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Dispersal and survival of a Pseudomonas syringae strain marked with resistance to rifampicin and nalidixic acid was studied in a maple (Acer rubrum) nursery. Experiments were conducted to monitor upward movement of the marked strain from inoculated perennial rye grass (Lolium perenne). This strain was detected during two of three experiments on medium in inverted petri plates positioned above the grass. Most colonies were detected 12 cm above the grass and several colonies were detected 33 cm above the grass. The marked strain was also isolated from maple leaves about 1 m above the inoculated grass. These data indicate that grasses can contribute a small but as yet unquantified amount of inoculum to trees. Epiphytic populations of the marked strain were established on maple twigs in July 1985 and recovered inconsistently thereafter until May 1986; the populations ranged from nondetectable to ca. 10^4 cfu/g on different sampling dates. These results show that P. syringae can

overwinter on maple twigs and potentially can be an immediate source of inoculum. Isolation of the marked strain from grass below inoculated trees at two different locations in the maple nursery indicated the marked strain dispersed downward from trees to grass.

Epiphytic populations of Pseudomonas syringae were monitored on maple trees, pear trees and grasses adjacent to them between July 1985 and September 1986. P. syringae was consistently isolated (ca. 10^5 cfu/g to 10^7 cfu/g) from perennial rye grass, orchard grass, red fescue grass, annual rye grass, and brome grass growing in plots beneath maple trees in a nursery. Perennial ryegrass between rows of trees in a pear orchard consistently yielded similar population sizes (ca. 10^4-10^7 cfu/g) of P. syringae over the same time period. In greenhouse pathogenicity tests, 55% of the isolates tested from grasses from the maple nursery were pathogenic to maple seedlings, and 29% of the isolates tested from perennial rye grass from the pear orchard were pathogenic to young pear trees. The number of P. syringae isolated from maple twigs and leaves was erratic (nondetectable to 10^5 cfu/g) over the sampling period, while the number isolated from pear was more stable and often higher (10^3 to 10^6 cfu/g) over the same time period. In greenhouse pathogenicity tests, 87% of the isolates tested from maple trees were pathogenic to maple seedlings, while 15% of the isolates tested from pear trees were pathogenic to young pear trees.

DNA profiles were used to distinguish among P. syringae isolates from different grass species and maple trees in a maple nursery. DNA profiles consisted of total bacterial DNA digested with the restriction endonuclease EcoRl and separated with gel electrophoresis. Heterogeneity among DNA profiles from 41 isolates suggested that populations of P. syringae in the nursery were genotypically diverse. Of 41 different isolates analyzed, 21 different DNA profiles were identified; 11 of the profiles were found in only one isolate, 10 of the different profiles were found in two or more isolates. Two antibiotic resistant Pseudomonas strains were inoculated to plants in a nursery and recovered over an eight month period following inoculation; all the recovered strains had DNA profiles identical to the released antibiotic resistant strains. DNA profile analysis appears to be a reliable technique to discriminate among P. syringae isolates from trees and grasses.

SURVIVAL AND DISPERSAL OF *PSEUSOMONAS SYRINGAE* IN A MAPLE NURSERY AND PEAR ORCHARD

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INTRODUCTION

The bacterium *Pseudomonas syringae* van Hall is one of nine species of fluorescent phytopathogenic *Pseudomonas*. *P. syringae* is Gram negative, polarly flagellated, obligately aerobic, and lacks cytochrome oxidase activity. The species *P. syringae* is composed of a diverse group of organisms that are distinguished primarily on the basis of host range (50). *P. syringae* is principally associated with aerial plant parts (50), although it has been isolated from air (37,53), plant debris (32) and soil (49,50). *P. syringae* is distributed widely as an epiphyte on many different plant genera and species; these include numerous gramineous and broadleaf annuals (20,32,37,38,39,54) and deciduous trees (8,10,39).

P. syringae causes widespread and economically important diseases of deciduous trees. *P. syringae* can cause serious damage, particularly to young trees, and may reduce yields from fruit trees (8,10,13,17). Common symptoms on deciduous trees are branch tip dieback, blossom blast, leaf spotting, cankers, death of entire branches or young trees, and gummosis (8,10). Disease development has been related to freezing temperatures

(45,55), rain (13), wind (13), fungal infection (48), wounds (13,17), and size of resident epiphytic P. syringae populations (13).

The disease cycle of bacterial canker of stone fruit trees has been studied more than any other disease of trees caused by P. syringae, and yet it is not completely understood. Crosse (13) describes bacterial canker of stone fruit trees as " a cyclic disease in which a winter stage in the bark of the stems and branches alternates with a summer phase in the leaves and other green tissues ". Branches and stems were susceptible to infection only between late autumn and early spring, and the summer phase of the disease began in spring as green tissues developed. Other reports showed that grasses and weeds harbored epiphytic P. syringae strains that were pathogenic to trees (19,32,46,54). However, the relative importance of potential inoculum associated with the winter stage, the summer phase and herbaceous plants has not been determined for bacterial canker and other diseases of trees caused by P. syringae.

Central to an understanding of the cycle for tree diseases caused by P. syringae is identification of the origin of cells that infect trees. P. syringae has been speculated to move from one part of a tree to another, e.g., from the winter bark phase to the summer leaf phase (8,13). It has also been suggested that epiphytic cells of P. syringae disperse to host trees from adjacent

plants, e.g., grasses, weeds, and other trees (17,32,54). None of these patterns of movement, however, has been reported conclusively to occur. Because P. syringae is so widely distributed, i.e., has been isolated from leaves, branches, cankers, flowers, herbaceous plants, water, soil, and air, it is difficult to determine the source of inoculum for infection. In addition, epiphytic P. syringae populations can be heterogenous (4,22,47) and it is not known what role the the different strains play in the disease cycle.

Evidence for sources of bacterial inoculum often has been presented either as dispersal or disease gradients (5,24,51) or by the identification of pathogen populations and host plants in proximal locations (19,32). These approaches are most appropriate when annual crops are involved and primary sources of inoculum can be identified. Gradients can be difficult to describe, and it can be hard to define the role of proximal pathogen populations for diseases of perennial crops caused by bacteria that are widely distributed. Under these circumstances, gradients can be obscured by spread of inoculum from many sources, including epiphtic populations of the pathogen on host plants.

Another approach to the study of inoculum sources involves monitoring the movement of genetically marked bacterial strains (36,55). The key to this approach is a genetic marker that: (1) can be readily selected for, (2)

does not impair pathogenic or environmental fitness and (3) is unique to the strain of interest. Antibiotic resistance is a commonly used genetic marker for the study of bacterial ecology that has been used effectively (6,16,18,55), however, instability of resistance to antibiotics and alteration of environmental fitness or pathogenicity in the mutant are potential problems that may limit its utility (16). DNA restriction fragment profile analysis is a more recently developed method that has been used to identify specific bacterial strains (15,28,43). A disadvantage of the DNA profile method is that there is no external marker or way to select specific strains on agar based medium, as can be done with antibiotic resistance markers. DNA profile markers, however, avoid the potential problems noted with antibiotic resistance, i.e., loss of antibiotic resistance and alteration of fitness, and can be applied to any bacterial isolate, not just designated marked strains.

The primary objective of this study was to determine whether epiphytic strains of P. syringae on grasses and resident strains of P. syringae on trees are sources of inoculum for maple and pear trees. To accomplish this objective, antibiotic resistance and DNA restriction fragment profiles were used to identify specific P. syringae strains and to monitor their survival and dispersal in a maple nursery. Population trends of epiphytic P. syringae on grasses and trees in a maple

nursery and pear orchard were also monitored, and the diversity of P. syringae isolates in the maple nursery was investigated by analyzing their DNA profiles.

1. Survival and Dispersal of an Antibiotic Resistant Strain of *Pseudomonas syringae* in a Maple Nursery

ABSTRACT

Dispersal and survival of a *Pseudomonas syringae* strain marked with resistance to rifampicin and nalidixic acid was studied in a maple (*Acer rubrum*) nursery. Experiments were conducted to monitor upward movement of the marked strain from inoculated perennial rye grass (*Lolium perenne*). This strain was detected during two of three experiments on medium in inverted petri plates positioned above the grass. Most colonies were detected 12 cm above the grass and several colonies were detected 33 cm above the grass. The marked strain was also isolated from maple leaves about 1 m above the inoculated grass. These data indicate that grasses can contribute a small but as yet unquantified amount of inoculum to trees. Epiphytic populations of the marked strain were established on maple twigs in July 1985 and recovered inconsistently thereafter until May 1986; the populations ranged from nondetectable to ca. 10^4 cfu/g on different sampling dates. These results show that *P. syringae* can overwinter on maple twigs and potentially can be an immediate source of inoculum. Isolation of the marked strain from grass below inoculated trees at two different

location in the maple nursery indicated the marked strain dispersed downward from trees to grass.

INTRODUCTION

Tip dieback and leaf spotting on maple (*Acer* spp.) (3,10) are common disease symptoms incited by *Pseudomonas syringae* van Hall. Incidence and severity of these symptoms vary from year to year in western Oregon. Reports from other regions have attributed outbreaks of diseases caused by *P. syringae* on deciduous trees to environmental factors (13,45) and to inoculum supply (13).

Based on earlier studies, there are at least two potential reservoirs of *P. syringae* inoculum in nurseries. One reservoir consists of the populations of *P. syringae* associated with buds, bark, wood, and leaves of host trees (6,9,17,22,33,41). These populations are suspected to be an immediate source of inoculum (13). The other reservoir includes epiphytic populations on plants, e.g., grasses, weeds, trees, and plant refuse near host trees (19,32,46,54). Grasses and weeds potentially contribute inoculum that is important to disease development on trees, however, evidence to verify suspected dispersal of *P. syringae* from grasses or weeds to trees has not been reported. The relative contribution of *P. syringae* inoculum from trees, grasses, and weeds is not known.

The purpose of this study was to investigate the potential role of grasses and host trees as sources of P. syringae inoculum for diseases of trees. This research had two primary objectives: 1) to determine if there is dispersal of P. syringae from grasses to maple trees in a nursey, and 2) determine if an antibiotic resistant strain of P. syringae survives on maple trees over a one year period.

MATERIALS AND METHODS

Selection of an antibiotic resistant strain. A P. syringae strain that was isolated in our laboratory from grass in an Oregon nursery was used for development of a marker strain. A spontaneous mutant of this strain with resistance to 100 ppm rifampicin (rif) and 150 ppm nalidixic acid (nal)(Sigma Chemical Co., St. Louis, MO) was obtained by spreading ca. 10^7-10^8 cells on antibiotic gradient plates. The antibiotics rifampicin and nalidixic acid have been used previously to mark P. syringae for ecological studies (6,18); gradient plates were made as described by Carlton and Brown (11). Initially, cell suspensions were spread on gradient plates containing King's medium B (KB medium) (29) with either a rif concentration gradient from 0 to 100 ppm or a nal gradient from ca. 0 to 150 ppm. Colonies growing nearest the side

of the plates with the highest antibiotic concentration were repeatedly subcultured onto fresh gradient plates until resistance was obtained to 100 ppm rif and 150 ppm nal. Then both antibiotics were mixed and mutants resistant to 100 ppm rif and 150 ppm nal were selected by the same procedure.

The mutant and parental strains were subsequently compared with the following determinitive tests (35). Hypersensitivity in tobacco (Nicotiana tabaccum L.`Samsun NN') was tested as described by Klement et al (30). Arginine dihydrolase activity was tested as described by Thornley (52). Fluorescense was tested by exposing colonies on KB medium to ultraviolet (UV) light at 350 nm. The antibiotic resistant P. syringae strain Pss2-3RNH will be referred to subsequently in this report as the "marked" strain.

Pathogenicity tests. The marked strain was inoculated to 6 yr old red maple trees (Acer rubrum cv Red Sunset) at the Botany and Plant Pathology Field Laboratory in October 1985. Inoculations were done with cells from 24 hr cultures suspended in water and adjusted to 10^8 colony forming units (cfu) per ml (A_{600} was 0.1 in a Spectronic 20 colorimeter, Bausch and Lomb Co, Rochester, N.Y.); 5 ul was applied at individual inoculation sites. Different trees were used for each of the following four inoculation methods: (i) 118 leaves were removed and

inoculum was applied immediately to the exposed leaf scars, (ii) 40 leaves were removed and each leaf scar was wounded by pushing the corner of a sterile razor blade 2-3 mm into the leafscar through a drop of inoculum, (iii) a drop of inoculum was applied to each of 80 leaf midveins about one third of the distance from the petiole to the apex, then a sterile razor blade was used to cut through the drop and midvein and, (iv) a drop of inoculum was placed on each of 80 petioles 1 cm from the twig and a sterile razor blade was used to cut through the drop of inoculum and petiole.

Axillary buds were counted that were killed adjacent to the inoculated leaves and leafscars, and infected wood adjacent to dead buds was sampled in May 1986 to detect the marked strain. Pieces of infected branches (1 to 2 cm long) were soaked in 10% commercial bleach 4 min, rinsed 5 min in sterile deionized water, and then split lengthwise and soaked in 1 ml sterile deionized water for 4 hr. Aliquots (0.1 ml) were spread onto KBRN medium (KB medium amended with 100 ppm rif dissolved in 6 ml DMSO, 150 ppm nal and 100 ppm chlorothalinil (Bravo)).

Experimental site. Experiments were done in a nursery plot of red maple trees (Acer rubrum L. cv. Red Sunset) interplanted with different grass species located at the Oregon State University Botany and Plant Pathology Field Laboratory about 0.8 km east of Corvallis, Oregon.

The plot contained three 60 m rows of 3-4 m tall maple trees that were planted in 1982. Trees were spaced at 0.5-1.0 m intervals in rows; rows were separated by 1.2 m. Four trees were inoculated for the survival study; the dispersal experiments were done in locations removed from the inoculated trees. The area between tree rows was planted with blocks of six different species of grass commonly planted in Oregon nurseries, perennial rye (Lolium perenne L. var Manhattan II), field brome (Bromus arvensis L.), annual rye (Lolium multiflorum Lam.), sudan (Sorghum sudanense (Piper)Stapf.), orchard (Dactylis glomereta L. var Potomac), and creeping red fescue (Festuca perenne L.). Each grass species was planted in two adjacent blocks, 10 to 15 m x 1 m, on 29 Aug. 1985; the seeding rate was ca. 40 g seed/m^2. Grasses were mowed periodically.

Inoculum preparation and application to trees. The marked strain was grown on KB medium for about 3 days at 26 C, cells were suspended in sterile deionized water and adjusted to ca. 10^8-10^9 cfu/ml. Three 1 m long branch segments were sprayed on each of four maple trees; two trees were inoculated on 28 July 1985 and two others on 5 September 1985. Inoculum suspensions were sprayed onto leaves and branches of trees with a hand-pump sprayer until all surfaces were wet. Plastic bags enclosed the branches while spraying to limit the spread of inoculum.

The trees sprayed with the marked strain will be referred to subsequently in this report as "inoculated" maple trees. All maple trees were inoculated before the grasses emerged in the adjacent plots.

Sampling and isolation from trees and grasses. Samples from trees and grasses were collected at 2 to 3 wk intervals between July 1985 and September 1986. Inoculated branches were sampled to monitor population trends of the marked strain. Branches on noninoculated maple trees that were 0.5 to 1.5 m from inoculated trees were sampled in the attempt to detect dispersal from tree to tree. Samples from all trees contained approximately equal amounts of leaves and twigs from July to October 1985 and from May to September 1986. Twigs only were sampled after leaf fall in November 1985 until April 1986. Leaves and twigs were removed from three zones (basal, middle, tip) between 1.5 and 2.0 m from soil level on all branches on each tree and were combined into one sample for each tree. Grasses in blocks were sampled from October 1985 to September 1986 in the attempt to detect dispersal from trees to grass. Grass was sampled by breaking whole grass plants off about 1.0 cm above soil level. The grass from each block was combined into one 2-4 g sample for each block of grass on each sampling date. One or two bulk samples were taken from each species. Between each sample hands and shears were cleaned with 95%

ethanol. Samples were placed into new plastic bags and most were processed within 2 hr; they were stored in darkness at 6 C if they could not be processed within 2 hr and were always processed within 5 hr.

Leaf and twig samples were washed in 125 ml erlemeyer flasks containing 40 ml sterile deionized water for 60-75 min on a rotary shaker (140 rpm). Serial dilutions up to 10^{-8} were prepared from the wash water and 0.1 to 0.4 ml of each dilution was spread in duplicate on KB and KBRN medium. When populations were high in previous samples, 0.1 ml was plated and the minumum detection limit was ca. 200 cfu per g. When populations were low, 0.4 ml were plated and the minumum detection limit decreased to ca. 50 cfu/g.

Bacterial colonies that grew on KBRN and KB medium, were fluorescent under uv light (29) and did not have cytochrome oxidase activity (31) were counted after 65-72 hr of incubation at 26 C and recorded as cfu per g tissue. Mean numbers of cfu/g recovered from each plant species were calculated with raw data and then transformed to $\log_{10}$ values before plotting. The standard errors were also calculated from raw data.

Marked P. syringae strains recovered from tree and grass samples were stored in sterile deionized water (14,27) at 6 C for further analysis. Four to six colonies from individual samples were placed into a single tube containing 1 ml sterile deionized water.

Apparatus for investigating upward dispersal.

Perennial ryegrass (*Lolium perenne*) was grown in 8 L pots (20 cm top diameter) in the greenhouse to be used as point sources of inoculum in dispersal experiments. The grass was grown in a mixture of sand, peat, soil, and perlite (1:1:1:2) and watered daily. The grass was sprayed weekly after emergence with suspensions (10^7-10^9 cfu/ml) made with ca. 48 hr cultures of the the marked strain to maintain epiphytic populations. When the experiments began the population of the marked strain was 10^7-10^8 cfu/g of grass tissue. Mean epiphytic populations were determined by washing and dilution plating one 2 to 4 g sample from each of three different pots. The pots were moved to the experimental sites immediately before the experiments began and arranged under and around the bottom rack of a wooden frame used to support petri plates. The wooden frame had three adjustable racks, ca. 12 cm, 33 cm, and 1.0 m above the potted grass, covered with poultry netting (2.5 cm wire mesh) (Fig. 1.1). Seven open and inverted petri plates containing KBRN medium were positioned on each rack so that a vertical path between the potted grass and the plates on each rack was unobstructed. The KBRN medium was amended with 50ppm cycloheximide to inhibit fungal growth. The setup with racks and pots of grass was replicated three times, and

the experiment was repeated three times (trials I, II, and III).

Experimental trials to detect upward dispersal. Trial I was done during the day on 3 September 1986 with the following protocol. Thirteen pots of grass under each wooden frame were bombarded with water droplets (1.3 cm per hr) from impact sprinklers for 5 hr. The inverted plates were replaced once with a new set during the experiment to minimize dessication of the medium. The concentration of viable marked cells in the air was measured with an Andersen particle fractioning sampler (Andersen Samplers Incorporated, Atlanta, Georgia) (2) at the lowest level of one wooden frame. The sampler was operated with an airflow of 25.6 L./min for three 30 min periods. Air flow was calibrated with an in-line gas flow meter (Mathison Gas Products, East Rutherford, New Jersey). Glass petri plates containing KBRN medium were placed on all six stages in the sampler. Twelve maple leaves were sampled individually before the pots of grass were placed out and 84 leaves were sampled after the experiment was completed; the leaves were collected from all four sides of each of the three dispersal frames. Contamination of leaves with the marked strain was guarded against by cleaning hands with 95% ethanol after collecting each sample and handling leaves by the petioles, which were removed before washing leaves.

Leaves were washed individually in test tubes containing 20 ml sterile deionized water for 4 hr on the shaker and then allowed to stand on a lab bench for an additional 22 hr to enrich small numbers of marked bacteria. After each washing period, 0.1 ml from each sample suspension was spread in duplicate on KBRN medium.

Dispersal trial II was conducted during the day on September 24, and dispersal trial III was done on the night and morning following trial II (September 24 to 25). Wind speed during trial II and III was measured with a Alnor Thermo-anemometer (Type 8500, Alnor Inst. Co., Niles, Il.). The experimental methodology was changed from trial I in the following ways: (i) 12 additional (25 total) pots of inoculated grass were placed under each rack, (ii) the Andersen sampler was not used, and (iii) bulk samples with six maple leaves in each from around dispersal frames were washed instead of single leaves, the bulk samples were washed in 40 ml sterile deionized water. Bulk samples were collected before trial II from two sides of each of the three wooden frames (six bulk samples, 36 total leaves) and one bulk sample was collected from the four sides of each frame (12 total bulk samples, 72 total leaves) after trial III. Additional bulk samples were collected from trees on all four sides of each wooden frame 6 and 13 days after trial III (12 samples with six leaves in each on each day).

Vertical flux of the marked strain from the potted grass was estimated by using the mean number of marked P. syringae colonies collected on medium in inverted petri plates at level 1 of the wooden racks. The following equation was developed to calculate the estimated vertical flux during each of the experimental trials.

$$\text{VERTICAL FLUX } (\text{cfu}/\text{m}^2/\text{min}) = (\text{cfu collected} * R)/T$$

T=time in min that the experiment was conducted
R=quotient of 1.0 m^2/total surface area of seven petri plates in m^2

RESULTS

Characteristics of strains. The marked strain, Pss2-3RNH, was compared to the wild-type parental strain, Pss2-3, with several key determinitive tests for P. syringae. Both strains induced a hypersensitive reaction in tobacco, had arginine dihydrolase activity, and were fluorescent under UV light on KB medium; neither strain had cytochrome oxidase activity.

Pathogenicity studies. Maple trees in the field that were inoculated with the marked strain during October showed symptoms of infection the following spring , i.e., death of axillary buds and blackening of bark. Symptoms developed after inoculation of leafscars, cut leafscars, and leaf midveins, but no symptoms developed where petioles were inoculated or where sterile distilled water

was used as a control (Table 1.1). The marked strain was recovered in May 1986 from internal tissues of inoculated maple branches that showed symptoms of P. syringae infection.

Population trends. Populations of the marked P. syringae strain were established on four maple trees, but recovery from inoculated twigs and leaves of maple was erratic over the duration of the experiment (Fig. 1.2). This strain was recovered from twigs on the maple trees over different periods of time, i.e., 6, 7, 9, and 10 months after the branches were inoculated. The marked strain was recovered from inoculated maple leaves up to 17 October 1985, which was about 2 months after inoculation and 1 wk before leaf drop. A total of 78 samples of inoculated leaves were processed, and the marked strain was isolated on KBRN medium from 19% of these samples, whereas miscellaneous P. syringae were isolated on KB medium from 23% of the samples. Out of 134 samples of inoculated twigs, the marked strain was isolated from 25% and miscellaneous P. syringae from 47%. The marked strain was not detected on leaves that emerged from inoculated branches in the spring of 1986.

All the fluorescent bacterial colonies that were isolated from leaves and twigs on KBRN medium appeared to have identical pigmentation, growth rates, and colony morphology. Therefore, this medium effectively prevented

growth of pseudomonads indigenous to the experimental sites. Small numbers of slow growing, white and orange, domed bacterial colonies occasionally grew on the KBRN medium but they were easily distinguished from P. syringae on the basis of pigmentation and colony morphology.

Dispersal from trees to grass and between trees. Over the experimental period from October 1985 to September 1986, 173 samples were collected from the grasses in the nursery plot and the marked strain was isolated from only eight samples (4.6%). Sudan grass yielded about 6.3×10^4 cfu of the marked strain per g on 15 October and 3×10^3 cfu/g on 17 October. Further sampling from sudan grass was not done because this grass died with the onset of cold weather. Between 250 and 3.2×10^4 cfu/g were isolated from perennial ryegrass on 15 and 17 October, 14 November, 5 and 17 January, and from annual ryegrass only on 17 October. The marked strain was not detected on grass after these dates. The perennial rye grass and sudan grass were directly below inoculated branches and the annual rye grass was about 4 m from inoculated branches. These data show that the marked strain dispersed downward from inoculated trees to grass, but lateral dispersal was very limited. No background level of P. syringae resistant to 100 ppm rif and 150 ppm nal was detectable on these same grass species before trees were inoculated nor from any of the other grass

species at any other time from October 1985 to September 1986.

Dispersal upward from potted grass. The climatic conditions during the three dispersal trials were as follows. During trial I the mean air temperature was 24 C between the rows of maple trees and the day was sunny. During trial II the day was cloudy with occasional rain showers, the mean air temperature was 13 C and wind speed was 1.2 to 2.1 m/sec. During trial III the sky was cloudy, the average air temperature was ca. 11 C and wind speed was 1.2-2.1 m/sec at the start and end of the experiment. Throughout much of trial III the wind and rain was noted visually and audibly to be stronger than it was at the beginning and end of the trial, however, the stronger wind and rain were not measured.

The marked strain of P. syringae was collected on KBRN medium placed above pots of grass, on maple leaves adjacent to the pots of grass and with the Andersen sampler, indicating that limited upward dispersal had occurred from the inoculated grass. Only small numbers of dispersed bacteria, however, were detected. Most of the cells of the marked strain, 64 in experimental trial I and 40 in trial III, were detected on medium in petri plates positioned on the lowest rack of the dispersal frame (13 cm above the potted grass). Only two colonies of the marked strain were detected at the level of rack 2 (33 cm

above the potted grass) and on only one plate during trial III. Only two colonies of the marked strain were detected on medium in the Andersen sampler. Both of those colonies were recovered on agar plates at the top stage of the sampler where particles $\geq$ 8 um are captured (2).

The numbers of viable cells detected at levels one and two of the wooden frame were used to estimate vertical flux of the marked strain. Estimated values for vertical flux at level 1 were 1.9 cfu/m^2/min and 0.39 cfu/m^2/min during trials I and III, respectively. Vertical flux at level 2 in trial III was estimated to be 0.06 cfu/m^2/min. Vertical flux at level 3 could not be estimated because no bacteria grew on the medium placed at level 3, which was ca. 1.0 m above the potted grass and at about the same level as the lowest maple branches.

The percent marked P. syringae that dispersed upward during the three experimental trials was calculated. The estimates for vertical flux at level 1 and the total population of marked bacteria present on potted grass under dispersal frames were used for the calculation. In all three experimental trials, the populations of the epiphytic marked strain on the potted grass were $1x10^8$ cfu/g, and the total population of the marked strain on all grass in all pots under each dispersal frame was ca. $1x10^{11}$ cfu. It was estimated that $1.29x10^{-8}$ percent of the total cells available for dispersal moved vertically through a square meter at level 1 in trial I, and $2.7x10^{-9}$

%/m^2/min. dispersed vertically during trial III. These estimates indicated that few of the cells dispersed upwards from the grass. In addition, there was no detectable quantitative difference in the populations of the marked strain on potted grass before and after the experiments; this is further evidence for a low rate of dispersal.

Upward dispersal of the marked strain from potted grass to trees occurred, but was observed only under the more severe weather conditions encountered during experimental trial III. The marked strain was isolated from 5 of 12 bulked leaf samples which were washed extensively (26 hr). Leaf samples collected adjacent to potted grasses 6 and 13 days after trial III yielded no marked P. syringae, even after 26 hr of washing. None of the 84 leaves washed after trial I yielded the marked strain. Additional sampling from maple trees adjacent to the dispersal frames and from other locations in the nursery before and after the experiment indicated that no background population of rif and nal resistant P. syringae was detectable.

DISCUSSION

Data from the experiments that were done to monitor survival and dispersal of the marked strain in a maple nursery suggested: (a) dispersal of P. syringae strains

from one location to another was infrequent and (b) epiphytic populations of P. syringae can survive on maple twigs from July to May and on leaves from July to leaf fall in October.

The fact that the marked strain was isolated only part of the 14 month sampling period after the trees were inoculated implies that either the marked strain no longer survived, or population levels fell below the lower limits of the detection methods, i.e., below 50 to 200 cfu/g. Survival of the marked strain on twigs from July to May shows that pathogenic P. syringae strains can overwinter on maple twigs and may provide an immediate source of inoculum for young susceptible tissues in the spring.

Data presented in this report indicate that grasses are a minor source of inoculum for infections on trees. The number of dispersed bacteria detected in this study was low. Several factors may account for this limited dispersal. For example: (i) P. syringae appeared to be adsorbed strongly to grass surfaces, as reported for P. syringae pv. lachrymans (23,34), and few cells may have been released; (ii) the distances between the potted grass and the higher level of inverted petri plates and maple tissues may have been so great that cells of the marked strain which were dispersed were diluted excessively or survived poorly during dispersal, thus making detection difficult; and (iii) the marked strain may have survived poorly on the leaf and twig surfaces and failed to

colonize these tissues after landing on them. The small amount of dispersal detected and a lack of detectable differences in populations of the marked P. syringae strain on grass before and after these experiments suggests the bacteria were strongly adsorbed to the grass and few were removed.

Bacterial inoculum is thought to be dispersed to plant surfaces primarily by aerosols and rainsplash (26). According to Harrison (32), there are three primary mechanisms that generate aerosols of phytopathogenic bacteria from plant tissues: (1) impaction of raindrops, (2) impaction of water from sprinkler irrigation systems, and (3) mechanical pulverization of plant tissues. Lindemann et al (37) reported that plant canopies can be a major souce of airborne bacteria on dry sunny days when no water or any known mechanical forces except wind disturbed the plant canopies. In this study, dispersal experiments were done during rainfall, during sprinkler irrigation and during a dry sunny period before sprinker irrigation had wet all the grass. In all cases few or no airborne bacteria were captured. Nearly all the airborne bacteria were detected only about 12 cm above the potted grass. These data suggest that most of the bacteria were dispersed in ballistic particles generated by rainsplash, and were not dispersed in aerosols. In addition, the bacteria collected on medium in the Andersen sampler were

on the top stage, which is where ballistic particles would most likely be captured (53).

To my knowledge, this is the first evidence reported that shows upward dispersal of P. syringae can occur from grasses to trees and to intermediate heights between grasses and trees. Several studies have collected data on dispersal of bacteria, but none of those studies was done in a nursery or orchard environment. Two of the studies reported results that contrast with those reported here. Graham et al (21) studied the generation of aerosols of Erwinia carotovora var atroseptica after impacting infected potato stem pieces with water drops in laboratory experiments and reported that water drop impaction was not an effective way to generate aerosols. Only about 0.003% of the bacteria available for dispersal on potato stems were detected as viable aerosol propagules during their 30 min experimental periods. In contrast, calculations with data reported here estimate the proportion of total bacterial populations converted from epiphytes to viable aerosol propagules was about 1.9×10^3 times lower (ca. 1.6×10^{-6}%/m^2/experimmental period). This lower percentage could be due to a combination of factors, for example the kind of plant tissue (37), variable adsorption to plant tissues by different species of bacteria and the experimental methodology for detection of dispersed bacteria. In experiments done near Wisconsin wheat fields (37), the vertical flux of P.syringae was measured with

Andersen samplers over a 24 ha wheat canopy to be 102 cfu/m^2/min. The vertical flux estimates from Wisconsin were about 50 to 250 times greater than those reported here, but different methods were used. The sets of potted grass used in experiments reported here were more protected from wind and were much smaller than the wheat field in Wisconsin; these differences might contribute to the contrasting values.

The data from this study suggest that active dissemination of detectable populations of bacteria in the maple nursery was limited. In addition to the limited dispersal from grass to trees, the following results suggest the marked strain tended to remain or survive in detectable numbers only where it was placed initially : (a) few bacteria that dispersed from trees to grass were detected, and even after some cells were detected they did not remain in detectable numbers on the grass beyond 1 to 4 months, (b) dispersal of bacteria from the inoculated maple trees to adjacent trees was not detected, and (c) the marked strain was not detected on new leaves that emerged from branches inoculated the previous growing season.

The results from this study support the hypothesis that both trees and grasses may contribute P. syringae inoculum for infection of trees. However, epiphytic populations on trees are potentially the most important source because they are an immediate source of inoculum.

The contribution from grasses appears to be small and is thus far unquantified. The relative importance of these two potential sources remains undefined. To conclusively prove that either grasses or trees contribute inoculum which is important to initiation of disease on trees, however, marked P. syringae strains that have been established on grasses and trees must be isolated from natural infections on trees. This confirmation remains to be done.

Table 1.1. Results of four methods for inoculation of field grown maple trees with *Pseudomonas syringae*

Inoculation Method[a]	Number of Sites Inoculated	% bud death[b]
leafscar	118	12.7
incised leafscar	40	55.0
midvein	80	8.8
petiole	80	0

(a) See materials and methods for description of inoculation methods.
(b) Axillary buds killed adjacent to inoculated leaves or leafscars.

Fig. 1.1. Apparatus used to detect upward dispersal of antibiotic resistant Pseudomonas syringae from potted grass. Open and inverted petri plates containing KBRN medium (KB medium amended with 100ppm rifampicin and 150 ppm nalidixic acid) were placed on each rack.

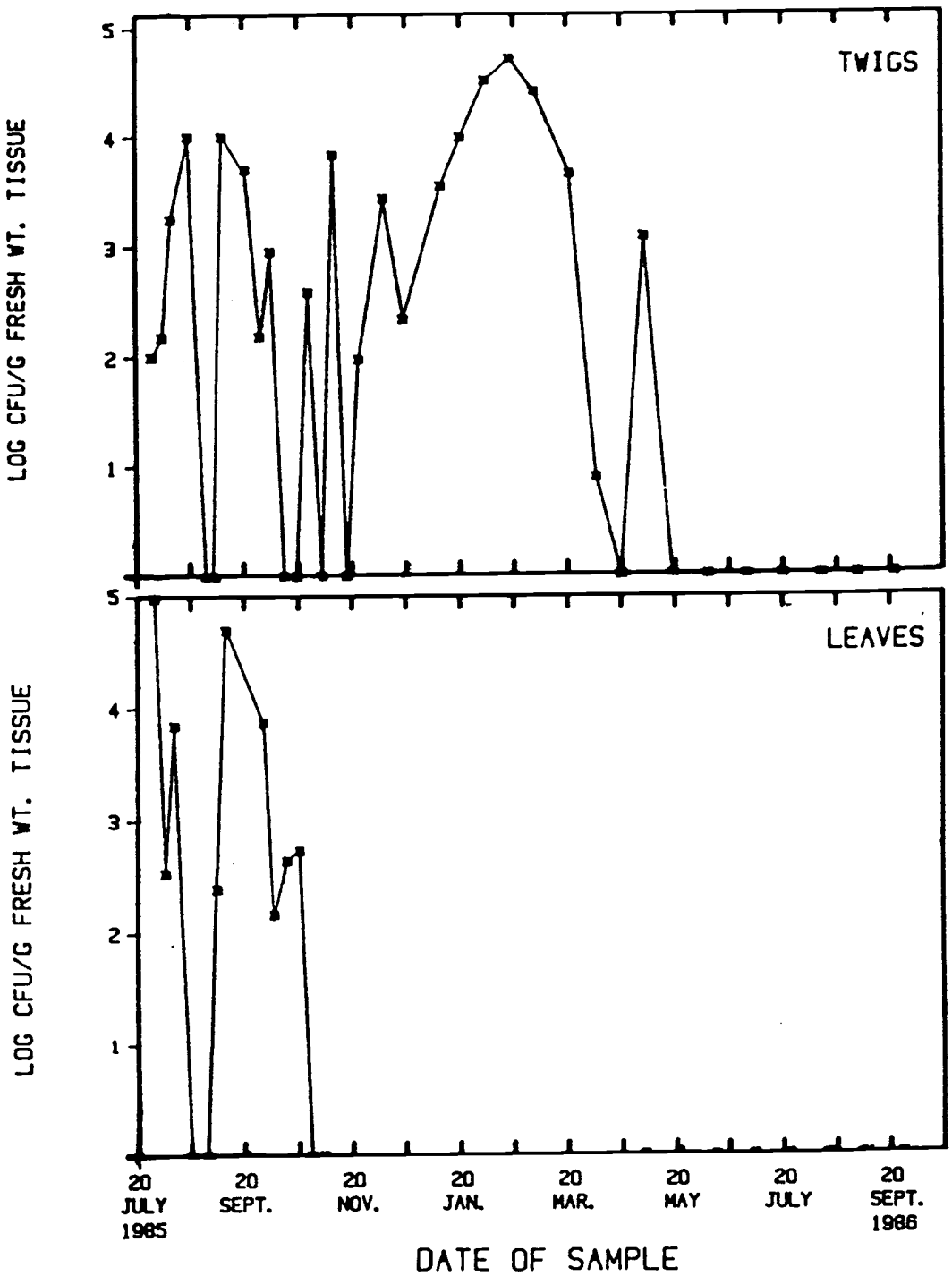

Fig.1.2. Number of epiphytic antibiotic resistant Pseudomonas syringae recovered from twigs and leaves of inoculated maple trees during the period July 1985 to September 1986. Samples were collected at 14-21 day intervals. Leaves fell from the trees between 24 and 31 October. The range of standard errors is 1.9 to 4.8 $\log_{10}$ colony forming units (cfu)/g.

2. Population Dynamics of *Pseudomonas syringae* on Maple Trees, Pear Trees and Grasses

ABSTRACT

Epiphytic populations of *Pseudomonas syringae* were monitored on maple trees, pear trees and grasses adjacent to them between July 1985 and September 1986. *P. syringae* was consistently isolated (ca. 10^5 cfu/g to 10^7 cfu/g) from perennial rye grass, orchard grass, red fescue grass, annual rye grass, and brome grass growing in plots beneath maple trees in a nursery. Perennial rye grass between rows of trees in a pear orchard consistently yielded similar population sizes (ca. 10^4-10^7 cfu/g) of *P. syringae* over the same time period. In greenhouse pathogenicity tests, 55% of the isolates tested from grasses from the maple nursery were pathogenic to maple seedlings, and 29% of the isolates tested from perennial rye grass from the pear orchard were pathogenic to young pear trees. The number of *P. syringae* isolated from maple twigs and leaves was erratic (nondetectable to 10^5 cfu/g) over the sampling period, while the number isolated from pear was more stable and often higher (10^3 to 10^6 cfu/g) over the same time period. In greenhouse pathogenicity tests, 87% of the isolates tested from maple trees were pathogenic to maple seedlings, while 15% of the isolates

tested from pear trees were pathogenic to young pear trees.

INTRODUCTION

Pear blast (Pyrus spp.) (8) and tip dieback and leaf spot of red maple (Acer rubrum) (3,10) are common diseases caused by Pseudomonas syringae van Hall in Oregon. These and other diseases of deciduous trees caused by P. syringae are economically important in Oregon nurseries and orchards (8,10), and nursery workers report that chemical control methods are often not successful. A recurring question related to control, both among nursery workers and researchers, concerns the sources of P. syringae inoculum. Grasses grown as a ground cover between rows of woody plants in nurseries and orchards are one potential source of inoculum (19,32,46). Epiphytic populations of P. syringae on host trees are suspected to be another source of inoculum (6,17,41). Neither the quantities of inoculum available on grasses and trees nor the amount of inoculum provided to host trees from these different potential sources has been determined.

This study was initiated to compare the quantity of P. syringae inoculum available on maple trees, pear trees and grasses over a one year period.

MATERIALS AND METHODS

Experimental sites. Experiments were performed at two different sites. Site A was the same nursery with maple trees and six species of grass described in Chapter 1. Eight maple trees were used to study populations of P. syringae. Site B was an orchard of mixed varieties of pear (Pyrus communis.) and apple (Malus spp.) trees at the Oregon State University Lewis-Brown Horticulture Farm located about 1.6 km east of Corvallis, Oregon. Epiphytic populations of P. syringae were monitored on three different pear trees, cultivars Eldorado, Honeysweet, and Winter Nelis. The pear trees were planted in 1980 and perennial ryegrass grew between rows of pear trees. Grasses at sites A and B were mowed periodically.

Sampling, Isolation, and Characterization of P. syringae. Samples from trees and grasses at sites A and B were collected at 2 to 3 wk intervals between 28 July 1985 and 18 September 1986. To isolate P. syringae, samples of leaves, twigs, and grasses were collected, washed and plated on King's medium B (KB medium) (29) as described in Chapter 1.

Bacterial colonies that were fluorescent under ultraviolet (UV) light (29) (wavelength 350 nm) were counted after 65-72 hr of incubation at 26 C. When the number of fluorescent colonies on an individual plate was 10 or less, all fluorescent colonies were tested for

cytochrome oxidase activity (31). When the number was greater than 10, 10 colonies with characteristic P. syringae morphology were tested for cytochrome oxidase activity, and the percent cytochrome oxidase negative colonies in a subsample was used to estimate the total number of fluorescent, cytochrome oxidase colonies for each sample. The fluorescent, cytochrome oxidase negative colonies were labelled as P. syringae. Mean number colony forming units (cfu) of P. syringae isolated from each plant species was calculated with raw data and then transformed to $\log_{10}$ values before plotting. The standard errors were also calculated with raw data.

P. syringae isolates from tree and grass samples were stored in sterile deionized water at 6 C for pathogenicity tests and further analyses (14,27). One to three colonies of each fluorescent, cytochrome oxidase negative colony type (which differed morphologically from all other types) were selected from each isolation plate. These colonies were stored together in a single tube with 1 ml sterile deionized water.

Pathogenicity tests. One hundred forty two P. syringae isolates from grasses, pear trees, and maple trees were inoculated to young maple or pear trees. Isolates selected for pathogenicity tests were chosen randomly from cultures that were stored at different times during the population studies. Pure cultures of P.

syringae were obtained by repeated subculturing of single colonies onto KB medium. Pure isolates for pathogenicity tests were grown on KB medium for 35 to 40 hr at 26 C and colonies were suspended in sterile deionized water; cell concentrations were adjusted to 10^7-10^8 cfu/ml (A_{600} was 0.04 to 0.05 in a Bausch and Lomb Spectronic 20 colorimeter).

Six month old red maple trees (Acer rubrum L. cv. Red Sunset) with ca. 0.5 cm caliper were used for pathogenicity tests with the isolates from maple trees and grasses in the nursery. The maple trees were planted in a pasteurized soil mix containing equal parts of sand, peat and loam, they were watered daily and grown in a greenhouse at 23 C day and 21 C night. Pear trees (Pyrus communis L. cv Magnus) on Old Home x Farmingdale rootstocks were used for pathogenicity tests with the isolates from pear trees and grasses from the orchard plot. Pear trees with 1.25 cm caliper were obtained as dormant bare-rooted whips in June 1986. Branches were pruned to 0.3-0.4 m length, roots were pruned to fit into 8 L plastic pots and the trees were planted in a mixture of sand, peat, soil, and perlite (1:1:1:2). Trees were grown outdoors until 2 wk before inoculations in October 1986 when they were moved into a greenhouse with temperature and watering as described for the maple trees. Four wk after inoculations the pear trees were moved outdoors into rainy and cool (10-15 C) weather in an

attempt to stress the plants and promote infection; after 10 days they were moved back into the greenhouse.

Stems of young maple and pear trees were inoculated in the greenhouse between 4 and 19 October 1986. The inoculum suspensions (10 ul) of individual P. syringae strains were applied to one leaf scar on three different maple or pear trees immediately after leaves were detached. A known pathogenic P. syringae strain and sterile deionized water were applied to leafscars on each inoculated tree as positive and negative inoculation controls, respectively. A sterile scalpel tip was then inserted through the drop of inoculum and 2-4 mm into the leaf scar, and the inoculation site was wrapped with Parafilm (American Can Company, Greenwich, CT). This inoculation procedure introduced 10^4 to 10^5 cfu of P. syringae at each site. Number of lesions (measure of pathogenicity) and size (measure of virulence) were recorded 21 and 44 days after inoculations to maple and pear, respectively.

Surface area of grass and twigs. The ratio of surface area to mass was estimated for grasses and twigs. This was done to compare the amount of surface area that is potentially available on each gram of each kind of tissue for colonization by P. syringae. Lengths of maple and pear twigs were measured with a ruler, and diameter was measured with a vernier caliper. Length was

multiplied by circumference to obtain total surface area of individual twigs. Nine composite samples consisting of 4-16 twigs (2 to 4 g) were measured and the mean surface area (cm^2) per gram was calculated for all nine samples. The surface area of annual rye and perennial rye was estimated with a △ T Area Meter (Delta-T Devices, Cambridge, England). Three flat objects with different dimensions and color intensities were used for calibration. The total surface area of six 0.3-1.0 g composite samples of both grass species were measured and the mean surface area (cm^2) per gram was determined for both species.

RESULTS

Population trends on maple and pear trees.

Epiphytic populations of P. syringae were monitored on maple trees and pear trees from July 1985 to September 1986. The number of P. syringae isolated from the eight maple trees was variable over this sampling period (Fig. 2.1). During the first 4 months of sampling, P. syringae was isolated only once (6 September) from one maple tree. On 7 Nov. 1985, however, two weeks after leaf fall, P. syringae was isolated from seven of the eight maple trees (mean number isolated was 228 cfu/g of twig tissue). The number of P. syringae isolated thereafter from maple trees remained at that level or increased until 5 June 1986 when

populations fell below detectable levels. Populations remained at nonndetectable levels until 28 August 1986 and then increased over the next two sampling times up to the last sampling on 18 September 1986.

In contrast to the maple trees, P. syringae was recovered from the pear trees more frequently and in higher numbers over the same period (Table 2.1, Fig. 2.2). During the period 5 September 1985 to 19 September 1986 the mean number of epiphytic P. syringae isolated from the three pear trees was consistently greater than 1.5×10^3 cfu/g. The populations of P. syringae on the pear trees never dropped to nondetectable levels as they did on the maple trees.

Disease symptoms were not observed on the maple trees at any time over the sampling period. Symptoms characteristic of bacterial blight caused by P. syringae, e.g., necrosis on leaves and branch tips, were observed in April on one of the three pear trees that were sampled (cv Eldorado).

Population trends on grasses. Brome grass, red fescue, perennial rye grass, orchard grass, and perennial rye grass consistently yielded high, usually 3×10^5 to 10^7 cfu/g, populations of P. syringae from October 1985 to September 1986; the population was always greater than 3×10^3 cfu/g (Figs. 2.3 and 2.4). The populations of P. syringae on all five species of grass were similar and

followed the same trends. Sudan grass died with the onset of cold weather and was not sampled after 14 November. Most of the samples from grass (96%) yielded P. syringae. (Table 2.1).

The isolation of P. syringae from grasses in the maple nursery was similar in quantity and consistency to isolation from pear (Table 2.1). The perennial rye grass in the pear orchard plot consistently yielded P. syringae (Table 2.1); the population size was usually 10^4-10^7 cfu/g.

Pathogenicity studies on maple and pear trees. The P. syringae isolates from maple were more pathogenic (more caused infections) and more virulent (incited larger lesions) on young maple trees in the greenhouse than the P. syringae isolates from the grasses in the maple nursery (Table 2.2). In contrast, few of the P. syringae isolates from pear trees or grasses in the pear orchard infected pear trees (Table 2.2). Unlike the infections on maple, no clear distinction could be made between levels of virulence for the pear inoculations. Consequently, the P. syringae isolates inoculated to pear were simply rated as pathogenic or nonpathogenic. No symptoms occurred on any noninoculated sites or where water was used for control inoculations on young maple or pear trees.

Surface area of twigs and grasses. The higher epiphytic populations recorded for grass may result in part from a greater ratio of surface area:mass for grass than twigs. Populations reported on a cfu/g basis do not reflect the surface area differences. The surface area per g for perennial rye grass, annual rye grass, maple twigs, and pear twigs was estimated to be 46±9, 36±8, 11±2, and 8±1 cm^2/g, respectively. The measurements for grass must be considered estimates because the small size and varying color intensity of grass pieces affect the accuracy of measurements recorded by the area meter. Surface area measurements provided a mean estimated ratio of 5.4 for grass surface area per g:maple twig surface area per g. Therefore, each gram of grass has ca. 5.4 times more surface area for P. syringae to populate than each gram of twig tissue. This value does not seem large enough, however, to account for the differences in populations of P. syringae isolated from the grasses and trees.

DISCUSSION

The population size of P. syringae on grasses was consistently higher and more stable than that on maple trees over the 11 month period when both were sampled simultaneously. The differences in populations of P. syringae on trees and grasses were most marked in the

summer. In summer, high populations occurred on grass when none were detected on maple leaves and twigs. The pathogenicity studies showed that maple trees and grasses both supported pathogenic P. syringae populations. Therefore, grasses supported much larger quantities of P. syringae inoculum than the maple trees over the time this study was conducted.

In contrast to the low populations on maple trees and the high populations on grasses in the nursery, the populations of P. syringae on pear trees and grasses in the orchard plot were at similar high levels. This suggests that pear trees and grasses provide a better habitat for survival and multiplication of P. syringae than maple trees over much of the year. Furthermore, the role of tree and grass inoculum reservoirs may be different for different kinds of trees.

In this study, samples of twigs and leaves from individual trees and plots of grass were bulked before assaying bacterial populations. Due to the lognormal distribution of epiphytic bacterial populations on individual leaves and twigs, bulked samples will overestimate the mean population on individual leaves and twigs (25). A more accurate estimate of the true population mean would likely result if leaves and twigs were sampled individually. Rough estimates of the P. syringae populations were desired in this study, however, and bulked samples can fulfill this objective (25). In

addition to analyzing individual leaves or twigs, the population estimate will be more accurate if the population variance is constant and the number of leaves or twigs is increased per bulk sample (25). The number of leaves in the samples from grass was much higher than the number of leaves or twigs in samples from maple trees. The populations recorded for grass may have been more constant than the populations on the trees because the population estimates approached the mean more closely for the grasses. Pear tissues were sampled the same as the maples but had populations of P. syringae that were similar to the grasses. This suggests that the variance of the populations (perhaps due to habitat differences) might have influenced the population means more than the number of leaves or twigs per sample.

The population fluctuation on maple and pear trees with a high peak in spring and a low level in summer is similar to that reported for P. syringae on stonefruit trees in Washington state, U.S.A. (22) and Victoria, Australia (57). Wimalajeewa (57) suggested that population levels of epiphytic bacteria are influenced most by the availability of substrates for growth, while rainfall and temperature have secondary effects. The availability of substrates probably increases in spring as dormancy breaks in the trees. In Victoria, low populations of epiphytic P. syringae in summer were attributed to low humidity, high temperatures, and high UV

irradiation (57). It is likely that the same environmental factors in Oregon could affect the epiphytic populations of P. syringae on trees in the summer.

In Oregon, the highest incidence of P. syringae infections occurs on woody nursery plants in the spring, which was the time when population levels of P. syringae were highest on maple and pear trees in this study. The significance of the high populations of epiphytic P. syringae on grasses throughout most of the year remains largely undefined. Results from another study (Chapter 1) indicate that P. syringae can disperse from grasses to trees, however, the effect of this dispersal on populations of P. syringae on trees has not been determined.

Table 2.1. Isolation frequency of epiphytic Pseudomonas syringae from maple trees, pear trees and grasses between July 1985 and September 1986

Plant/location	Number of Samples	% that yielded P. syringae
maple[a]/nursery	262	38
grass[b]/nursery	173	96
pear[a]/orchard	90	95
grass/orchard	60	100

(a) Samples from maple and pear trees consisted of twigs between November and April and a mixture of leaves and twigs at other times.
(b) This included samples from all six species of grass planted in the maple nursery plot.

Table 2.2. Pathogenicity and virulence of *Pseudomonas syringae* isolates from maple trees, pear trees and grasses on young maple and pear trees in the greenhouse

Source of isolates/ location	Number of isolates tested	Pathogenic[a] isolates (%)	Virulence rating of isolates[a,b] 0	1	2+
maple/nursery	47	87.2	12.8	42.6	44.6
grass/nursery	42	54.8	45.2	35.8	19.0
pear/orchard	39	15.4	33	0	6
grass/orchard	14	28.6	10	0	4

(a) Pathogenicity and virulence of isolates from the maple nursey and pear orchard were tested on stems of young maple and pear trees, respectively.
(b) Levels of virulence correspond to size of lesions; 0: no necrosis at inoculation site; 1: brown necrosis limited to 1 mm around inoculation site; 2+: black sunken lesions, >1 mm around inoculation site.

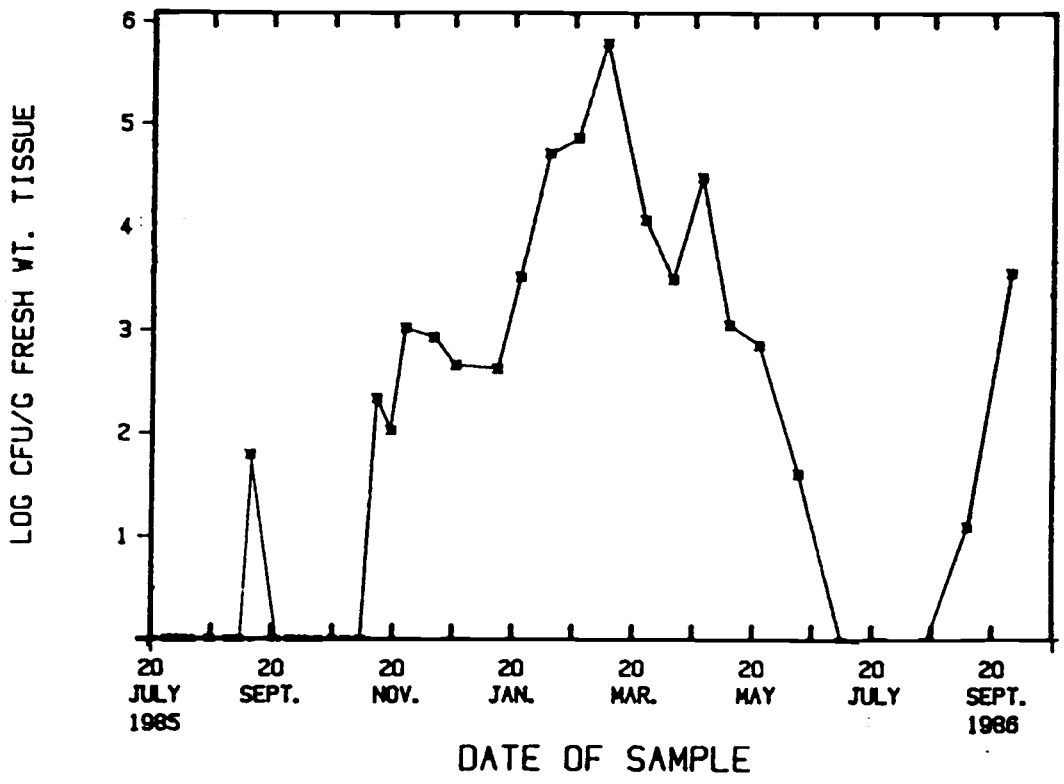

Fig. 2.1. Number of epiphytic Pseudomonas syringae isolated from leaves and twigs of maple trees during the period July 1985 to September 1986. Samples were collected at 14-21 day intervals. No leaves were present on trees from November to May. The range of standard errors is 1.1 to 5.5 $\log_{10}$ colony forming units (cfu)/g.

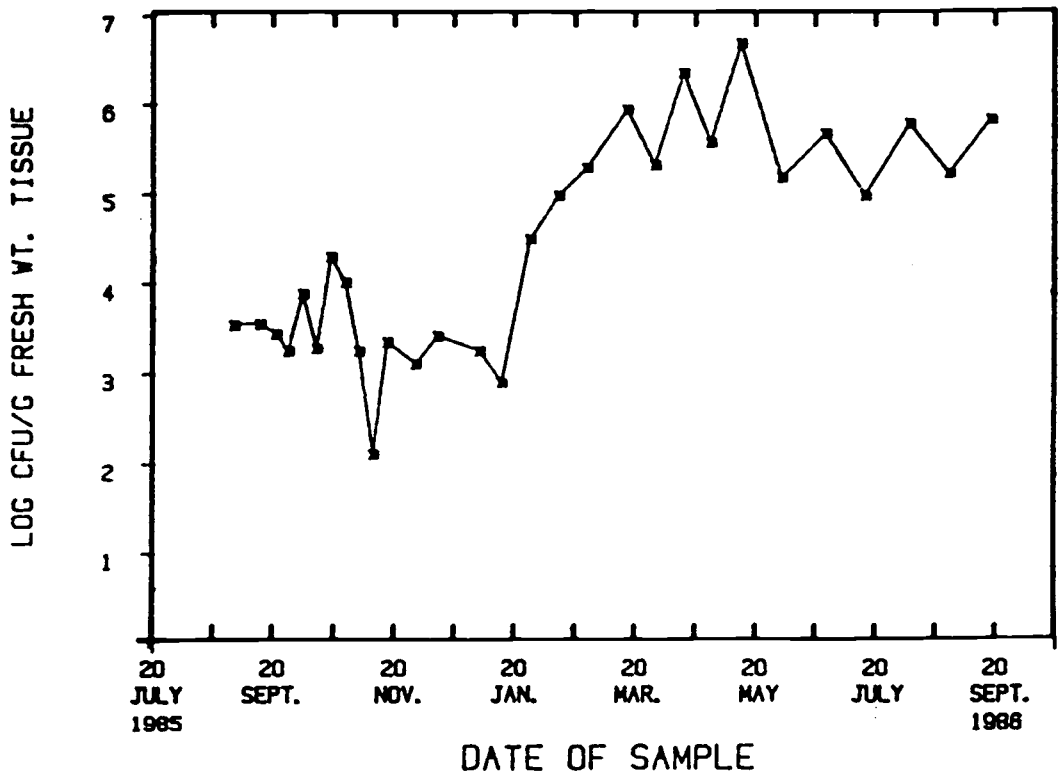

Fig. 2.2. Number of epiphytic Pseudomonas syringae isolated from leaves and twigs of pear trees during the period September 1985 to September 1986. Samples were collected at 14-21 day intervals. No leaves were present on trees from November to April. The range of standard errors is 2.1 to 6.9 $\log_{10}$ colony forming units (cfu)/g.

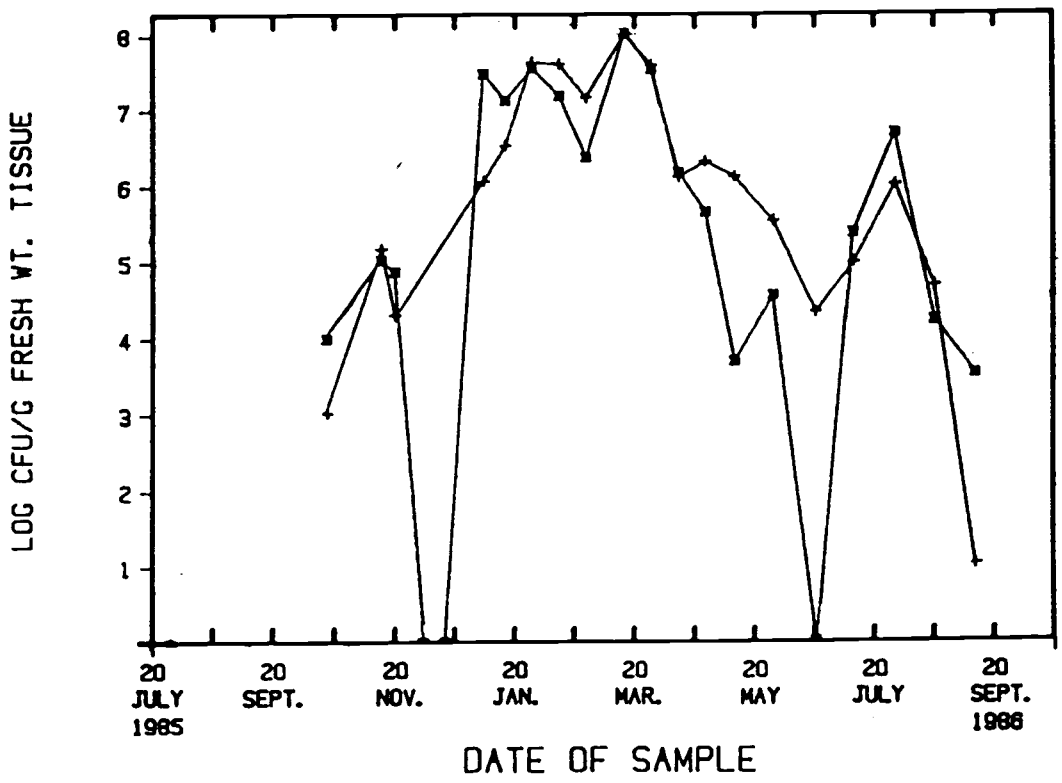

Fig. 2.3. Numbers of epiphytic Pseudomonas syringae isolated from leaves of brome grass (-■-) and red fescue grass (-+-) from plots located in a maple nursery during the period October 1985 to September 1986. Samples were collected at 3 to 4 week intervals. The range of standard errors is 2.3 to 7.6 $\log_{10}$ colony forming units (cfu)/g.

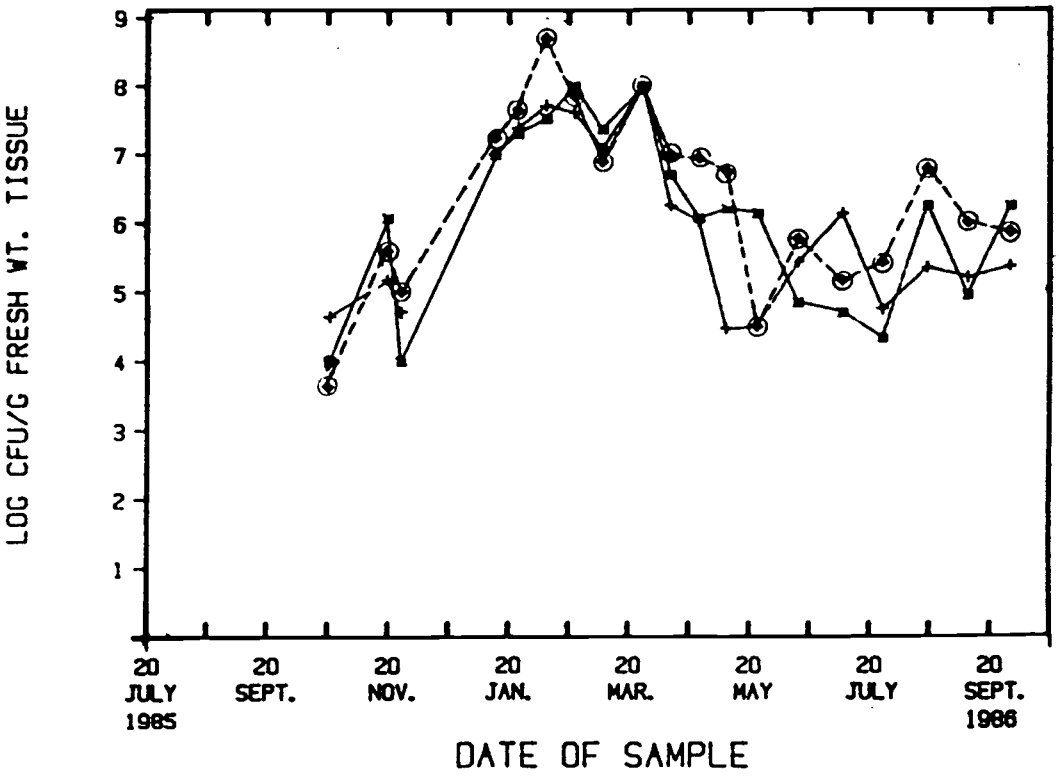

Fig. 2.4. Numbers of epiphytic Pseudomonas syringae isolated from leaves of perennial ryegrass (-■-), orchard grass (-+-) and annual ryegrass (-⊕-) from plots located in a maple nursery during the period July 1985 to September 1986. Samples were collected at 3 to 4 week intervals. The range of standard errors is 3.6 to 8.3 $\log_{10}$ colony forming units (cfu)/g.

3. Diversity of *Pseudomonas syringae* in a Maple Nursery as Determined with DNA Restriction Fragment Profiles

ABSTRACT

DNA profiles were used to distinguish among *P. syringae* isolates from different grass species and maple trees in a maple nursery. DNA profiles consisted of total bacterial DNA digested with the restriction endonuclease EcoR1 and separated with gel electrophoresis. Heterogeneity among DNA profiles from 41 isolates suggested that populations of *P. syringae* in the nursery were genotypically diverse. Of 41 different isolates analyzed, 21 different DNA profiles were identified; 11 of the profiles were found in only one isolate, 10 of the different profiles were found in two or more isolates. Two antibiotic resistant *Pseudomonas* strains were inoculated to plants in a nursery and recovered over an eight month period following inoculation; all the recovered strains had DNA profiles identical to the released antibiotic resistant strains. DNA profile analysis appears to be a reliable technique to discriminate among *P. syringae* isolates from trees and grasses.

INTRODUCTION

Reduction of inoculum populations is an important part of management practices for control of diseases caused by Pseudomonas syringae van Hall on trees. To achieve the greatest success, however, inoculum sources should be identified. This is a problem in nurseries and orchards that is complicated by phenotypic diversity and apparent ubiquity of P. syringae populations. When there are mixed populations of P. syringae distributed throughout an orchard or nursery it is difficult to determine which of those populations are responsible for infection on trees. The occurrence of phenotypically heterogenous populations of P. syringae in nurseries and orchards has been shown with various biochemical, physiological and pathogenicity tests (22,47, L. W. Moore, personal communication). Evidence for widespread, i.e., apparently ubiquitious, distributions of P. syringae has been obtained by isolating this bacterium from gramineous plants and broadleaf annuals (20,38,39), woody plants (8,10,39), plant debris (32) and soil (49).

This diversity and apparent ubiquity of P. syringae emphasizes the need for a method to distinguish between strains if specific sources of inoculum are to be identified. Restriction endonuclease analysis of total cellular DNA has been reported to be an accurate method to distinguish between different bacterial strains (7,15,43).

For example, this technique has been used to characterize strains of Rhizobium (43), and has allowed differentiation of Erwinia carotovora pv. carotovara strains to the subpathovar level (15). The distribution of several bacterial pathogens of humans and animals has also been successfully followed with restriction endonuclease analysis (7,28,42,43).

The objectives of this study were: a) to use DNA restriction fragment banding pattern analysis (hereafter referred to as DNA profiles) to distinguish among P. syringae isolates from maple trees and grasses in a nursery and b) to determine if DNA profile analysis is a useful tool to study survival and dispersal of P. syringae. In addition, DNA profiles were used to investigate genomic diversity of indigenous P. syringae strains and to verify the identity of antibiotic resistant P. syringae strains recovered from trees and grasses. In this paper the term 'isolate' refers to any single culture of P. syringae recovered from the maple nursery; different isolates can be genotypically the same or different. The term 'strain' refers to any pure culture of P. syringae that can be distinguished from other pure cultures on the basis of at least one known characteristic, e.g., DNA profiles or surface antigens.

MATERIALS AND METHODS

Bacterial strains. The P. syringae cultures used for DNA profile analysis were isolated between September 1985 and May 1986 from maple trees and grasses in the nursery plot described in Chapter 1. Five different groups of Pseudomonas isolates (94 total isolates) were analyzed. Group I was composed of 41 P. syringae isolates collected on King's medium B (KB medium) (29). Group II contained 18 strains isolated on KB medium supplemented with 100 ppm rifampicin (rif) and 150 ppm nalidixic acid (nal) (KBRN medium). The group II strains were recovered from trees that were inoculated initially with the antibiotic resistant P. syringae strain (Pss2-3RNH), or from grasses adjacent to inoculated trees (Chapter 1). Group III was composed of 15 strains recovered on KB medium (without antibiotics) that had DNA profiles identical to the antibiotic resistant strain (Pss2-3RNH) released into the nursery (Chapter 1). The isolates in groups IV and V were not species P. syringae; they were included in the analyses for testing uniqueness and stability of DNA profiles. Group IV contained 16 oxidase positive, fluorescent pseudomonad strains that were resistant to 50 ppm rif and 100 ppm tetracycline-hydrochloride (tet). The group IV isolates were recovered between October and April from grasses that were spray inoculated initially with the pseudomonad strain PAN8/82RT that was resistant to these

antibiotics. The antibiotic resistant strain, PAN8/82RT, was selected as a spontaneous mutant from suspensions of the wild-type strain PAN8/82 by using the methods described in Chapter 1. Group V contained four cytochrome oxidase positive, fluorescent Pseudomonas isolates from grass and maple trees in the maple nursery.

Preparation and growth of bacteria. Colonies of fluorescent, oxidase negative pseudomonads isolated from tree or grass tissues were stored at 6 C in tubes of sterile deionized water (27) until the time of DNA analysis. The isolates were then streaked repeatedly onto KB medium for purity. Representative isolates distinguishable on the basis of pigmentation and colony morphology were selected for DNA profile analysis.

Single colonies from pure cultures were transferred into 6 ml of Yeast-Dextrose-Peptone (YDP) liquid growth medium (0.4% yeast extract, 2.0% glucose, 0.4% peptone II, 0.5% ammonium sulfate; pH=7.0) (44) and incubated on a rotary shaker (140 rpm) at 27-29 C for 24 hr. After this growth period the A_{600} was ca. 0.9-1.2., as measured in a Bausch and Lomb Spectronic 20 colorimeter.

DNA extraction. Total cellular DNA was extracted from the bacterial strains using a procedure described by D. Drahos (Monsanto Corp., personal communication). DNA extraction was done in autoclaved 1.5 ml polypropylene

micro centrifuge tubes (American Scientific Products, McGaw Park, IL.). About 0.9 ml of bacterial liquid culture was transferred to micro centrifuge tubes and spun 2 min at 15,000 rpm in an Eppendorf micro centrifuge (Brinkman Insts., Westbury, N.Y.) at 6 C. The cell pellet was resuspended in 0.25 ml Tris-EDTA-glucose (TEG) buffer (10 mM Tris-HCl, pH=8.0; 50 mM EDTA, pH=8.0; 1% glucose) with a vortex mixer (Van Waters & Rogers Co., U.S.A.). Ten ul of 10% sodium dodecyl sulfate (Bio-Rad Labs., Richmond, Calif.) was added to the cell suspensions before mixing by inversion. This mixture was heated at 65-70 C for 10 min, and then extracted twice with 0.3 ml of a 1:1 (v/v) mixture of phenol (Mallinckrodt Chem. Co., Paris, Kentucky) and chloroform (J.T. Baker Chemical Co., Phillipsburg, N.J.) saturated with Tris-EDTA (TE) buffer (10 mM Tris-HCl,1 mM EDTA; pH=8.0) (40). The suspension was agitated briefly in a vortex mixer to form a milky emulsion. The emulsion was microfuged 2 min to separate the aqueous and organic phases, then the aqueous top phase was transferred to a sterile tube and extracted with the phenol:chloroform mixture. The top phase from this last extraction was transferred to another tube and the DNA was precipitated by adding 1.0 ml 95% ethanol; the ethanol was poured off after the DNA precipitated. The ethanol wash step was repeated twice with a 1.0 ml of 70% ethanol and again with 1.0 ml 95% ethanol. The DNA precipitate was pelleted by microfugation for 2 min. After removal of the

ethanol with a sterile micropipette the DNA pellet was dried in a vacuum oven at room temperature for 30-60 min (ca. 10 in/Hg). The dried DNA was resuspended in 25-35 µl TE buffer and this DNA preparation was stored at +5 C for short term storage or -20 C for long term storage.

DNA digestion. The DNA preparations were digested with the restriction endonuclease EcoRl (Bethesda Research Laboratories, Gaithersburg, Maryland; and United States Biochemical Corporation, Cleveland, Ohio). Fifteen units of EcoRl and 1.5 ul 10X EcoRl digestion buffer (40) (1.0 M NaCl, 10 mM dithiothreitol, 500mM Tris-HCl, 100 mM $MgCl_2$) were added to 4-10 µl of the DNA preparation (ca. 0.8 µg DNA, determined by measuring A_{260} in a spectrophotometer (Bausch and Lomb Co., Rochester, N.Y.)). The DNA was incubated at 37 C for about 3 hr to achieve complete digestion. RNase A (Sigma Chem. Co., St. Louis, MO), 1.0 µl of a 10 mg RNaseA/ml soln., was added 10 min before the end of the 3 hr incubation period. Digestion was terminated by adding 1.5 µl of 0.15 M EDTA (pH 7.5) to obtain a final concentration of 10 mM EDTA.

Electrophoresis of DNA. After the DNA digestion, 6 µl of gel loading buffer Type II (0.25% bromphenol blue, 0.25% xylene cyanole, 15% Ficoll,type 400) (40) was added and mixed before 4-6 µl of the DNA-loading buffer mixture was loaded into agarose gels. The digested DNA from P.

syringae strain Pss2-3RNH was loaded into each gel as a DNA profile standard. Gels were 2 mm thick and prepared with 0.7% agarose (Ultra-pure DNA grade agarose, Bio-Rad) on a 12 x 15 cm glass plate. Wells (3 x 1.5 mm) were formed with a 30 well comb. The DNA restriction fragments were separated by submarine gel electrophoresis (Wide-Mini Sub Cell, Bio-Rad) in Tris-acetate electrophoresis (TAE) buffer (0.04 M TRIS base, 0.002M EDTA, 0.57% (v/v) glacial acetic acid) (40) at 60 V (31 mA) for the first 10 min then at 30V (=2 V/cm)(18 mA) for 4 hrs 50 min.

Staining and photography of DNA in gels. Gels were stained 30-45 min in ethidium bromide (0.75 mg/L TAE buffer). After staining, the gels were photographed over an ultraviolet transilluminator (Chromato-vue, Ultra-violet Products Inc., San Gabriel, Calif.) with Polaroid type 55 pos/neg film (Polaroid Corp., Cambridge, Mass.) and a yellow Kodak wratten #9 ultraviolet filter (Eastman Kodak Co., Rochester, N.Y.).

Analysis of DNA profiles. Banding patterns in the DNA profiles were compared visually. Only profiles with clear banding patterns were analyzed. In addition, only those DNA profiles from gels containing recognizable banding patterns from the DNA profile standard strain (Pss2-3RNH) were analyzed. DNA profiles from the different isolates were compared with the negatives of the

profiles and with black and white enlargements made from each negative. Profiles were cut from the enlargements and taped individually onto index cards, thus allowing direct side-by-side comparison of profiles. To reduce bias in the analysis, all profiles were compared without knowing the identity of the isolate from which the DNA profiles were obtained.

Testing stability of DNA profiles. The effects of isolating and digesting DNA from sub-cultures of the same strain on different days and electrophoresing this DNA in different gels were investigated. DNA was extracted from 10 different sub-cultures of P. syringae strain Pss2-3RNH and electrophoresed in eight different gels. DNA was isolated from four sub-cultures of a P. syringae isolate from annual ryegrass and electrophoresed in four different gels. DNA was isolated from two sub-cultures of an isolate from red fescue grass and electrophoresed in one gel. The DNA profiles from the different subcultures of each isolate were compared after the different treatments.

Stability of DNA profiles from marked bacteria exposed to environmental conditions in the maple nursery for 6 to 8 months was investigated. In this experiment, the DNA profiles were analyzed from the antibiotic resistant Pseudomonas strains in the groups III and IV.

RESULTS

Repeatability of DNA profiles. The DNA profiles from several P. syringae strains remained identical regardless of whether the DNA was isolated, digested and electrophoresed on different days. For example, under these varying conditions, all the DNA profiles from 10 subcultures of strain Pss2-3RNH were identical even though they were electrophoresed in 8 different gels. Similarly, the profiles from four subcultures of the rye grass isolate were identical in four gels, and the profiles from two subcultures of the fescue grass isolate were identical when electrophoresed in the same gel.

DNA profiles from P. syringae isolates. Forty one P. syringae isolates from the maple nursery (Group I) were analyzed by DNA profiles, and 21 different DNA profiles were identified on the basis on restriction fragment banding patterns (Table 3.1, Fig.3.1). Eleven DNA profiles were found only in single isolates (Table 3.1, DNA profiles K-U). Each of ten DNA profiles were found in more than one isolate (Table 3.1, DNA profiles A-J). The DNA profiles from four cytochrome oxidase positive pseudomonad isolates (not spp. P. syringae) from the maple nursery plot were analyzed and their DNA profiles were different than all profiles from P. syringae isolates.

The DNA profiles showed that heterogenous populations of P. syringae existed on individual plants and different

plant species in the maple nursery. With the exception of annual ryegrass, all plant species, i.e., maple trees and five species of grass, yielded two to eight different strains with different DNA profiles.

When plant samples are assayed, P. syringae colonies with different morphologies are usually isolated. These different morphologies can suggest that different strains reside on the tissue sample. In this study, however, several isolates with different colony morphologies had identical DNA profiles, i.e., isolates with DNA profiles B, E, F and I in Fig. 3.1. Thus, colony morphology may depend on differential gene expression more than genomic variation.

Stability of DNA profiles from strains in the environment. DNA profiles from 18 P. syringae strains resistant to rif and nal (Group II) that were recovered from the nursery plot between September 1985 and May 1986 were identical to each other (Fig.3.2). Furthermore, they were identical to the DNA profiles from strains PSS2-3RNH and Pss2-3 (the antibiotic resistant strain released in the nursery and the parental wild-type strain, respectively) which had been maintained in culture (Fig.3.2). Similarly, the DNA profiles from 16 Pseudomonas isolates from the nursery plot with rif and tet resistance (Group IV) were identical to each other. They were also identical to DNA profiles from the mutant

strain that was released in the nursery (PAN8/82RT) and the wild-type parental strain (PAN8/82), which had been maintained in culture over the same time period (Fig.3.3). In addition to providing evidence for stability of DNA profiles, these data also verified the survival and identity of the two antibiotic resistant Pseudomonas strains recovered from the field.

The P. syringae strain Pss2-3RNH was recovered from the maple nursery plot and positively identified with DNA profiles, without taking advantage of its key unique phenotypic trait, i.e., antibiotic resistance. Fifteen P. syringae isolates were recovered on KB medium (Group III) and had DNA profiles identical to strain Pss2-3RNH. All 15 isolates were spread onto medium containing 100 ppm rif and 150 ppm nal. Fourteen were resistant to these antibiotics. One isolate was not resistant, nor would it grow on medium containing either just rif or nal, suggesting that this isolate had reverted back to sensitivity to the antibiotics.

DISCUSSION

The DNA profiles were identical when DNA from one strain was isolated on different days, then digested and electrophoresed at other times. The results show that variation between profiles results primarily from genomic differences between P. syringae strains and not from

inherent problems with the technique. This illustrated the accuracy of the method, despite the difficulty of controlling precisely such variables as chemical purity of DNA and concentration of DNA

When using the DNA profile method to investigate the ecology of a particular bacterial strain, it is critical that DNA profiles be stable over time to insure positive identification of the strain. It was established that the DNA profiles in two Pseudomonas strains remained stable for at least 6 to 8 months after they were released into a maple nursery. Although no changes in DNA profiles were noted in this study, such changes could occur by several processes. For example, insertions of foreign DNA, deletions of part of the genome and random mutations can alter specific nucleotide sequences (GAATTC) recognized by the restriction endonuclease EcoRl. Consequently, the size and number of nucleotide fragments generated during incubation with EcoRl and the DNA profiles would change. The results reported here indicate that if such changes occurred in the genome of the antibiotic resistant strains that were analyzed, they did not cause significant changes in the DNA profiles

There are two results from this study that suggest the DNA profiles from P. syringae are strain specific. First, DNA profiles from the two antibiotic resistant strains used in these experiments were different from DNA profiles obtained from all other P. syringae isolates from

the nursery plot. Second, 21 different profile types were identified among the 41 indigenous P. syringae isolates from grasses and maple trees in the nursery plot. It is likely that most isolates with the same DNA profile were the same strain because all grouped isolates except those in three groups came from a single bulk sample of tissue.

Analysis of DNA profiles suggests that heterogeneous populations of P. syringae are present on individual trees and grasses and distributed throughout the maple nursery. This diversity among the DNA profiles corroborates that the diversity of P. syringae strains found (with respect to hypersensitivity in tobacco, ice nucleation activity, biochemical tests and pathogenicity) found in nurseries and orchards (4,47) is based on genotypic and not just phenotypic variation.

DNA restriction fragment profile analysis is a useful tool to compare the identity of individual strains of P. syringae that cannot be distinguished on a morphological basis. However, a limitation of this technique is the time required to analyze large numbers of isolates. The time factor is relatively not a problem when populations of bacteria are composed of one or a few strains. In contrast, when the population contains numerous strains of the bacterium (as in this study) many isolates must be analyzed to study a particular strain. Alternatively, a rapid procedure to group related isolates, e.g., simple serological tests, should be used before DNA profile

analysis to limit the number that must be analyzed. DNA profile analysis may have its greatest value as a confirmatory test to supplement other methods, such as with the use of the antibiotic resistant mutant in this study.

Table 3.1. DNA profile types among epiphytic *Pseudomonas syringae* isolates from twigs, leaves and grasses in a maple nursery

DNA profile[a]	Number of isolates with each profile	Origin of isolates[b]
A	5	PR(2)[c], F(1), Or(2)
B	2	AR
C	3	T
D	5	F
E	3	B
F	2	B
G	2	T
H	3	PR(2), T(1)
I	2	T(1), PR(1)
J	3	S
K	1	T
L	1	T
M	1	Or
N	1	S
O	1	T
P	1	T
Q	1	PR
R	1	PR
S	1	Or
T	1	Or
U	1	B

(a) Each DNA profile has a different DNA restriction fragment banding pattern.
(b) 'Origin' is plant species from which *P. syringae* was isolated. Abbreviations correspond to red maple trees and grasses arranged as shown in Fig. 1 of Chapter 1. Red maple trees are designated as T; isolates were from 6 different trees. Grasses are abbreviated as follows, PR:perennial ryegrass, F:red fescue grass, Or:orchardgrass, AR:annual ryegrass, B:brome grass, and S:sudan grass.
(c) Number in parentheses is the number of colonies isolated from that plant with a particular DNA profile.

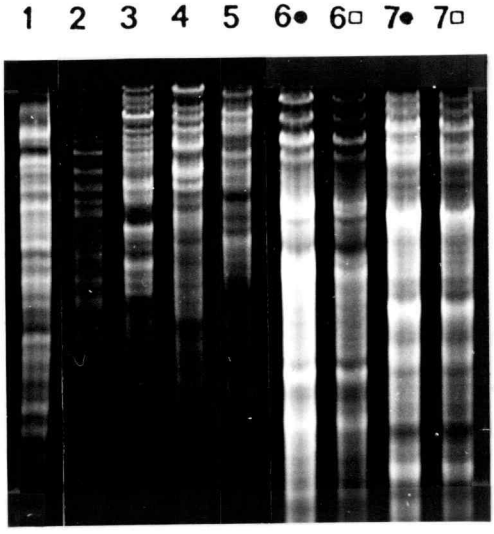

Figure 3.1. Lanes from two agarose gels showing DNA profiles from seven different Pseudomonas strains. Lanes 1,2,5,6 and 7 show DNA profile types H,C,I,F and A, respectively, (see Table 3.1) from different P. syringae strains isolated from a maple nursery plot. The • and □ lanes for 6 and 7 show the result when different quantities of DNA are loaded; lane • contains twice as much DNA as lane □ . The DNA profile in lane 3 is from a cytochrome oxidase negative, fluorescent Pseudomonas isolate, i.e., not P. syringae. The DNA profile in lane 4 is from the P. syringae strain Pss2-3RNH that was used as a DNA profile standard.

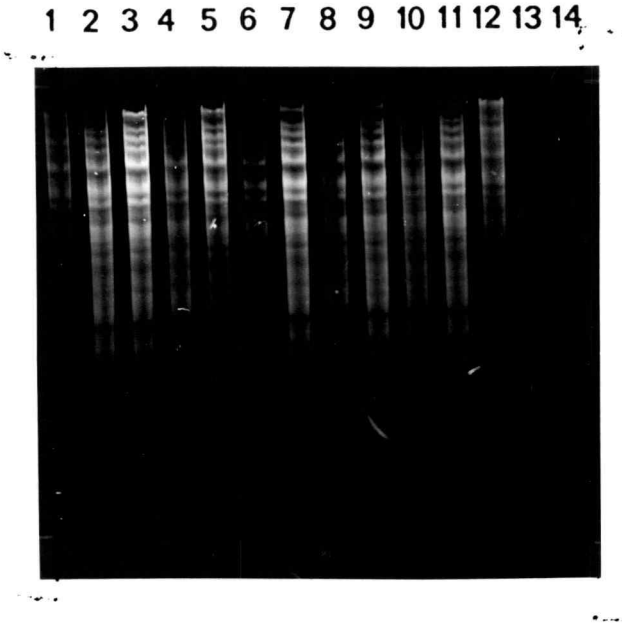

Figure 3.2. DNA profiles from 12 isolates of the antibiotic resistant Pseudomonas syringae strain Pss2-3RNH with identical banding patterns. These isolates (lanes 1-12) were recovered between September 1985 and May 1986 from maple trees that were inoculated once with strain Pss2-3RNH. DNA profiles in lanes 13 and 14 are from two wild-type P. syringae isolates from the same maple nursery plot.

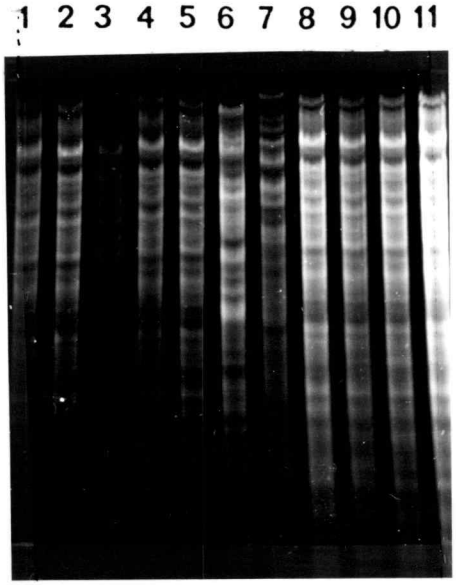

Figure 3.3. DNA profiles from seven isolates of the antibiotic resistant Pseudomonas strain PAN8/82RT and four other Pseudomonas strains. The antibiotic resistant isolates (lanes 1,2,3,8,9,10,and 11) were recovered between October 1985 and February 1986 from grasses in a nursery plot that were inoculated once with this strain. The DNA profiles in lanes 4 and 5 are from the strains PAN8/82RT and it's parental strain PAN8/82, respectively, which were maintained in culture. DNA profiles in lanes 1-5 and 8-11 have identical banding patterns. The DNA profile in lane 6 is from a wild-type P. syringae isolate from the same nursery plot. The DNA profile in lane 7 is from the P. syringae strain Pss2-3RNH that was used as a DNA profile standard.

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