Epidemiology of epiphytic Pseudomonas syringae on barley
by Dimitrios G Georgakopoulos

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Plant Pathology
Montana State University

Abstract:
Epiphytic populations of P. syringae from 24 barley cultivars and lines planted in Montana in 1986 were determined by dilution plate assay of 10-leaf samples on BCBRVB, a modified King's B selective medium. Leaf symptoms were recorded at each sampling. P. syringae colonies were tested for ice nucleation activity (INA) by a dropfreezing technique and the percentage of INA+ bacteria determined. Populations were low in the beginning of the study and increased up to log 6 cfu/leaf by the end of the growing season. Populations from some entries were consistently 100% INA+ bacteria. There was no correlation between leaf symptoms and population levels. Significant differences in population levels were observed among the entries. Six entries were reexamined in the field in Arizona during the winter of 1987, and in Montana during the summer of 1987, and the differences in population levels, and no-correlation of symptoms and population seemed to persist. The second time, populations were again almost 100% INA+ bacteria, but the third time they were lower. An experiment on diurnal population changes showed only small changes in a 24-hour period. Dissemination experiments included a study of plant-to-plant dissemination and two studies of the movement of marked strains. Plant-to-plant dissemination was studied by planting a 1:8 mixture of a high-population line with a low-population cultivar and comparing the population of P. syringae on the "low" cultivar in the mixture with those of the control (" low" cultivar alone). No significant differences were observed. The marked strain dissemination studies included the creation of double marked strains by spontaneous mutation and the inoculation with these of barley cultivars and lines. In the first study, the inoculum did not survive very well epiphytically. In the second study, one line was inoculated with a marked INA+ strain and another line with a 1:1 mixture of marked INA+ and INA- strains.

In both cases the inoculum survived epiphytically, and the INA- strain did not eliminate the INA+ strain, or vice-versa. The INA+ strain was disseminated short distances during sprinkler-irrigation, and up to 70 m during rain.

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Dimitrios G. Georgakopoulos

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Dec 8, 1987
Date

David C. Smith
Chairperson, Graduate Committee

Approved for the Major Department

Dec 8, 1987
Date

D E Mathes
Head, Major Department

Approved for the College of Graduate Studies

December 9, 1987
Date

Henry L Parsons
Graduate Dean

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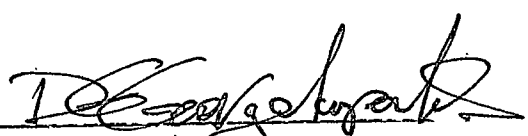
Signature 

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ABSTRACT

Epiphytic populations of P. syringae from 24 barley cultivars and lines planted in Montana in 1986 were determined by dilution plate assay of 10-leaf samples on BCBRVB, a modified King's B selective medium. Leaf symptoms were recorded at each sampling. P. syringae colonies were tested for ice nucleation activity (INA) by a drop-freezing technique and the percentage of INA+ bacteria determined. Populations were low in the beginning of the study and increased up to log 6 cfu/leaf by the end of the growing season. Populations from some entries were consistently 100% INA+ bacteria. There was no correlation between leaf symptoms and population levels. Significant differences in population levels were observed among the entries. Six entries were reexamined in the field in Arizona during the winter of 1987, and in Montana during the summer of 1987, and the differences in population levels, and no-correlation of symptoms and population seemed to persist. The second time, populations were again almost 100% INA+ bacteria, but the third time they were lower. An experiment on diurnal population changes showed only small changes in a 24-hour period. Dissemination experiments included a study of plant-to-plant dissemination and two studies of the movement of marked strains. Plant-to-plant dissemination was studied by planting a 1:8 mixture of a high-population line with a low-population cultivar and comparing the population of P. syringae on the "low" cultivar in the mixture with those of the control (" low" cultivar alone). No significant differences were observed. The marked strain dissemination studies included the creation of double marked strains by spontaneous mutation and the inoculation with these of barley cultivars and lines. In the first study, the inoculum did not survive very well epiphytically. In the second study, one line was inoculated with a marked INA+ strain and another line with a 1:1 mixture of marked INA+ and INA- strains. In both cases the inoculum survived epiphytically, and the INA- strain did not eliminate the INA+ strain, or vice-versa. The INA+ strain was disseminated short distances during sprinkler-irrigation, and up to 70 m during rain.

INTRODUCTION

The ability of the bacterium Pseudomonas syringae to cause ice crystals to form in supercooled liquids and water vapor (ice nucleation activity, INA) has led to a number of studies during recent years. Most focus on the relationship between INA and frost damage on a number of crops, where the bacterium lives epiphytically. Considerable controversy has arisen concerning the release of a strain of P. syringae developed by recombinant DNA techniques for biological control of frost damage. However, less effort has been devoted to the possible involvement of ice nucleating bacteria in atmospheric phenomena such as the condensation of rain and ice crystals in clouds, thus affecting precipitation. Ice nucleating bacteria, especially P. syringae, are the most efficient ice nuclei in nature, active at -1 to $-2^{o}C$ as opposed to dust particles ($-15^{o}C$) which are considered the main source of atmospheric ice nuclei.

It has been proposed that a "bioprecipitation cycle" may exist in nature: ice nucleating bacteria leave the plant surface, enter the atmosphere, catalyze the formation of rain in the clouds and thus create more moisture for plant growth and more "substrate" for bacterial growth. The accelerating desertification process in dry areas of the world, such as the Sahara and the Sahel in Africa, and decrease of precipitation in South America has been attributed to overgrazing, burning and injudicious farming practices. An

explanation for this observation could be the break caused in the "bioprecipitation cycle", because these acts destroy the vegetative substrates of bacterial growth, resulting in a decrease in condensation and ice nucleation in the atmosphere. Subsequently, a drastic reduction of precipitation occurs, accompanied by greater precipitation runoff. This occurs due to land erosion that results from the destruction of natural plant communities. Could the selection of crops that support high populations of ice-nucleating bacteria affect and even counteract this process?

The following research was done to answer two primary questions:

1. Can barley support a high population of P. syringae and are there differences in the population size among cultivars?
2. Does P. syringae enter the atmosphere from a barley field?

Barley, a major crop in Montana, was chosen as the model plant because it is drought-tolerant and one of the most important crops in arid areas of the world. The answers to these questions may provide additional evidence for the existence of a "bioprecipitation cycle" in nature, and perhaps facilitate future research.

LITERATURE REVIEW

The leaf surface is a favorable environment for the survival and growth of microorganisms. Epiphytic microorganisms are microorganisms that live and multiply on the leaf surface. The survival and population dynamics of the epiphytic microflora depends on a number of factors, such as temperature, relative humidity, water on leaves, nutrients, host, but also interspecific interactions (parasitism, competition, antibiotic production) (Blakeman, 1982; Hirano and Upper, 1983; Morris and Rouse, 1985). Bacteria form a major component of the epiphytic microflora; many are saprophytic, belonging to the genera Erwinia, Pseudomonas, Xanthomonas, Flavobacterium, Lactobacillus, Bacillus and many others, not identifiable at the species level (Blakeman, 1982). Crosse in 1959 was the first to report that phytopathogenic bacteria are a component of the microflora of apparently healthy leaves. He isolated Pseudomonas syringae pv. morsprunorum in large numbers from healthy cherry leaves and stems and suggested that these populations could provide inoculum for the infection of stems and branches. His technique was leaf and stem washings, a technique largely used in studies of epiphytic bacteria. It consists of shaking individual leaves, or leaves pooled in samples, or other plant material in water, for some time, and subsequent dilution plating. Leaf washing is the best method for quantitative studies. Populations are usually expressed in terms of log 10 of colony-forming

units per gram fresh or dry weight of tissue, per unit area, or per leaf. A concern in quantitation of epiphytic bacteria is associated with the utilization of bulked samples. Crosse found that epiphytic populations of Pseudomonas syringae pv. morsprunorum vary greatly from leaf to leaf and from branch to branch in cherry. Hirano et al. in 1982 reported that for any given canopy at any given time, total epiphytic bacterial populations and selected components thereof can be described by the lognormal distribution (i.e. the logarithm of bacterial populations on individual leaves is normally distributed).

Other methods for studying epiphytic bacteria are microscopy and leaf imprinting. Microscopic techniques have been useful primarily for determination of the spatial distribution or preferential localization of bacteria on leaf surfaces. Leaf imprinting has been successful in isolating or detecting a specific component of the epiphytic microflora. Both methods, however, are qualitative (Hirano, 1983).

Cells of epiphytic bacteria, both saprophytic and pathogenic adhere on the leaf surface. Haas and Rotem (1976) inoculated cucumber leaves with precise numbers of the pathogen Pseudomonas syringae pv. lachrymans. One minute after inoculation, leaves were shaken for ten minutes and bacterial populations counted with dilution plating. They showed that a constant proportion of bacteria (7%), independently of inoculum concentration, were removable, the great majority being adsorbed on the leaf surface. They also showed that this adsorption does not involve specific sites on the leaf. In a similar study, but using different techniques, Leben and Whitmoyer (1979) showed that not

only pathogenic but also saprophytic bacteria adhere on the leaves. Preece and Wong (1981) further demonstrated that pathogens attach themselves much more effectively to their host plants (52-92% attachment) than to non-hosts (11-30%). Only about 20% of saprophytic bacteria became attached to leaf surfaces. Mew and Kennedy in 1971 published similar results for Pseudomonas syringae pv. glycinea on soybean leaves.

By scanning electron microscopy (Mariano and McCarter, 1985) and leaf imprints (Luisetti and Gaignard, 1984), it was shown that bacterial epiphytic populations are localized as microcolonies on sites more or less hidden: epidermal cell junctions, along veins, around the base of trichomes, and occasionally within stomates. It is believed that bacterial adsorption involves the adhesive properties of extracellular polysaccharide (Blakeman, 1982).

The environment on the leaf surface fluctuates very rapidly. Changes can be quick and unpredictable; e.g. temperature, relative humidity, leaf wetness, or more gradual; e.g. stage of the leaf. Bacterial populations respond to these changes, both in number and composition of the microbial community. Of all factors, the most influencing the growth and survival of microorganisms is relative humidity (RH) at the plant surface. Epiphytic bacterial populations tend to increase when plants are wet (after rain, overhead irrigation, or high RH in controlled situations). Free water is essential for bacterial growth, because nutrients that affect growth are dissolved in it. It can also be important for the movement of epiphytic bacteria, either by their own motility or mechanical dissemination

such as aerosols, or leaf runoff water (Hirano and Upper, 1983; Khodair and Ramadani, 1984; Blackeman, 1985).

There is little question that epiphytic phytopathogenic bacteria provide inoculum for disease. A general observation is that increased inoculum results in increased disease incidence. But quantitative relationships such as the minimum population size required for disease development, have been established in only a few cases: Erwinia amylovora and fire blight; ice nucleation-active bacteria and frost damage; Pseudomonas syringae pv. syringae and brown spot on snap beans; and P. syringae pv. coronafaciens and halo blight on oats (Hirano et al., 1981; Hirano, 1983; Lindow, 1983; Lindemann et al., 1984).

Pseudomonas syringae is a major pathogen on many crops. Several studies on epiphytic P. syringae and plant disease have been published, both on annual and perennial crops. The bacterium lives as epiphyte on many species (Lindow and Upper, 1977; Lindow et al., 1978; Lindow, 1983a) beyond its host range as a pathogen. Epiphytic populations of P. syringae are influenced from the same factors mentioned for all epiphytic bacteria, relative humidity and free moisture on the leaves being the most important. Cool temperatures seem to be the most suitable for epiphytic growth of this bacterium. In the case of P. syringae pathovars that are ice nucleation-active, frosts also result in an increase in epiphytic populations. Sources of inoculum can be seeds, plant debris, dormant tissues, or weeds. Dispersal is also favored by moisture; rainsplash, rain and irrigation-generated aerosols and even airborne bacteria are effective ways of

dissemination (Leben et al., 1970; Ercolani et al., 1974; Venette and Kennedy, 1975; Smitley and McCarter, 1982; Gross et al., 1983; Hirano, 1983; Baca and Moore, 1984; Latorre et al., 1985; Wimalajeewa and Fleet, 1985).

Pseudomonas syringae causes two diseases on cereals: halo blight on oats and leaf blight on wheat and barley. Leaf blight was first observed in South Dakota in 1965, on spring and winter wheat, and it was first reported by Otta in 1972. On wheat, symptoms appear generally from the boot to the early heading stage as numerous, very small, water soaked spots on the flag leaf and on the first and second leaf below it. Within 2-3 days these spots will expand and often coalesce into large, greyish-green dessicated areas (Otta, 1974). The disease has been reported also in Montana (Scharen et al., 1976; Sands et al., 1977) and Minnesota (Sellam and Wilcoxon, 1976). It is not one of the major diseases of wheat and barley, but it can cause yield losses as reported by Scharen et al., in 1976. Leaf necrosis and the leaf spot stage of basal glume rot of wheat, incited by Pseudomonas atrofaciens have a similar symptomatology. In his 1977 article, Otta found little, if any, difference between isolates of P. syringae and P. atrofaciens. Reports indicate differences in susceptibility of wheat cultivars to the bacterium (Otta, 1974, Sellam and Wilcoxon, 1976, Scharen et al., 1976). However, epiphytic populations of P. syringae did not differ significantly on seedlings of susceptible, moderately susceptible, and resistant wheat cultivars under controlled conditions, according to Fryda and Otta (1978). The same authors reported that the bacterium moved from the seed to the seedling and

survived on healthy leaves under greenhouse, growth chamber, and field conditions. These results indicate that P. syringae can survive as an epiphyte on wheat and that seedborne P. syringae can be an important source of inoculum.

Research on epiphytic bacteria became more important after the discovery by Maki et al. (1974), that isolates of P. syringae from decaying alder leaves were found to be ice nucleation-active at very warm (-1.8 to -3.8°C) temperatures. Many pathovars of P. syringae, certain strains of Erwinia herbicola, P. fluorescens, P. viridiflava, and Xanthomonas translucens are also ice nucleation active (Lindow et al., 1978b; Lindow, 1983a; Kim et al., 1987).

The principle of ice nucleation is based on the fact that water does not necessarily freeze at the melting point. It can be supercooled to several degrees below 0°C and still be in the liquid phase. It will freeze only upon the presence of a suitable catalyst for the liquid-solid phase transition. These catalysts are called ice nuclei. The mechanism of ice nucleation involves the ordering of water molecules into an ice-like lattice around a nucleus with lattice structure similar to ice (Lindow, 1983a). Other materials possessing ice nucleation activity are dust particles (active below -10 to -15°C), silver iodide, used as a cloud seeding agent (-8°C), and crystals of several organic compounds (-5°C) (Mason and Hallet, 1957; Zettlemoyer et al., 1961; Lindow 1983a). But ice nucleation-active bacteria and especially P. syringae are the most efficient ice nuclei, active at -1.8°C.

The ice nucleation-active factor has been identified and purified for P. syringae and P. fluorescens. It is a protein located on the outer cell membrane, of 153kD molecular weight for P. syringae and 180kD for P. fluorecens (Wolber et al.; 1986, Corotto et al., 1986). These two proteins have very similar structures and properties. The genes coding for these proteins have also been cloned in Escherichia coli and sequenced. The amino acid sequence predicted from the DNA sequence consists of interlaced 8, 16, and 48-amino acid repeats (in ascending order of fidelity). The repeated unit is hydrophilic and particularly rich in serine and threonine. The primary sequence suggests that the protein folds into a regular structure built up from the 48-amino acid repeat, and that this structure presents H-bonding side chains in a manner which mimics an ice lattice. The fact that the 48-amino acid repeat is built up from 3 less perfect 16-amino acid repeats, which are in turn built up from two least perfect 8-amino acid repeats, suggests that the protein structure is formed by a hierarchy of folded domains (Orser et al., 1984; Green and Warren, 1985; Corotto et al., 1986; Wolber and Warren, 1986). Other reports indicate that phospholipids are also determinants of the ice nucleation activity (Kozloff et al., 1984; Govindarajan and Lindow, 1984).

In vitro cultural conditions, such as medium composition, solid versus liquid growth medium, aeration, and growth temperature were found to affect the ice nucleation efficiency of cells of many ice nucleation-active strains of P. syringae and E. herbicola, as well as the temperature at which ice nucleation is expressed in these cells (Maki et al., 1974; Paulin and Luisetti, 1978; Lindow et al., 1978a,b;

Yankofsky et al., 1981; Lindow et al., 1981; Lindow, 1983a; Hirano, 1985).

The presence of epiphytic ice nucleation-active (INA) bacteria on frost sensitive plants increases their sensitivity to frost damage at temperatures slightly below 0°C. Normally plant tissue can supercool to -7°C without the formation of ice, but epiphytic INA bacteria catalyze the formation of ice in, or on plant tissue, causing mechanical disruption of cell membranes (Arny et al., 1976).

Even before the discovery of the role of INA bacteria in frost damage, reports indicated that many diseases induced by P. syringae require, or are favored by, ice formation on plants prior to disease development (Panagopoulos and Crosse, 1964; Weaver, 1978; Sule and Seemuller, 1987). As most bacteria, including P. syringae, cannot invade plant tissue, it is possible that P. syringae evolved with the capacity to predispose plant tissue to ice damage and subsequent penetration and disease development (Lindow, 1983a).

Populations of INA P. syringae undergo seasonal variations, as observed for all epiphytic bacteria. They are usually low in young, vegetative tissue. Colonization and survival on plants also vary with the host (Lindow, 1985). Hirano et al. (1984), reported large diurnal changes (up to 2.8 log cfu/leaf) of P. syringae populations on bean leaflets, as well as diurnal changes in their ice nucleation activity. The host seems to affect not only the population size but also the ice nucleation activity and pathogenicity of P. syringae (Gross et al., 1984; Lindow, 1986; Baca et al., 1987).

After the discovery of INA bacteria, frost damage was regarded as a "plant disease" that can be "cured" by eliminating INA bacteria from the plant surface. Three strategies have been used: application of chemicals (bactericides and ice nucleation inhibitors), selection and use of naturally occurring antagonistic bacteria, and use of genetically engineered ice nucleation deficient ("ice-minus") bacteria.

Bactericides and ice nucleation inhibitors (usually salts of heavy metals that do not kill the bacteria but inactivate their ice nucleation activity) provided significant frost control in experimental applications on several crops. It seems that they are more effective as protectants (before bacterial populations establish on the leaf surface),because even dead bacteria can nucleate ice formation as long as the cell is intact (Lindow 1982, 1983b).

The degree of competition among epiphytic microorganisms on the leaf surface is insufficient to prevent buildup of significant populations of INA bacteria. Thus, it was attempted to select for bacteria antagonistic to the INA ones, and alter the epiphytic microbial community, in order to reduce the populations of INA bacteria during periods of low temperatures, and therefore reduce the probability of frost injury. Antagonistic bacteria that have been tried as in vivo competitors of INA bacteria, include non-INA strains of E. herbicola, P. fluorescens and P. putida with variable results. The mechanism of antagonism seems to be site exclusion rather than production of antimicrobial compounds (Lindow, 1981; Lindow et al., 1983a,b; Cody et al., 1987).

The most recent approach to prevent frost injury of plants by application of antagonistic bacteria, concerns the use of genetically engineered "ice-minus" P. syringae and P. fluorescens, with considerable controversy arising about the safety of such a release in the environment. The proposed advantage of "ice-minus" bacteria versus natural antagonists lies in their potential for establishment on the leaf surface: being near-isogenic with the wild types, they should occupy the same sites on the leaf, use the same nutrients, and outnumber the naturally occurring INA bacterial populations, (Lindow, 1985; Lindemann et al., 1985a; Lindemann and Suslow, 1987). Recently it was reported that the use of "ice-minus" bacteria reduced frost damage on plants up to 80% (Time 11/9/87, data not published).

Recent work has shown that significant numbers of bacteria, including species of INA, can leave the plant surface, enter the atmosphere, and disseminate from one point of a field to another. Such phenomena occur not only during wet conditions (rain, overhead irrigation) but also during dry days. Bacterial concentrations are higher in the atmosphere over plants than over soil, suggesting that plant canopies constitute a major source of airborne bacteria including INA (Lindemann et al., 1981; Lindemann et al., 1982; Andersen and Lindow, 1985; Dow and Maki, 1985; Lindemann and Upper, 1985). Similar results were obtained by Bovallius et al (1978a). The same authors (1978b), and Mandrioli et al (1984), give evidence for long range transport of biological particles, including bacteria, in the atmosphere, over distances as far as 1800 km and as high as 6 km.

Earlier work indicated that biological ice nuclei in the atmosphere originated from decomposing vegetation (Schnell and Vali, 1972; Schnell and Vali, 1973; Schnell and Vali, 1976) but these nuclei were not further characterized or identified as bacteria. The demonstrated presence of microbes in the atmosphere in raindrops and snow flakes, along with the discovery of the ice nucleating properties of P. syringae (Maki et al., 1974), led Vali and Schnell (1976) to suggest that INA bacteria may play a more or less important role in atmospheric precipitation processes. Parker (1970) reported the presence of organic substances of biological origin in raindrops and clouds (vitamins and other nutrients) and suggested that the clouds might be viewed biologically, as atmospheric ecosystems having significant numbers of functioning microorganisms. In 1978, Maki and Willoughby conducted successful ice nucleation experiments in controlled cloud chambers by using freeze-dried cultures of INA P. syringae and P. fluorescens isolated from decomposing plant material, water from streams and lakes, and from snow and rain. Sands et al. (1982) reported the isolation of INA P. syringae from raindrops in rainstorms at elevations from 180 to 2500 m above cropland, and suggested that these epiphytic bacteria "are components of a cycle involving rainfall induction, followed by enhancement of vegetation, leading to increased production of INA bacteria". They named this phenomenon "bioprecipitation cycle" and suggested that the enhancement or decrease of this cycle "may result in increased vegetation and biomass productivity in a geographical area or decreased productivity and desertification".

MATERIALS AND METHODS

Variability in P. syringae Population Size Among Barley Cultivars

The scope of these experiments was to determine possible differences in epiphytic population sizes of P. syringae among barley cultivars, and to select for one or more cultivars supporting high epiphytic populations of the bacterium. The susceptibility to bacterial leaf blight of the plant material examined was also investigated by recording leaf blight symptoms throughout the course of the experiments, and correlating symptoms to populations of P. syringae.

Plant Material

The epiphytic growth of P. syringae was studied on 24 barley lines and cultivars. Twenty of these were six-row lines that originated from a breeding program for dryland barley at the University of Arizona, Tucson. The other four were commonly grown barley cultivars in Montana (Table 1). The epiphytic populations of P. syringae were monitored on all entries during the summer of 1986 and on six selected during the winter of 1987 (Marana Agricultural Experiment Station, Arizona) and the summer of 1987 in Bozeman.

Table 1. List of the 24 barley lines and cultivars examined for epiphytic populations of *P. syringae* in the field, Bozeman, 1986.

AR1	AR7	AR13	222-9
AR2	AR8	AR14	BOLD
AR3	AR9	AR15	STEPTOE
AR4	AR10	AR16	KLAGES
AR5	AR11	AR17	CLARK
AR6	AR12	222-1	ERSHABET

Planting

All entries were planted in four randomized replications. Plots consisted of four rows, three m long and 30 cm apart. Each row received five g of seed planted with a cone seeder. The seed was previously sterilized in water at 51°C for 10 minutes. Planting for the 1986 Bozeman experiment was done on May 28, for the 1987 Arizona experiment in November 1986, and for the 1987 Bozeman experiment on May 31, 1987. The plots of the 20 dryland lines in Bozeman, 1986 were not irrigated. The plots of the four "Montana" cultivars and all plots in Bozeman, 1987 were irrigated once or twice a week by sprinkler irrigation. The plots in Arizona, 1987 were irrigated by flood irrigation.

Leaf Sampling

Hirano et al. (1984) reported that epiphytic population sizes of *P. syringae* on bean leaves change with the time of the day. Thus, a standard time of sampling (8-10 a.m.) was established in order to minimize the possible effect of this factor on the results. From each entry and replication, 5 flag and 5 lower leaves were sampled at random with a pair of forceps sterilized in 70% ethanol. The leaves

were put in a Ziploc plastic bag, transported to the laboratory and stored in a cold room at 4°C until processing. The time between sampling and processing never exceeded 2 1/2 hours. During every sampling, and for each entry and replication, symptoms were recorded on the flag leaf by using a scale from 0-5.

Leaf Samples Processing

In every plastic bag containing 10 leaves, 50 ml of sterile distilled water were added. The bag was shaken briefly by hand, left for 15 minutes, and then shaken again. Three to five tenfold serial dilutions were performed by using an automatic pipette (Pipetman), and plastic sterile pipette tips. From each dilution, 0.1 ml was plated on a BCBRVB plate (Sands, et al., 1980) a modified King's B (King, 1954) selective medium, which mainly allows the growth of fluorescent pseudomonads.

Bacterial Colony Identification

Plates were incubated for five days in the dark at 21°C. Then, for each leaf sample (entry and replication) the number of colonies that produced a fluorescent pigment under long wave ultraviolet light was counted, at the plate and dilution where colonies grew normally, and expressed their typical characteristics. At least 20% of the colonies of that plate were tested for oxidase reaction and ice nucleation activity (INA). Fluorescent and oxidase-negative colonies were initially characterized as *P. syringae*-like (Palleroni, 1984; Sands, et al., 1970, 1980). From the number of *P. syringae*-like colonies at a dilution, the number of *P. syringae*-like colony forming

units (cfu) per leaf was calculated. The INA of the colonies was tested with a variation of Lindow's drop-freezing technique with an aluminum foil "boat" (Lindow et al., 1978a). A piece of aluminum foil was pressed against the surface of an ELISA plate, sprayed with an inert paraffin (Pledge, S.C. Johnson and Son, Inc.) and wiped with a piece of tissue paper, in order to create uniform indentations and a hydrophobic surface. A 0.03 ml sterile distilled water droplet was placed in each indentation with an automatic pipette and sterile pipette tips. One droplet per colony was inoculated with a P. syringae-like colony, until it became cloudy (concentration of bacteria 10^6-10^7/ml). A few droplets were not inoculated. The aluminum foil "boat" was placed on a liquid (water-ethylene glycol 1:1) circulating cooling bath (model RM 20, Brinkmann Co.) set at -4° C. After 5 minutes the number of frozen inoculated droplets were recorded. The solid or liquid state of the droplets was determined visually and physically by touching with a bacteriological loop. For each plate tested, the percentage of INA positive (INA+) colonies was calculated. Thus, the percentage of epiphytic INA+ P. syringae-like bacteria for each leaf sample (entry and replication) was determined.

Collection of P. syringae Isolates

From the 1986 population study, 48 colonies from all entries were purified by streaking on King's B medium. After 5 days of incubation in the dark at 21°C, they were tested for fluorescent pigment production, oxidase activity, as previously, and for INA. For

the latter, 10 single colonies were tested per isolate, with the aluminum "boat" technique. Test tubes, containing 4 ml of Kings' B broth were inoculated with one single colony each. After three days of incubation in the dark at 21°C, 2 ml of an 80% solution of glycerol in sterile water was added in each tube and the tubes were stored in the freezer at -10°C.

All isolates were tested for arginine dehydrolase activity (Thornley, 1960), hypersensitivity in tobacco leaves (Klement, 1963) and utilization of alpha-ketoglutarate and D(-)tartrate, from 2-day old cultures at 28°C in the dark. For the first test, cultures were stabbed into tubes of Thorley's medium 2A, plugged with a layer of sterile mineral oil, and incubated for three days at 28°C. P. syringae gives a negative reaction to this test. An oxidase-positive, fluorescent saprophytic Pseudomonas sp. was used as a positive control. The second test consists of injecting an aqueous suspension of bacteria into the intercellular space of a tobacco leaf cv. Burley with a 26 1/2 gauge needle and syringe. The same oxidase-positive Pseudomonas sp. was injected as a negative control. Results (complete collapse of the tissue) were recorded after 24 hours. The third and fourth tests consist of streaking aqueous bacterial suspensions on plates of Ayers' medium supplemented with D(-)tartrate and alpha-ketoglutarate (Ayers et al., 1919). Results were recorded after 3, 7, and 14 days. Positive tests were repeated once. As controls, bacterial suspensions were streaked on plates of Ayers' medium alone, and Ayers' medium supplemented with glucose. P. syringae utilizes

alpha-ketoglutarate, but not D(-)tartrate (Palleroni, 1984, Sands et al., 1970, 1980).

Analysis of Results

For every entry and replication in all experiments, the mean population was determined as the area under the population curve, divided by the total time of sampling, in days (Figure 2, see Results). Population values were converted to logarithmic. The statistical analysis was performed by using the AVMF mode of the MSUSTAT program.

Diurnal Population Changes

Leaf samples were taken every four hours from AR13 in four repetitions starting at 8 a.m. on July 24, 1987 and ending at 8 a.m. on July 25, 1987. Sampling, plating, and incubation, were performed as previously. Colony identification was performed by fluorescence and oxidase reaction.

Plant-to-Plant Dissemination

The scope of this experiment was to determine if P. syringae moves from plant to plant.

Planting

A 1:8 mixture of the entries AR13 and CLARK was planted in 1987. The plot consisted of 40 rows, three m long and 30 cm apart, planted with a cone seeder. Each row received five g of seed mixture. As

control plots of AR13 and Clark (planted separately), the same plots for the epiphytic populations study were used. The seed was previously sterilized in water at 51°C for 10 minutes. The reasons for choosing AR13 and Clark were the substantial difference in the mean populations of P. syringae that they supported during the 1986 experiment, and the difference in appearance: AR13 is an early, high-population, six-row line while Clark is a later, low-population, two-row cultivar. Also, AR13 has wider leaves and fewer tillers, while Clark has narrower leaves and more tillers.

Leaf Sampling

Leaf samples were taken from 8-10 a.m. From the plot planted with the seed mixture, four plants of AR13 were chosen at random and, pulled out. Their leaves then were cut with a pair of forceps sterilized in 70% ethanol, counted, and put in a Ziploc plastic bag. The leaves of the two Clark plants that were flanking each AR13 plant were sampled in the same way. As controls, the leaves of four plants of AR13 and four plants of Clark from the control plots, chosen at random, were sampled. The samples were transported in the laboratory, stored in a cold room at 4°C, and processed within 2 1/2 hours. The experiment was performed twice.

Leaf Samples Processing

In every plastic bag containing one sample, 100 ml of sterile distilled water were added. Serial dilutions, plating, incubation of the plates and colony identification were performed as in the experiment on diurnal population changes.

Analysis of Results

For each plant sampled, the population of P. syringae per leaf was determined, since the number of leaves per plant was recorded. The population values were transformed to logarithmic, and the populations on the Clark plants from the treatment plot were compared with the populations on the Clark plants from the control plots.

1986 Dissemination Experiment with Marked Strains

The scope of this experiment was to create strains of P. syringae resistant to two antibiotics (double-marked) and to test their ability to survive epiphytically in the field.

Marking Procedure

The procedure to create double-marked strains of P. syringae was performed in three rounds:

1st Round: The selection for marked strains was performed with the "disk" method. A sterile filter paper disk, 1/2 inch in diameter (Schleicher and Schuell, Inc.) was immersed in a filter-sterilized solution of an antibiotic and placed in the middle of a Petri dish containing King's B medium, plated with a suspension of a P. syringae strain (from 24-hour culture on King's B slants at 21°C). The plates were incubated at 21°C in the dark for five days and spontaneous antibiotic-resistant mutants appeared as single colonies in the zone of inhibition around the paper disk. The antibiotics used were rifampicin (0.1, 1, 10, 100, 1000 ppm), erythromycin (10, 100, 1000

ppm), and streptomycin (10, 100, 1000 ppm) (Table 2). Sixteen isolates of P. syringae were used in this round (Table 3).

2nd Round: Colonies selected from the 1st round were suspended in sterile distilled water and plated on Petri dishes containing King's B medium, amended with 1000 ppm rifampicin, or 1000 ppm streptomycin, in order to select for resistant strains to these high concentrations of the antibiotics. The plates were incubated in the dark at 21°C for five days.

3rd Round: In this round, the double-marking was attempted: the selection of strains resistant to two different antibiotics. Colonies selected from the 2nd round were again suspended in sterile distilled water and plated on Petri dishes containing King's B medium amended with one of the following: rifampicin (1000 ppm), streptomycin (1000 ppm), tobramycin (100, 500 ppm), tetracycline (100, 500 ppm), trimethoprim (50, 100, 500 ppm), kasugamycin (50, 100, 500 ppm), and novobiocin (50, 100, 500 ppm). They were incubated in the dark at 21°C for five days.

Planting

Four entries (Klages, Clark, 222-1, and 222-9) were planted in a field of approximately 0.4 hectares at the A.H. Post Research Farm, west of Bozeman, Montana. The field was divided in four equal parts, one for each entry. Planting was performed with a cone planter and each cultivar was planted at a rate of approximately one g of seed/m. The seed was previously sterilized in water at 51°C for 10 minutes.

Table 2. Antibiotics and concentrations (ppm) tested for marking isolates of P. syringae, 1986, 1987.

1986		1987	
1st Round ("Disk" Method)	Streptomycin: 10,100,1000 Erythromycin: 10,100,1000 Rifampicin: 0.1,1,10,100,1000	1st Round (Plating)	Streptomycin: 500 Rifampicin: 100
2nd Round (Plating)	Streptomycin: 1000 Rifampicin: 1000	2nd Round Double Marking (Plating)	Str.: 500-Rif.: 100 Str.: 500-Kan.: 10 Rif.: 100-Kan.: 10
3rd Round Double Marking (Plating)	Streptomycin: 1000 Rifampicin: 1000 Tobramycin: 100,500 Tetracyclin: 100,500 Trimethoprim: 50,100,500 Kasugamycin: 50,100,500 Novobiocin: 50,100,500		

Table 3. List of P. syringae isolates used in the experiments to create antibiotic-resistant (marked) strains.

1986 Experiment:	DG113, DG154, DG167, DG173, DG175 DG178, DG184, DG187, DG198, DG201 DG205, DG206, DG214, DG218, DG219 DG260
1987 Experiment:	DG100, DG101, DG102, DG103, DG104, DG105 DG109, DG112, DG114, DG115, DG116, DG117 DG118, DG119, DG120, DG121, DG122, DG123 DG124, DG125, DG126, DG127, DG128, DG129 DG130, DG131, DG132, DG133, DG134, DG135 DG136, DG138, DG139, DG140, DG141, DG142 DG143, DG144, DG145, DG146, DG147, DG148 DG149, DG150, DG151, DG152, DG153

Inoculum Production and Inoculations

Test tubes containing 5 ml of a liquid medium with nutrient broth and glycerol (hereafter abbreviated NBG) were inoculated, each one with one double-marked strain. They were incubated at room temperature (25°C) in a shaker. After 48 hours, 1 ml from each culture was pipetted in a 2-liter Erlenmeyer flask containing 1 l of the same liquid medium (one culture per flask). Flasks were put in a shaker at room temperature, and after 24 hours all cultures were mixed with 60 liters of distilled water (non-sterile), resulting in an inoculum concentration of approximately 1×10^6 cfu/ml (determined with serial dilutions). All four entries were inoculated the evening of the same day, from 8:45-11:00 with a backpack sprayer. Klages and Clark were at the boot stage; 222-1 and 222-9 were at the early heading stage.

Leaf Sampling

Leaf samples were taken as in the epiphytic population study. From each entry, 3 samples were taken from sites chosen at random and maintained throughout the experiment. Samples were taken in the morning of the day of inoculation, in order to determine the background population of *P. syringae* naturally resistant to the two antibiotics of the double-marked strains (if any).

Leaf Samples Processing

Leaf samples were processed as in the study for epiphytic populations. The medium used was King's B amended with 100 mg/l

cychloheximide (antifungal compound, Sigma Co.) and the antibiotics to which the strains of the inoculum were resistant. The plates were incubated for seven days at 21°C in the dark. Colonies of P. syringae were identified as in the study for diurnal population changes.

1987 Dissemination Experiment with Marked Strains

The scope of this experiment was to create double marked strains of P. syringae, inoculate barley cultivars, and follow the dissemination of the strains through the air (over distance).

Marking Procedure

In this experiment, the double-marking of P. syringae isolates was performed in two rounds:

1st Round: The selection for marked strains was performed by direct plating of bacterial suspensions in sterile distilled water on Petri dishes containing King's B medium amended with rifampicin (100 ppm), or streptomycin (500 ppm), or kanamycin (10, or 20 ppm). The bacterial suspensions originated from 24-hour cultures of 48 P. syringae isolates (Table 3) on King's B slants at 28°C in the dark. The plates were incubated at 21°C in the dark for five days.

2nd Round: Colonies selected from the 1st round were suspended in sterile distilled water and plated on Petri dishes with King's B medium amended with rifampicin (100 ppm) and streptomycin (500 ppm), or rifampicin (100 ppm) and kanamycin (10 ppm), or kanamycin (10 ppm) and streptomycin (500 ppm) in order to select for double-marked strains. The plates were incubated as previously stated (Table 2).

Doubling Times and INA of Double-Marked Strains

The doubling times (DT) of the double-marked strains were compared with the doubling times of the parental strains, in order to select for one or more that would have DT as close as possible to their parental strains, and thus survive better epiphytically.

This study was performed by using a Klett-Summerson photoelectric colorimeter with a red filter. This instrument estimates the bacterial concentration in a liquid culture or suspension by measuring the optical density. So, it was necessary to determine the regression between bacterial concentration and Klett units. In order to do this, seven strains of P. syringae (DG100, DG101, DG103, DG104, DG105, DG146, DG148) were grown on King's B slants for 24 hours at 21 and 28°C. Sterile distilled water suspensions of these cultures were prepared and five-fold dilutions were performed in all. Each dilution was plated on King's B plates and a reading on the Klett was taken immediately after. The experiment was repeated in the same way by using liquid cultures in room temperature of four double-marked strains in NBG. These cultures were each grown in a 500 ml conical flask with a side-arm, special for growth rate studies (Bellco), containing 100 ml of NBG, under constant shaking.

The doubling times of 32 double marked strains, isolated from single colonies, and of their parental strains were calculated. The cultures were first grown in test tubes containing 5 ml of NBG, in room temperature, under constant shaking. After 48 hours, 1 ml from

each culture was pipetted into a 500 ml side-armed flask, containing 100 ml of NBG. The flasks were put on a wrist-action shaker, at room temperature. Readings on the Klett colorimeter were taken every two hours, after bacterial growth was visible ("cloudy" cultures).

The INA of all double-marked strains was tested from 48 hour cultures on King's B medium amended with the necessary antibiotics. Aqueous suspensions of approximately 10^8 cells/ml were prepared, and eight droplets from each strain (0.03 ml) were tested with the standard method of the aluminum foil "boat".

Planting

Two fields at the Horticultural Research Farm, Bozeman, Montana, one 0.12, and the second 0.09 hectares (approximately) were planted, the larger with AR13 and the smaller with AR15, on May 31st, and June 3rd, 1987, at a rate of 5 g seed/3 m. The seed was previously sterilized in water at 51°C for 10 minutes. A cone planter was used. Sprinkler irrigation was provided once or twice a week.

Inoculum Production and Inoculations

They were performed as in the 1986 experiment with marked strains. AR13 was inoculated with an INA+ marked strain (inoculum concentration 1.8×10^7 cells/ml), and AR15 was inoculated with a 1:1 mixture of an INA+ and an INA- strain, carrying different markers (inoculum concentration 2.6×10^7 cells/ml). Both fields were irrigated prior to inoculations. One piece, at the SW corner of every field was left uninoculated.

Leaf Sampling

Leaf samples were taken from 8-10 a.m. from four sites, selected in random in every field, with the standard methodology. One sample was also taken from the uninoculated plots. Samples were taken in order to determine any background P. syringae population naturally resistant to the antibiotics of the double-marked strains, as in the 1986 experiment.

Leaf Samples Processing

The leaf samples were taken in the laboratory, stored in the cold room at 4°C, and processed within 90 minutes, with the standard methodology (addition of sterile distilled water, shaking, serial dilutions). Dilutions from AR13 samples were plated onto BCBRVB and King's B amended with the marking antibiotics and cycloheximide, in order to determine the populations of total and marked P. syringae. Similarly, dilutions from AR15 samples were plated onto BCBRVB and King's B with cycloheximide the appropriate marking antibiotics for each strain sprayed on AR15. The plates were incubated for 5 days at 21°C in the dark. Fluorescence, oxidase reaction and INA tests were used to identify colonies of P. syringae.

Air Dissemination of P. syringae

The scope of this experiment was to detect any aerial dissemination of P. syringae from the inoculated fields of AR13 and AR15, especially the conditions under which this occurred, and the distance of migration. A total of 30 samples to detect airborne

bacteria, were taken from 7/29/87 until 9/4/87, by using mainly two techniques:

Use of an Air Pump

An LVM 110 electric air pump, powered from a car battery was used to sample airborne marked P. syringae (the INA+ strain), approximately 30 cm above the canopy level. The output of the pump was connected to a plastic tube carrying a Millipore filter with a 0.2-microns membrane at the end. Similar devices (Anderson 2000 viable airborne particles sampler) have been used in other studies (Venette and Kennedy, 1975, Lindemann et al., 1982). The membrane filters were put in test tubes containing 5 ml of sterile distilled water, sonicated for five minutes in a ME 4.6 Ultrasonic cleaner (Mettler Electronics Corp.). Serial dilutions were then performed and plated on King's B amended with the appropriate antibiotics, in order to isolate the INA+ marked strain. Only two samples were taken with this technique.

Display of Petri Dishes

Twenty-two sites at various distances from the fields of AR13 and AR15 were selected and King's B Petri dishes, amended with the appropriate antibiotics (for the isolation of the INA+ marked strain) and cycloheximide were displayed, fixed on stakes or fence posts (Figure 1). Such samples were taken under five types of conditions:

1. Petri dishes during the day (morning or afternoon) for up to two hours (five samples).

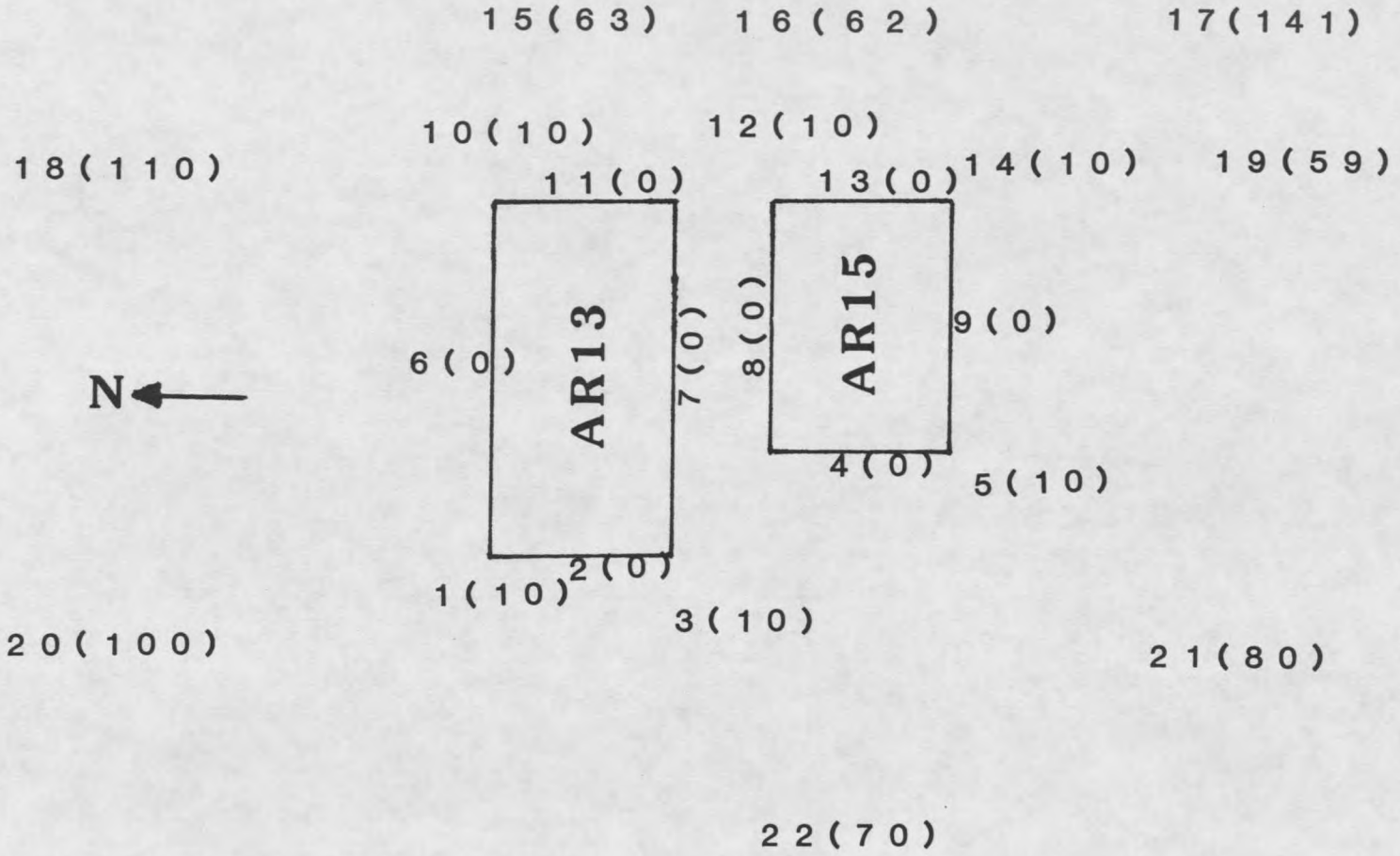

Figure 1. Petri dish display sites around the inoculated fields of AR15 and AR13. In parenthesis, closest distance to the fields, in m.

2. Petri dishes overlayed with 10 ml of sterile distilled water, during the day (morning or afternoon) for up to seven hours (12 samples).
3. Petri dishes in the evening, during and after irrigation, for up to two hours (five samples).
4. Petri dishes during rain (day) for up to 10 hours (two samples).
5. Petri dishes, with or without water, overnight, displayed after sunset and collected before sunrise, in order to avoid the effect of ultraviolet light (four samples).

In cases where water was still present in the plate after collection, plates were transported carefully to the laboratory and dried in the clean air hood.

This experiment was designed to study only the dissemination of the INA+ marked strain of P. syringae.

RESULTS

Variability in P. syringae Population Sizes Among Barley Cultivars

Significant differences in the mean population of epiphytic P. syringae were observed among the 24 entries examined, which were classified as low, intermediate, and high, in regard to the mean P. syringae population (Table 4). Populations were low (log 0-3 cfu/leaf) in all entries except AR13, before heading. An increase in population sizes was generally observed throughout the time of the experiment, and at the end they reached log 3-6 cfu/leaf (Figures 2, 3-26, 33, 34, Tables 5-28).

Differences among the entries were also observed in the percentages of INA+ bacteria in the populations of P. syringae (Table 29). Some entries supported almost consistently 100% INA+ bacteria (AR6, AR13) while others supported lower percentages (Steptoe, Clark).

There was no correlation between population levels and leaf blight symptoms ($r = 0.13$, $r^2 = 0.02$). Symptoms were low (1-2 of the symptom rating scale) and appeared mostly at the end of the growing season.

Six entries: AR4, AR5, AR6, AR13, AR15, and Clark were selected for further study, in order to see if the differences in epiphytic populations of P. syringae would be consistent. These