties. The hypothesis of independent evolution would be supported if resistant strains shared a common DNA fingerprint (banding pattern of PCR products) within a county but differed between counties. The argument would be further strengthened if genetic similarities were greater between streptomycin-resistant and -sensitive strains within than between counties. Regarding the introduction of *E. amylovora* into the nursery, the budwood hypothesis would be supported if strains of *E. amylovora* from the infected nursery trees showed fingerprints identical to those of strains from the budwood orchard, but different from those of strains from rootstock sources or neighboring commercial orchards.

The success of strain differentiation based on DNA fingerprinting requires that genetic heterogeneity between strains is detectable after several years of geographic

separation, and that strains from one location are relatively genetically homogeneous; that is, they share a characteristic DNA fingerprint. The genetic structure of *E. amylovora* as a species is poorly understood. Biochemical, physiological, and morphological analyses have been contradictory with some indicating homogeneity (3, 7) and others heterogeneity (1, 8, 11) within the species. REP- and ERIC-PCR technology would be valuable in assessing the genetic structure of populations of *E. amylovora* even if the criteria required for discrimination among strains are not satisfied.

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