The University of Reading
Department of Agricultural Botany

DETECTION AND EPIDEMIOLOGY OF BACTERIAL CANKER (*Pseudomonas syringae*) ON WILD CHERRY (*Prunus avium*)

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Thesis submitted for the Degree of Doctor of Philosophy

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ABSTRACT

Bacterial canker has been severely affecting wild cherry trees (*Prunus avium*) and restraining the planting of this valuable hardwood for the last 13 years. The disease has been attributed to the bacterium *Pseudomonas syringae* pv. *morsprunorum*, which has caused bacterial canker in sweet cherry since at least the beginning of this century.

Twenty-four wild cherry sites were investigated. From eight it was possible to isolate a total of 23 cultures. These were used to test rapid diagnostic techniques in conjunction with 52 other cultures obtained from different sources. Two of the wild cherry cultures were obtained from a nursery, which is of grave concern.

The diagnostic techniques tested were based on nutritional tests (Biolog system), nucleic acids (DNA hybridisation probe and REP-PCR), and immunology (slide immunofluorescence and conjugated *Staphylococcus aureus* agglutination).

The Biolog system could identify the bacteria at the species level and allowed a numerical taxonomy study. In this, three clusters were seen, one of *P. s.* pv. *syringae* isolates, another of *P. s.* pv. *morsprunorum* isolates, and a last one of intermediate isolates, including most of the cultures isolated from wild cherry. These cultures were also intermediate in classical nutritional tests that usually discriminate the two pathovars. It is suggested that the wild cherry cultures are an intermediate form, not yet stabilised. They should be included in the *P. syringae* pv. *syringae* rather than in *P. s.* pv. *morsprunorum* until more taxonomic work is done.

A DNA hybridisation probe obtained from other workers failed to react with some *P. s.* pv. *morsprunorum* cultures, possibly because only part of the original probe could be used. Again almost none of the cultures from wild cherry hybridised. Although REP-PCR was too variable to allow identification of *P. s.* pv. *syringae*, it could be used to distinguish it from typical *P. s.* pv. *morsprunorum*. Again the wild cherry cultures had very different patterns from the ones obtained from *P. s.* pv. *morsprunorum*.

The immunofluorescence did not have enough specificity to discriminate pathovars or even species but the same antiserum worked well in the conjugated *S. aureus* agglutination test.

The spatial and temporal spread of *P. s.* pv. *morsprunorum* replicated in simulated plantations containing single inoculated trees was evaluated. The results suggest that epiphytic forms were ubiquitous and that the spacing between trees was unimportant. One year was enough for a plantation, which was initially free of the bacteria to acquire a population almost equivalent to an inoculated plantation. Single isolates of *P. s.* pv. *morsprunorum*, with REP-PCR patterns stable in culture, apparently gave rise to isolates with different patterns within one year of inoculation.

The strategy of planting clonal cherry material, supposedly resistant to bacterial canker, can become very risky because of the high phenotypic and genetic variation of *P. syringae* isolated from wild cherry trees, which was demonstrated by the diagnostic techniques.

To the memory of my father

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CHAPTER 1
INTRODUCTION

Wild cherry (*Prunus avium* L.) has one of the highest value timbers that can be produced in Britain with a relatively short rotation period (55-70 years) and this can probably be shortened by genetically improved clones and tissue culture techniques (NICOLL, 1993). Furthermore, since the First World War the demand for this timber has greatly exceeded the supply and it is one of the most sought after in the trade. Because it is also rapid to establish, PRYOR (1988) deduced that cherry is probably the most financially rewarding hardwood crop to grow.

Moreover, the fruit is important for wild birds and the attractive blossom and the blazing reddish autumnal colours are beneficial elements for landscaping. All these features are indispensable if the intention is to maintain a multifunctional forest, where wildlife and human visitors would cohabit with timber production.

Although variation has been noted no attempt at describing subspecies or races of *P. avium* has been made in Britain, unlike in other European countries. It can be expected to have the usual complex variation associated with species which can reproduce vegetatively, with reduced variation between trees within a stand or wood, but with greater differences apparent among trees in different woods (PRYOR, 1988).

Cherry has occasionally been planted from the 1950s onwards, and plantations can be found in most regions of Britain. Nevertheless, it occurs naturally throughout Britain although it is less common in the northern part of Scotland as it is essentially a lowland species (PRYOR, 1988).

Recently, the European Union has been strongly encouraging its members to divert land from agricultural production and afforest with high value hardwood. Therefore, in 1988 and 1992, the UK government introduced the Woodland Grant Scheme and the Farm Woodland Premium Scheme, respectively, and other countries have recently created similar forestry grant schemes. Horticultural Research

International (HRI) has also created the Farm Woodlands Programme of which a strong component is the genetic improvement of wild cherry trees to obtain superior phenotypes with enhanced resistance to bacterial canker. In the latter programme cherry was adopted as a model species for several projects, because of its economic potential, high-value timber, resistance to squirrel damage, and its ability to substitute for tropical hardwoods (NICOLL, 1993).

The most important problem of cherry trees is bacterial canker, which was first detected in fruiting cherries. According to an ADAS survey, the area under cultivation with sweet cherry in England and Wales decreased by approximately 50% in the period between 1951 and 1966 (FREIGOUN, 1974). It is irrefutable that bacterial canker has been an important factor in this decline.

The first record of bacterial canker in wild cherry trees, in Britain, which was registered by the Forestry Authority, was in June of 1984 (R.G. STROUTS, unpublished data). Since then, several others have been made and it is now seen as the major constraint on wild cherry cultivation. Hence, the Forestry Authority have been recommending a maximum limit on the proportion of cherry trees, of approximately 15%, in the new plantations because they may eventually suffer from the disease and die (J.N. GIBBS, personal communication, 1994).

Because of the increased planting of wild cherry trees in the recent past, the incidence of bacterial canker has become more apparent and in certain years, when the weather conditions are conducive to canker development, damage could become very serious locally (PRYOR, 1988).

In other European countries the disease has also been prevalent. In central Italy, in surveys conducted in a wild cherry plantation during 1992 and 1995, bacterial canker was detected in 17 of the 18 clones examined (SCORTICHINI *et al.*, 1995). In France, the disease has been found, since the last 10 years in the *Picardie* and *Pas-de-Calais* areas, in several cherry plantations of uneven age (BIENFAIT *et al.*, 1995).

More rational disease control strategies may become apparent from a better quantitative understanding of the ecology of epiphytic phytopathogenic bacteria populations and of the epidemiology of the diseases they cause. For example: 1. Disease hazard and the timing of pruning or other treatments may be predicted on the basis of epiphytic population levels on symptomless leaves. 2. Resistant cultivars and wild relatives as well as symptomless susceptible cultivars are potential reservoirs of epiphytic bacteria for dispersal (HIRANO & UPPER, 1983) but their importance is unknown. 3. Disease may come from nurseries and there may be a need to verify

planting material as clean. Reliable, quick methods of diagnosis will also be needed to study these questions.

This Thesis is divided into 10 chapters. After this introductory chapter, chapter 2 will review the most important taxonomic traits of the causal agent, then its symptomatology, epidemiology, life cycle, and control.

Chapter 3 describes the general techniques and procedures used throughout the work.

In chapter 4, the cultures used are described and characterised by the classical nutritional tests. Also, a brief description of the wild cherry sites from which cultures were isolated is made.

In chapters 5 to 8, the diagnostic techniques used during this work are described and the results obtained discussed. Chapter 5 deals with nutritional profiles obtained with a commercial kit and allows some taxonomical thoughts with the aid of numerical analysis. Chapter 6 is concerned with DNA technology, using a repetitive primer that provides the possibility of generating unique genomic fingerprints. In chapter 7, also using a DNA technology, a previously designed probe to detect the causal agent is tested, permitting identification without prior isolation. Chapter 8 studies the possibility of using a polyclonal antiserum with two different assays, immunofluorescence and conjugated *Staphylococcus aureus* agglutination, to assist in the rapid diagnosis of the disease.

In chapter 9, several aspects related to the epidemiology of the disease in wild cherry trees, that have never been investigated, like the spread dynamics of the causal organism from a known source of inoculum and the risk distances in a uniform planting are studied. The potentiality of bud overwintering and survival in summer are also aims of the epidemiological part of this research. An important unanswered question related to control, concerns the sources of bacterial inoculum for wild cherries. The host tree can be implicated as the main potential inoculum source, but other sources may also be important in the disease cycle.

The final conclusions and remarks are made in the chapter 10, where the main questions that had been raised throughout the work and the main conclusions are discussed.

The ultimate purpose of this research is to obtain a better quantitative understanding of the epiphytic bacterial populations on wild cherry to improve the disease control strategy. To achieve this aim it is necessary that reliable diagnosis

allows a precise identification of the bacteria and this was the main focus of the Thesis. The diagnostic work also contributes to clarify the confused taxonomy of the bacteria responsible for the disease.

CHAPTER 2
BACTERIAL CANKER ON CHERRY TREES

2.1 Causal agent

Since the beginning of this century the symptoms of dieback and gummosis in cherry and plum have been attributed to plant pathogens. They were first attributed to various fungi, without successfully fulfilling Koch's postulates, and afterwards to bacteria, *Bacillus spongiosus* Aderhold & Ruhland 1905 and *Pseudomonas cerasus* Griffin 1911 (FREIGOUN, 1974; BRADBURY, 1986). WORMALD (1931 and 1932) was the first to thoroughly describe the symptoms and etiology of the disease in plum trees, and later in sweet cherry trees (WORMALD, 1937 and 1938). In addition, WORMALD (1938) studied the cultural and morphological characters of the causal organism, and named it *Pseudomonas morsprunorum* Wormald 1931. At the same time he differentiated this species, in certain particulars, from *P. syringae* van Hall 1902 (=*P. prunicola* Wormald 1930), which was known to cause bacterial shoot wilt of plum trees (WORMALD, 1930).

Several authors questioned the validity of *P. morsprunorum* as a separate species because its distinguishing characters were not stable *in vitro*, nor sufficient to keep it as a different species (FREIGOUN, 1974). LOVREKOVICH *et al.* (1963) found no differences between the antigenic structures of the two species and considered this evidence that bacteria regarded as *P. morsprunorum* actually belong to the species *P. syringae*. The view that *P. morsprunorum* was different from *P. syringae* was sustained by work of a few authors (CROSSE & GARRETT, 1963 and 1966; GARRETT *et al.*, 1966). This idea was based not only on physiological and morphological differences, but also on phage sensitivity reactions, infection through leaf scars (GARRETT & CROSSE, 1975), and the end of the canker phase in spring. Cankers resulting from *P. syringae* became inactive earlier and bacteria in them died more rapidly than was the case with *P. morsprunorum*. For these authors all the stone fruit isolates corresponded to *P.*

morsprunorum, and the pear and citrus isolates were referred to *P. syringae* on the grounds of their similarity with the lilac strains.

Finally, in 1978, they were considered too similar to warrant separation as species and were included in *P. syringae* van Hall, the incitant of lilac blight, as different pathovars, *morsprunorum* (Wormald) Young, Dye & Wilkie 1978 and *syringae* van Hall 1902 (YOUNG *et al.*, 1978 and 1996).

The cherry strains of *P. s.* pv. *morsprunorum* were divided into two races recognised by their distinctive colony structure and some physiological and pathological characters (FREIGOUN, 1974; FREIGOUN & CROSSE, 1975). There was also some evidence of interactions between races and cultivars of sweet cherry (FREIGOUN, 1974). Race 1 is the most common and longest established form (GARRETT, 1982).

2.2 Symptoms

Regardless of biological differences between pathovars, they cause virtually indistinguishable symptoms (PRUNIER & COTTA, 1985; LELLIOTT & STEAD, 1987). The first sign of the infection is, in early spring, twigs and fruiting spurs that have failed to flush, or have flushed but soon afterwards wilted and died. Infection of fruits is rare and of little economic importance. The bacterial invasion progresses rapidly in the branch and considerable areas of the bark may ultimately be invaded.

Externally, the lesions can be detected by the reddish-brown colour of the bark but especially by the amber coloured gum that exudes copiously (Fig. 2.1), sometimes even without foliar symptoms (STROUTS & WINTER, 1994). If at any time a canker girdles a branch, stem dieback results, which is usually noticed during summer. Symptoms of dieback appear during the growing season depending on the age of the branch and how early it was girdled. On young cherries where cankers are common on the stem, outright death of the tree can occur (Fig. 2.2).

From the end of May, on developing or immature leaves the bacteria cause necrotic round spots of 1-2 mm in diameter (*P. s.* pv. *morsprunorum*) or angular spots of 3-5 mm in diameter (*P. s.* pv. *syringae*); in cases of severe infection the distal margin and apex of the leaf may be killed as a result of multiple infection (PRUNIER & COTTA, 1985). Usually, the leaf spots crack around the margins and fall out leaving small holes producing a "shot-hole" effect.

Figure 2.1
Bacterial canker (*Pseudomonas syringae* pv. *morsprunorum*) symptom showing amber coloured gum on a three year old sweet cherry tree.

2.3 Epidemiology and life cycle

The disease is characterised by a defined seasonal cycle in which an autumn/winter parasitic phase alternates with a summer epiphytic phase. The winter phase (bacterial canker) begins in autumn with the invasion of the cortical and phloem tissues of the stems via the scars left by the falling leaves (Fig. 2.3). Other common sites of infection are the crotch of the tree and the angles between the branches (GARRETT, 1982).

Like most bacterial diseases, bacterial canker is favoured by wet weather. Furthermore, rain, mainly wind driven, is known to be an important factor to carry the bacteria and promote their entrance into the stomata causing a temporary water congestion of the intercellular spaces (CROSSE, 1956). Increased amounts of disease caused by *P. syringae* pathovars have frequently been associated with rain (HIRANO & UPPER, 1994).

Figure 2.2
Wild cherry plantation severely affected by bacterial canker.

After being washed from leaves by rain water and entering the plant through leaf scars the bacteria are drawn into the xylem vessels by negative pressure and eventually migrate into medullary rays and other living tissue (ROOS & HATTINGH, 1987a). The cankers begin to form in winter, after a long period of incubation (four to five months), but make little progress until early spring when they develop quickly. Soon after petal fall the progress of cankers is arrested and the bacteria die in the tissues, because the bark becomes resistant in summer. Cankers are not, therefore, normally perennial. CROSSE & GARRETT (1966) showed that cankers resulting from *P. s.* pv. *syringae* became inactive earlier and bacteria in them died more rapidly than was the case with *P. s.* pv. *morsprunorum*.

The bacterium can also infect the leaves on extension shoots and fruits, but without economic importance (GARRETT, 1982).

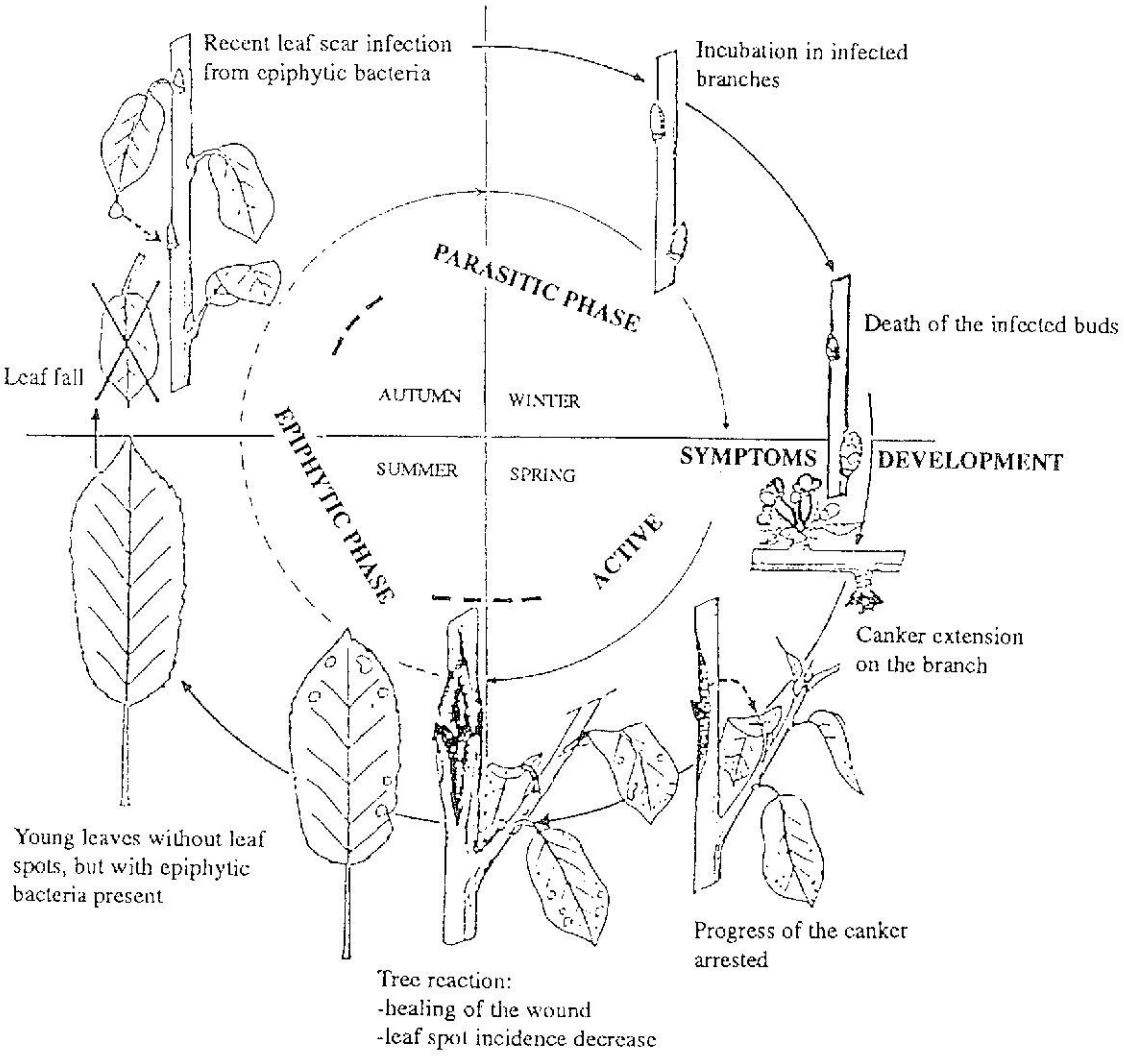

Figure 2.3
Disease cycle of bacterial canker caused by *Pseudomonas syringae* pv. *morsprunorum* (translated from PRUNIER *et al.*, 1985).

Trees are immune to canker infection during summer but the bacteria invade the foliage as resident epiphytic populations, frequently inducing leaf spot. According to CROSSE (1959 and 1963), bacteria are present on the leaf surfaces during the growing season in large numbers and provide the main source of inoculum for the new infections. A direct relationship between the epiphytic population and the incidence of the disease was not established, although a general trend of increased disease associated with increased population was observed (CROSSE, 1963). The infection of a leaf scar during the autumn depends on the arrival of a contaminated drop of water in which the concentration of inoculum exceeds the threshold concentration for that scar

at that particular time. Moreover, the peak periods of susceptibility coincide with the peak epiphytic population levels. GARRETT (1982) also states that injury to bark can provide entry for the bacteria and if it occurs between September and May is likely to induce cankers.

2.4 Control

There are three main possible ways to control the disease, suggested by several authors, namely genetic improvement to enhance resistance, avoidance of wounds during the susceptible period, and protection of leaf scars by use of bacteriostatic sprays.

Developing trees with enhanced resistance to the disease using conventional breeding and methods of genetic transformation is one of the aims of research into trees genetics and physiology which started in 1990 at HRI, East Malling, and a few years before in France at the INRA, Orleans. These genetic improvement programmes eventually are intended to supply multiclone cherries for woodland plantations with resistance to *P. syringae* (NICOLL, 1993).

FREIGOUN (1974) states that, contrary to expectations, no experimental evidence was obtained to suggest that time of pruning is an important measure for reducing the amount of disease. Nonetheless, several workers assert the importance of limiting the wounds extension and number to avoid further infection. STROUTS & WINTER (1994) suggest that the pruning wounds should be treated with a protectant, like octhilinone.

The economically important phase of the disease can be substantially reduced, particularly on young trees, by an autumn Bordeaux mixture spray schedule. These sprays reduce leaf surface populations of bacteria, and thereby the numbers available for leaf scar infection (GARRETT, 1982). Streptomycin is more effective against leaf spot but less potent in controlling canker infection than Bordeaux mixture (FREIGOUN, 1974).

Control of the canker phase has been erratic and usually unsatisfactory. Most control practices have been based on the application of protective bactericides. Copper compounds and antibiotics have been recommended in various spray schedules. These have been ineffective in about half the cases, and have produced from 10 to 80% control in the other half (CAMERON, 1970). The erratic results from present control measures may be partially explained as follows: in trees where the systemic existence of the pathogen is negligible, a protective spray may be quite effective in reducing the entrance of the bacteria from the surface into wounds, bud

scales, or other avenues of infection. If the pathogen is already systemic in the host, then the effectiveness of the protectant is decreased. This probably accounts for the fact that protective copper sprays applied to external surfaces have not reduced the incidence of cankers in many orchards (CAMERON, 1970).

CHAPTER 3
GENERAL PROCEDURES

3.1 Isolation and growth media

Nutrient Sucrose Agar (NSA)

Nutrient agar (Oxoid, CM3) with 5% w/v sucrose was sterilised by autoclaving at 121°C for 15 minutes. Crystal violet (2 ppm) and actidione (50 ppm) were added to suppress respectively Gram-positive bacteria and fungi (FREIGOUN, 1974). The actidione stock solution (10 $mg.ml^{-1}$) was filter sterilised and added to the molten agar when needed.

After incubation for three to five days at 25°C on this medium *Pseudomonas syringae* produces large, domed almost hemispherical, mucoid, white-cream, glistening colonies, which make it easily distinguishable from the majority of saprophytes (WORMALD, 1932). This medium is normally used for the isolation of pseudomonads and to check for levan production in the LOPAT (Levan type colonies, Oxidase reaction, Potato rot, Arginine dihydrolase, Tobacco hypersensitivity) scheme for grouping fluorescent pseudomonads (LELLIOTT *et al.*, 1966). Levan is a polymer synthesised from fructose, and its formation is responsible for the characteristic domed mucoid colonies of some *Pseudomonas* spp. and *Erwinia* spp. on media high in sucrose (HILDEBRAND *et al.*, 1988). These levan type colonies are semi-transparent to translucent initially and become opaque after two-three days.

Luria Bertani (LB)

bacto-tryptone (Difco)	10 g
bacto yeast extract (Difco)	5 g
NaCl	10 g
agar	15 g
distilled water	1 l

The pH was adjusted to 7.0 with 5N NaOH and the medium autoclaved at 115°C for 20 minutes. LB broth was obtained without adding agar to the medium.

This medium has been used in molecular biology, since the early works of LURIA & BURROUS (1957), because of the genetic stability that bacteria present on it. Thus, this medium was used when molecular biology worked was involved, namely to culture *Escherichia coli* for transformation and to culture *P. syringae* prior to DNA hybridisation and PCR techniques.

3.2 Isolation procedures

A loopful (1 μl) of a suspension of macerated plant tissue in sterile water was streaked on the surface of the agar medium to obtain separation of individual colonies. Single representative colonies were subcultured several times until an apparent pure culture was obtained. The presumptive identification of *P. syringae* was done throughout on NSA. Incubation was done at 25°C, unless otherwise stated, because results showed this to be the optimum temperature for growth (WORMALD, 1932).

Bacterial counts

Plates were dried after pouring the medium by removing the lids in a laminar flow cabinet for 30 minutes. Bacteria were counted by plating on NSA serial dilutions in sterile distilled water. An amount of 200 µl from chosen dilutions was spread over the surface of the agar with a bent glass rod. Two replicates were prepared from each of these dilutions. The mean of the dilution containing between 30-300 colonies per plate was calculated and transformed to cfu (colony forming units) per ml.

3.3 Preservation of cultures

The colonies were allowed to grow on NSA, for two days at 25 °C, and three loopfuls of 1 μl were transferred to 5 ml of sterile distilled water in a glass vial. The cells were dispersed in the water, and then the vials were stored at 5°C. To recover the cultures a loopful of the suspension was streaked on the surface of a NSA plate.

This method had been well proved by IACOBELLIS & DEVAY (1986) where even after 24 years of storage the bacterial populations of *P. s.* pv. *syringae* had not greatly decreased. The isolates maintained their pathogenicity and their ability to produce

toxins like syringomycin, indicating genetic stability for these traits. This method also provided easy access for re-establishing the original cultures.

CHAPTER 4
CULTURE ACCESSION

4.1 Culture accession and study sites

Culture accession

The 75 cultures of *Pseudomonas syringae* investigated in this work were obtained from four different sources. Forty four isolates were offered by the Horticultural Research International (HRI); five isolates acquired from the National Collection of Plant Pathogenic Bacteria (NCPPB); three isolates were donated from the working collection of MAFF's Central Science Laboratory (CSL); and 23 cultures were isolated from wild cherry trees, the large majority from woodlands, during my work in 1994 and 1995. The codes, hosts, and origin of the acquired cultures are listed in Table 4.1.

Study sites

During the years of 1994 and 1995, 24 wild cherry tree sites were investigated for the presence of bacterial canker. The cultures were isolated by the author onto NSA medium, with 2 ppm of crystal violet and 50 ppm of actidione added, on which the pathogenic pseudomonads form levan type colonies. The isolation methods are presented in section 3.2. Colonies were selected at random from primary isolation plates and purified by restreaking. Almost all the isolations were made during late winter, spring, or early summer. Only from eight sites was it possible to isolate *P. syringae*, 15 of these isolates being from cankers, either located in the trunk or in branches, and the rest from leaf washings. The culture codes, and the characteristics of the wild cherry trees from where the 23 cultures were isolated during this work, are listed in Table 4.2.

Table 4.1

Plant material from which the cultures were isolated, and origin of the *Pseudomonas syringae* cultures used in this work.

Host and culture	Isolation method	Country	Year	Worker
Pseudomonas syringae				
Sweet cherry (*Prunus avium*)				
Ps214	———[a]	UK	1983	———
Ps5355	———	France	1978	———
Ps5356	———	UK		———
Cherry laurel (*P. laurocerasus* L.)				
Ps73	Leaf spot	UK	1982	———
Pseudomonas syringae pv. *morsprunorum*				
Sweet cherry				
Psm617 (=NCPPB 617)	———	Netherlands	1955	A. Fuchs
Psm798 (=NCPPB 1781)	———	Italy	1965	G. Ercolani
Psm881 (=NCPPB 626)	———	Netherlands	1959	P. Matthews
Psm889 (=CSL 889)	———	———	—	———
Psm1462 (=NCPPB 1462)	Leafwash	UK	1960	J. Crosse
Psm2206 (=NCPPB 1463)	———	UK	1961	J. Crosse
Psm5238 (=NCPPB 1459)	Leafwash	UK	1957	J. Crosse
Psm5239 (=NCPPB 1460)	Leafwash	UK	1957	J. Crosse
Psm5240	Leaf spot	UK	1958	———
Psm5241	———	UK	—	———
Psm5243 (=NCPPB 1462)	Leafwash	UK	1960	J. Crosse
Psm5244	Seedling stem canker	UK	1960	———
Psm5249	Branch inoculation	UK	1964	———
Psm5250	Leaf spot	UK	1971	———
Psm5252	———	UK	1973	———
Psm5254	———	UK	1980	———
Psm5255	———	UK	—	———
Psm5257	Branch canker	UK	1978	———
Psm5260	———	UK	—	———
Psm5280	Branch inoculation	UK	1964	———
Wild cherry (*Prunus avium*)				
Psm680 (=NCPPB 680)	———	UK	1959	R. Lelliott
Psm5265	Leafwash	UK	1990	C. Garrett
Psm5266	Leafwash	UK	1990	C. Garrett
Psm5267	Leafwash	UK	1990	C. Garrett
Psm5268	Leafwash	UK	1990	C. Garrett
Psm5269	Branch	UK	1990	C. Garrett
Psm5270	Branch canker	UK	1990	C. Garrett
Psm5271	Leafwash	UK	1990	C. Garrett
Psm5272	Leafwash	UK	1990	C. Garrett
Psm5273	Leafwash	UK	1990	C. Garrett
Psm5274	Leafwash	UK	1990	C. Garrett
Psm5275	Branch canker	UK	1990	C. Garrett
Psm5276	Leafwash	UK	1990	C. Garrett
Psm5277	Leafwash	UK	1990	C. Garrett
Plum (*P. domestica* L.)				
Psm797 (=NCPPB 1095)	———	UK	1960	E. Billing
Psm2928	———	Switzerland	1965	———
Psm5281	Leaf spot	UK	1961	———
Psm5282	Leaf spot	UK	1964	———
Psm5299	———	UK	—	———
Psm5300	———	UK	—	———

Table 4.1 (continuation)

Plant material from which the cultures were isolated, and origin of the *Pseudomonas syringae* cultures used in this work.

Host and culture	Isolation method	Country	Year	Worker
Pseudomonas syringae pv. *syringae*				
Lilac (*Syringa vulgaris* L.)				
Pss281 (=NCPPB 281) Pathotype strain	———	UK	1950	K. Sabet
Pear (*Pyrus communis* L.)				
Pss5340	Shoot	UK	1959	———
Pss5342	Blossom	UK	1959	———
Sweet cherry				
Pss310 (=NCPPB 310)	———	UK	1950	J. Crosse
Pss2942	———	New Zealand	1901	———
Pss5357	Leaf spot	UK	1979	———
Wild cherry				
Pss5264	Leafwash	UK	1990	C. Garrett
Portuguese laurel (*Prunus lusitanica* L.)				
Pss420 (=CSL420)	———	———	—	———

[a] The cells with a strikethrough line means that no information was available from the source.

The major characteristics of the wild cherry tree sites studied, in 1994 and 1995, are now described briefly.

Study sites:

1. Watlington, Oxfordshire (SU 682 948). A few trees in a house garden severely affected with heavy gumming and extensive branch lesions. Several trees were already dead because the problem had been detected in June 1989. One culture (M1) was isolated from a branch canker.

2. North Aston, Oxfordshire (SU 475 288). Several nursery potted trees, 3 years old, were affected with presence of clear gum oozing from the trunk and leaf spots. Two cultures (NA5 and NA7) were isolated from this nursery.

3. Banbury, Oxfordshire (SP 430 416). Several trees showing dieback but without clear symptoms of bacterial canker.

4. Compton Wynyates Estate, Warwickshire (SP328 417). Almost all of around 300 trees have died since 1985. No cultures could be obtained from the material sampled.

5. Reading, Berkshire (SU 732 715). One isolated tree, around 30 years old, located in a park had the shoots severely affected with heavy transparent gumming. Two cultures (R4 and R8) were isolated from small cankers located in branches.

Cultures from leaf washings did not yield *P. syringae*. It is very important to note that these cultures were isolated in mid July (11.07.94).

6. Mansel Lacy, Herefordshire (SO 405 473). Small area of very severely affected trees around 50 years old. Very large cankers could be seen at the base of the trunk.

7. Mansel Lacy, Herefordshire (SO 395 478). Area of around 20 trees eight years old in a mixed plantation. Three trees were exhibiting leaf curl and some dieback. In the trunk small dark brown lesions under the bark, full of gum were detected. These lesions were connected with the presence of plastic tree shelters completely closed around the trees up to a height of 1.8 m. The dead areas of the bark coincided with the areas where there was accumulation of leaf litter. From these lesions only bacteria from the group *Erwinia herbicola* (Lönhis) Dye – *Pantoea agglomerans* (Beijerinck) Gavini *et al.* were isolated.

8. Alice Holt Forest, Hampshire (SU 812 426). Four year old pure plantation of 0.5 ha severely affected with production of gum. Only 13% of the trees were alive. All the dead and very affected trees were still inside the corrugated plastic trunk guards. Four cultures [1AH(a), 1AH(b), 1AH(d), and 1AH(c)] were isolated from cankers of different affected trees.

9. Alice Holt Forest, Hampshire (SU 810 409). Three year old pure plantation of 0.5 ha without visible symptoms of bacterial canker.

10. Alice Holt Forest, Hampshire (SU 813 409). Twelve years old mixed plantation mainly with *Quercus robur* L. with a total area of 1 ha. Of a total of 59 trees, two were dead and one was affected by severe dieback. Two cultures [3AH(a) and 3AH(c)] were obtained from leafwashings.

11. Chawton Park Wood, Hampshire (SU 678 365). Site constituted by very tall trees 39 years old. Around the car parking area and scattered around the woods there were a few trees exhibiting dieback but their height made the bark tissue collection impossible. The leaf samples did not harbour *Pseudomonas syringae*.

12. Tugley Wood, Surrey (SU 985 336). Small area (0.2 ha) planted nine years ago, pure in the centre and mixed with other broadleaf trees in the borders. Twelve trees were dead and 59 were alive. Several trees were very weak because of the corrugated trunk guards. On the dead or almost dead trees, it was possible to see a different colour in the bark and a very rough texture up to the height of the tree shelters. Only one tree was exuding gum but *P. syringae* was quite possibly the cause of

Table 4.2

Characteristics of the wild cherry plantations from which the 23 *P. syringae* cultures were isolated during the years of 1994 and 1995.

Culture reference	Age (years)	Isolation method	Tree location (BNG)	Date of isolation
M1	6-20	Branch canker	Watlington, Oxfordshire (SU 682 948)	15.03.1994
NA5	3	Trunk canker	North Aston, Oxfordshire (SP 475 288)	15.03.1994
NA7	3	Trunk canker	North Aston, Oxfordshire (SP 475 288)	15.03.1994
R4	≈ 30	Branch canker	Reading, Berkshire (SU 732 715)	11.07.1994
R8	≈ 30	Branch canker	Reading, Berkshire (SU 732 715)	11.07.1994
1AH(a)	4	Trunk canker	Alice Holt Forest, Hampshire (SU 812 426)	02.05.1995
1AH(b)	4	Trunk canker	Alice Holt Forest, Hampshire (SU 812 426)	02.05.1995
1AH(d)	4	Trunk canker	Alice Holt Forest, Hampshire (SU 812 426)	02.05.1995
1AH(e)	4	Trunk canker	Alice Holt Forest, Hampshire (SU 812 426)	02.05.1995
3AH(a)	12	Leafwash	Alice Holt Forest, Hampshire (SU 813 409)	04.05.1995
3AH(c)	12	Leafwash	Alice Holt Forest, Hampshire (SU 813 409)	04.05.1995
11L(b)	5	Branch canker	Liphook, West Sussex (SU 836 292)	23.05.1995
11L(e1)	5	Branch canker	Liphook, West Sussex (SU 836 292)	23.05.1995
11L(e2)	5	Branch canker	Liphook, West Sussex (SU 836 292)	23.05.1995
11L(f2a)	5	Branch canker	Liphook, West Sussex (SU 836 292)	23.05.1995
11L(f2b)	5	Branch canker	Liphook, West Sussex (SU 837 293)	23.05.1995
11L(f3)	5	Branch canker	Liphook, West Sussex (SU 837 293)	23.05.1995
12AF	4	Leafwash	Abinger Forest, Surrey (TQ 147 453)	25.05.1995
13EF (a)	41	Leafwash	Effingham Forest, Surrey (TQ 104 505)	15.06.1995
13EF(b)	41	Leafwash	Effingham Forest, Surrey (TQ 104 505)	15.06.1995
13EF(c)	41	Leafwash	Effingham Forest, Surrey (TQ 104 505)	15.06.1995
13EF(d)	41	Leafwash	Effingham Forest, Surrey (TQ 104 505)	15.06.1995
23SW	40	Leafwash	Sulham Wood, Berkshire (SU 651 734)	11.05.1995

BNG - British National Grid

21. Effingham Forest, Surrey (TQ 094 508). Mixed plantation (0.5 ha), 38 years old. Almost all the trees seemed healthy and no cultures were isolated from the leaf samples taken.

22. Ashley Hill Forest, Berkshire (SU 823 813). Pure plantation (1.8 ha), 42 years old. Several trees were heavily defoliated and with severe dieback. There were a few very tall trees dead and others had been cut down already. No cultures were isolated from branch cankers in the suckers or from leaf samples.

23. Ashley Hill Forest, Berkshire (SU 825 809). Small mixed plantation (0.2 ha) of 41 years old. This site was almost in the same state as the previous one, but the trees were slightly less damaged and the plantation was mixed with Q. *robur* and *Betula* sp.

24. Sulham Wood, Berkshire (SU 651 734). Small mixed plantation (0.3 ha) of 40 years old. No bacterial canker symptoms could be detected but from the leaf samples one *P. syringae* culture (23SW) was isolated.

4.2 Characterisation of the culture accession

The initial characterisation of the isolates was made by cultural characters and by hypersensitivity reaction on tobacco.

4.2.1 Cultural characters

The most important distinguishing characters between *P. s.* pvs. *morsprunorum* and *syringae* (Table 4.3) are growth characteristics in nutrient sucrose broth (NSB), aesculin hydrolysis, gelatin liquefaction, presence of fluorescence or presence of a brown diffusible pigment on King's medium B, and recovery from 5% nutrient sucrose agar (NSA) after six days (LELLIOTT & STEAD, 1987). These cultural tests were developed by WORMALD (1932) and GARRETT *et al.* (1966). In addition, other cultural tests have been considered differentiating such as the use of D,L-lactic acid and L-leucine (GARRETT *et al.*, 1966; BURKOWICZ & RUDOLPH, 1994).

Test methods

All tests were made at least once on every culture. Two day old cultures on NSA were used as the inoculum for each test. The incubation temperature was 25°C and media were sterilised at 121°C for 15 minutes, except in the test for the utilisation of D,L-lactic acid and L-leucine. Seventy five isolates were tested.

Table 4.3
Characters distinguishing *P. s.* pv. *morsprunorum* from *P. s.* pv. *syringae* (GARRETT *et al.*, 1966; LELLIOTT & STEAD, 1987).

Character	pv. *morsprunorum*	pv. *syringae*
Growth in 5% NSB	White	Yellow
Aesculin hydrolysis	–	+
Gelatin liquefaction	–	+
Brown diffusible pigment on King's medium B	±	–
Fluorescence on King's medium B	±	+
Use of D,L-lactic acid	–	+
Use of L-leucine	–	+

Growth in NSB. Tubes of Oxoid nutrient broth containing 5% sucrose were inoculated and the colour of the growth recorded after three days. WORMALD's (1932) method was used.

Aesculin Hydrolysis. Aesculin utilisation indicated by a progressive blackening within three-four days, assumed to result from β-glucosidase activity, was examined on slopes of the following medium: peptone (Oxoid L37), 10 g; aesculin, 1 g; ferric citrate scales, 0.5 g; agar (Oxoid No.3) 12 g; distilled water, 1 l. The culture was streaked on to a slope. The production of a dark brown to black colour in the medium was scored as a positive reaction.

Gelatin liquefaction. Gelatin hydrolysis was tested on nutrient gelatin containing: yeast extract, 3 g; peptone, 5 g; gelatin, 120 g; distilled water, 1 l; pH ≈ 7.0. After allowing the solids to dissolve for 15 minutes the medium was heated to dissolve, and dispensed into tubes to a depth of approximately 5 cm. The inoculation was made by stabbing and a noninoculated control was used. After seven days, before recording the results, the tubes were cooled at 4°C for 30 minutes. Liquefaction was scored positive when the medium flowed readily when tilted.

Fluorescent pigment production. Plates of the medium B of KING *et al.* (1954) were used. Cultures were examined for a greenish diffusible pigment in ultraviolet light for up to 7 days.

Utilisation of D,L-lactic acid and L-leucine. Use of these organic compounds was assessed by the results obtained from the wells E6 and G3 of the Biolog's GN MicroPlate (see section 5.2 and Fig. 5.1).

Recovery from 5% NSA. Plates of NSA (see section 3.1) were streaked and recovery was performed after six days in tubes of NSB and on plates of NSA.

Results and discussion

A summary of the reactions by the cultures to most of the tests is given in Table 4.4.

The distinctive cultural pattern of *P. s.* pvs. *morsprunorum* and *syringae* were confirmed in this study. After three days growth in NSB almost all the *P. s.* pv. *morsprunorum* cultures from sweet cherry and all from plum appeared white and opalescent. Only Psm881, Psm5250, and Psm5252 showed a yellow tinge. These three cultures also showed different responses to other tests, and should not be considered typical forms of *P. s.* pv. *morsprunorum*.

The aesculin hydrolysis test was very clear to interpret and gave distinct results for the two pathovars. In *P. s.* pv. *morsprunorum* group only Psm881 and nine isolates from wild cherry presented a positive response for this test. In the *P. s.* pv. *syringae* group all the isolates showed a positive reaction except Pss5342.

Gelatin liquefaction activity was not detected with isolates of *P. s.* pv. *morsprunorum*, except in Psm5250 and in seven isolates from wild cherry. In addition, almost all the pv. *syringae* isolates were positive in this test, except Pss2942 and Pss5342.

Almost none of the isolates of *P. s.* pv. *morsprunorum* fluoresced on King's medium B, except Psm881 and seven isolates from wild cherry. A brown diffusible pigment appeared in 72% of the cultures that did not show fluorescence. All the *P. s.* pv. *syringae* isolates fluoresced.

Use of D,L-lactic acid gave the most discriminatory results of all the tests. All the *P. s.* pv. *morsprunorum* isolates were negative, except four of the intermediate forms from wild cherry, and all the *P. s.* pv. *syringae* isolates were positive. Other authors have also agreed that this test can differentiate the pathovars rather well (BURKOWICZ & RUDOLPH, 1994).

Use of L-leucine did not give distinct results, which could be explained by the short incubation period (24 h) of the Biolog plates.

Table 4.4
Differential phenotypic features among the isolates.

Isolate	Growth in NSB	Aesculin hydrolysis	Gelatin liquefaction	Fluorescence on King's medium B	Use of D,L-lactic acid	Use of L-leucine
P. syringae						
Sweet cherry						
Ps214	yellow	+	+	+	–	±
Ps5355	yellow	+	–	–	+	±
Ps5356	yellow	+	–	–	+	+
Wild cherry						
1AH(a)	yellow	+	+	+	–	–
1AH(b)	yellow	+	+	+	–	–
1AH(d)	yellow	+	+	+	–	–
1AH(e)	yellow	+	+	+	–	–
3AH(a)	white	+	+	+	–	–
3AH(c)	white	+	+	+		
11L(b)	yellow	+	+	+	–	–
11L(e1)	yellow	+	+	+	–	–
11L(e2)	yellow	+	+	+	–	–
11L(f2a)	yellow	+	+	+	–	–
11L(f2b)	yellow	+	+	+	–	–
11L(f3)	yellow	+	±	+	–	–
12AF	yellow	–	–	–	±	–
13EF(a)	white	–	–	+	–	–
13EF(b)	white	+	+	+	–	–
13EF(c)	white	+	+	+	–	–
13EF(d)	yellow	+	+	+	–	–
23SW	yellow	+	+	+	–	–
M1	white	+	+	+	–	–
NA5	white	+	+	+	–	–
NA7	yellow	+	+	–	–	–
R4	yellow	+	+	+	–	–
R8	yellow	+	+	+	±	–
Cherry laurel						
Ps73	yellow	+	+	+	+	–
P. s. pv. *morsprunorum*						
Sweet cherry						
Psm617	white	–	–	– (bp)	–	–
Psm798	white	–	–	– (bp)	–	–
Psm881	yellow	+	–	+	–	–
Psm889	white	–	–	– (bp)	–	–
Psm1462	white	–	–	– (bp)	–	–
Psm2206	white	–	–	–	–	–
Psm5238	white	–	–	– (bp)	–	–
Psm5239	white	–	–	– (bp)	–	–
Psm5240	white	–	–	– (bp)	–	–
Psm5241	white	–	–	–	–	–
Psm5243	white	–	–	– (bp)	–	–
Psm5244	white	–	–	– (bp)	–	–
Psm5249	white	–	–	– (bp)	–	–
Psm5250	yellow	–	+	– (bp)	–	–

Table 4.4 (continuation)
Differential phenotypic features among the isolates.

Isolate	Growth in NSB	Aesculin hydrolysis	Gelatin liquefaction	Fluorescence on King's medium B	Use of D,L-lactic acid	Use of L-leucine
Psm5252	yellow	–	±	–	–	–
Psm5254	white	–	–	– (bp)	–	–
Psm5255	white	–	–	–	–	–
Psm5257	white	–	–	–	–	–
Psm5260	white	–	–	–	–	–
Psm5280	white	–	–	– (bp)	–	–
Wild cherry						
Psm680	white	–	±	–	–	–
Psm5265	yellow	+	+	+	–	–
Psm5266	white	–	–	– (bp)	–	–
Psm5267	yellow	+	+	– (bp)	+	–
Psm5268	yellow	+	+	+	–	–
Psm5269	white	–	–	– (bp)	–	–
Psm5270	white	–	–	–	–	–
Psm5271	yellow	–	–	–	–	–
Psm5272	yellow	+	+	– (bp)	+	–
Psm5273	yellow	+	+	+	–	–
Psm5274	yellow	+	+	+	+	–
Psm5275	yellow	+	+	+	±	–
Psm5276	yellow	+	±	+	±	–
Psm5277	yellow	+	–	+	+	–
Plum						
Psm797	white	–	–	– (bp)	–	–
Psm2928	white	–	–	– (bp)	–	–
Psm5281	white	–	–	– (bp)	–	–
Psm5282	white	–	–	– (bp)	–	–
Psm5299	white	–	–	– (bp)	–	–
Psm5300	white	–	–	– (bp)	–	–
P. s. pv. *syringae*						
Lilac						
Pss281	yellow	+	+	+	+	–
Pear						
Pss5340	yellow	+	+	+	+	–
Pss5342	yellow	–	–	+	+	±
Sweet cherry						
Pss310	yellow	+	+	+	+	±
Pss2942	yellow	+	–	+	+	–
Pss5357	yellow	+	+	+	+	–
Wild cherry						
Pss5264	yellow	+	+	+	+	–
Portuguese laurel						
Pss420	yellow	+	+	+	+	±

bp - presence of brown diffusible pigment
± - borderline

The results of recovery from NSA were quite unexpected. Only two cultures of *P. s.* pv. *morsprunorum* from sweet cherry (Psm617 and Psm5239) and two from plum (Psm5299 and Psm5300) showed the characteristic death after six days on NSA. All the other cultures were easy to recover after six days. Other authors have also considered this test unsatisfactory even after 8 days on NSA (BURKOWICZ & RUDOLPH, 1994).

The culture inducing "shot-hole" in cherry laurel (Ps73) identified only as *P. syringae*, can undoubtedly be included in the *P. s.* pv. *syringae* group.

Most of the cultures isolated from wild cherry in 1990 and identified as *P. s.* pv. *morsprunorum* did not actually show a clear signature of that pathovar. Only three cultures (Psm5266, Psm5269, and Psm5270) out of 13 showed the expected pattern. Three cultures had a characteristic *P. s.* pv. *syringae* pattern (Psm5274, Psm5275, and Psm5276) and are presumably misidentified. The other seven cultures of this group had an intermediate behaviour between the two pathovars but closer to pv. *syringae*.

The group of cultures from wild cherry isolated during my work, in 1994 and 1995, identified only as *P. syringae* clearly showed an intermediate behaviour between the two pathovars, 13 of them being very similar to pv. *syringae* [1AH(a), 1AH(b), 1AH(d), 1AH(e), 11L(b), 11L(e1), 11L(e2), 11L(f2a), 11L(f2b), 11L(f3), 13EF(d), 23SW, and R4] and one (R8) that could be identified as *P. s.* pv. *syringae*.

Notwithstanding the differences between the pathovars, several workers have previously reported the existence of intermediate forms (LATORRE & JONES, 1979a; MANSVELT & HATTINGH, 1986). The abundant evidence for the existence of intermediate forms between the two pathovars have been confirmed since the work of CROSSE & GARRETT (1963) and GARRETT *et al.* (1966), where they stated that further investigation of isolates from a wider range of plants may well reveal the occurrence of those on a much wider scale than is apparent. The wide distribution and host range of isolates of *P. s.* pv. *syringae* suggest that this may be the more general, parasitically less specialised type, from which the pv. *morsprunorum* may have been derived under selection pressures existing in horticultural regions (GARRETT *et al.*, 1966). Several tests demonstrated that pv. *morsprunorum* is a rather homogeneous pathovar with a narrow host range, in contrast to the more heterogeneous pv. *syringae* (BURKOWICZ & RUDOLPH, 1994). The pv. *morsprunorum* occurs worldwide, but infects only *Prunus* species, mainly fruit trees (BRADBURY, 1986), in contrast to a very wide host range of the pv. *syringae*. This could be explained by the higher virulence of pv. *morsprunorum*

towards sweet cherry compared to pv. *syringae* isolates (BURKOWICZ & RUDOLPH, 1994).

Nevertheless, BURKOWICZ & RUDOLPH (1994) believe that well-defined groups may still exist despite deviations of particular isolates from the standard pattern in one or very few characteristics. These authors believe that the suggestions of BILLING (1970), who speaks of a positive reaction to a test by a group of strains when 80-100% of the strains respond positively, should be followed.

4.2.2 Tobacco hypersensitivity test

The hypersensitive reaction (HR) in response to plant pathogenic bacteria was first recognised, for pseudomonads, on bean pods by KLEMENT & LOVREKOVICH (1961 and 1962) and later on tobacco by KLEMENT (1963) and KLEMENT *et al.* (1964).

The HR test is often used in diagnosis as a test for the pathogenic potential of necrogens (BILLING, 1987). Some authors state that the hypersensitive reaction on tobacco is not reliable as a criterion for establishing pathogenicity, because some isolates induce a reaction on tobacco but failed to infect the specific host plant and other isolates infect the host plant but not tobacco (LATORRE & JONES, 1979a). The hypersensitive reaction might be just one common property of a group of bacteria (HIRANO & UPPER, 1990). Nevertheless, since the HR symptoms are elicited almost solely by plant pathogenic bacteria and not by non-pathogenic saprophytes, HR in tobacco leaves or other tissues has become a standard test for plant pathogenicity of bacterial isolates. Saprophytic bacteria alone do not have the capacity to initiate either pathogenesis or HR (GOODMAN & NOVACKY, 1994). Furthermore, because a clear distinction can often be made between compatible and incompatible strains of necrogenic pathogens, this test is widely used in research on plant-pathogen interactions (BILLING, 1987).

BURKOWICZ & RUDOLPH'S (1994) studies revealed a strict correlation between the HR of tobacco leaves and the pathogenicity tests on the original hosts. Only two *P. s.* pv. *syringae* isolates from a total of 216 isolates of *P. s.* pvs. *syringae*, *morsprunorum* and *persicae*, mainly originating from fruit trees, were HR-positive but negative in the virulence tests. The reverse, positive in virulence tests but HR-negative on tobacco, was never observed.

Tobacco is the preferred plant since it is easy to cultivate and maintain, its large cavities beside leaf veins make it relatively easy to infiltrate inoculum and its reaction to many pathogens is well known (LELLIOTT & STEAD, 1987). The inoculation

technique developed (KLEMENT, 1963) enables easy infiltration of plant tissues with a known concentration of bacteria. Following infiltration of the bacteria, the sequence of events can be divided into three stages (BILLING, 1987). First, the induction period lasting about 3-4 h at the end of which the reaction is irreversible. There is then a delay, the latent period, before visible change in the structure of the plant cell is seen. This is soon followed by death of the plant cell and tissue collapse, the necrotic stage. Cell death is usually accompanied by browning of the tissue due to oxidation of phenolic substances released from dying cells. Tissues desiccate, often to dryness, within 24 h at most 48 h. Only tissue inoculated with live incompatible bacteria is killed while the remainder is spared, if the water transport is not interrupted by the collapsed tissue (GOODMAN & NOVACKY, 1994).

The pathogenicity of the culture accessions gathered in my work was confirmed by the tobacco hypersensitive reaction test.

Test method

Potted tobacco (*Nicotiana tabacum* L.) plants cv. White Burley, grown under ordinary glasshouse conditions until they had large leaves suitable for the test, were used with the method described by KLEMENT (1963). Bacteria were grown on Nutrient Agar (Oxoid) for 24 h and suspended in sterile distilled water to a concentration of 10^8 cfu.ml^{-1}. After calibration, a turbidimeter (Biolog) was used routinely to estimate the cell density. A hypodermic needle with an external diameter of 0.6 mm was used to inject the mesophyll with the suspension, near the lateral vein of the abaxial surface of the leaf. Injection of sterile distilled water was used as a control. The inoculated areas were labelled by using adhesive labels. Around eight to ten inoculations were made in each leaf and 72 isolates, with one repetition, were tested by this method.

The plants were left in the glasshouse and the results assessed two days after the inoculation. A positive HR reaction was given by a rapid collapse of inoculated tissue after 24-48 h, confirmed by a light-brown necrosis of the tissue within 3 days (Fig. 4.1). Yellowing or browning without collapse was not considered a positive reaction.

Results and discussion

The hypersensitive reactions of the various isolates on tobacco are presented in Table 4.5.

Table 4.5

Reactions induced by *P. syringae* isolates in the tobacco leaf.

Isolate	HR reaction
P. syringae	
Sweet cherry	
Ps214	–
Ps5355	+
Ps5356	+
Wild cherry	
1AH(a)	+
1AH(b)	+
1AH(d)	+
1AH(e)	–
3AH(a)	+
3AH(c)	+
11L(b)	+
11L(e1)	+
11L(e2)	+
11L(f2a)	+
11L(f2b)	+
11L(f3)	+
12AF	–
13EF(a)	+
13EF(b)	+
13EF(c)	+
13EF(d)	+
23SW	+
M1	–
NA5	+
NA7	+
R4	–
R8	+
Cherry laurel	
Ps73	+
P. s. pv. *morsprunorum*	
Sweet cherry	
Psm617	+
Psm798	+
Psm881	+
Psm889	+
Psm1462	+
Psm2206	+
Psm5238	+
Psm5239	+
Psm5240	–
Psm5241	+
Psm5243	+
Psm5244	+
Psm5249	+
Psm5250	+
Psm5252	+
Psm5254	–
Psm5255	–
Psm5257	–
Psm5260	–
Psm5280	+
Wild cherry	
Psm680	+
Psm5265	+
Psm5266	+
Psm5267	+
Psm5268	+
Psm5269	+
Psm5271	+
Psm5272	+
Psm5273	+
Psm5274	+
Psm5275	+
Psm5276	+
Psm5277	+
Plum	
Psm797	+
Psm2928	–
Psm5281	–
Psm5300	+
P. s. pv. *syringae*	
Lilac	
Pss281	+
Pear	
Pss5340	+
Pss5342	+
Sweet cherry	
Pss310	+
Pss2942	+
Pss5357	+
Wild cherry	
Pss5264	+
Portuguese laurel	
Pss420	+

Five of the *P. s.* pv. *morsprunorum* isolates from sweet cherry and two from plum showed a negative HR. However, all the isolates from wild cherry identified as *P. s.* pv. *morsprunorum* and all the *P. s.* pv. *syringae* isolates were positive. Of the wild cherry group isolated in my work and just identified as *P. syringae*, 83% were HR-positive. The HR-negative isolates were regarded as saprophytic pseudomonads,

although host pathogenicity tests in cherry stems should be performed before discarding completely these isolates as non-pathogenic. Saprophytic pseudomonads in lesions caused by other organisms are by no means uncommon in plant diseases (GARRETT *et al.*, 1966).

Figure 4.1

Hypersensitivity reaction on tobacco. Eight isolates and the water control were tested in one leaf. The isolates were: four *Pseudomonas syringae* pv. *morsprunorum* isolates from wild cherry (Psm 5272, Psm5273, Psm5274, and Psm5276), one from sweet cherry (Psm5241), and one from plum (Psm5300); one *P. s.* pv. *syringae* isolate from pear (Pss5342); and one *P. syringae* culture (Ps 5400) isolated from myrobalan plum (*Prunus cerasifera*) not belonging to the culture accession.

4.3 Conclusions

It was only possible to obtain *P. syringae* cultures from eight of 24 wild cherry sites visited. The frequent presence of bacteria of the group *Erwinia herbicola – Pantoea agglomerans* made the isolation of *Pseudomonas syringae* quite difficult. The hemispheric colonies because of levan production by those saprophytes, or weak pathogens (BRADBURY, 1986), caused several misidentifications on the primary plates. In some cases the black lesions in the trunk were apparently caused by *E. herbicola*

when the trees were in severe stress caused by the plastic trunk guards fixed tightly around the tree trunk.

Almost all the sites visited had different degrees of bacterial canker and severe dieback and defoliation, sometimes with gum exudations, were frequent symptoms.

Generally, the distinct cultural patterns of *P. s.* pvs. *morsprunorum* and *syringae* were confirmed but a few of the cultures examined seemed to have been misidentified.

The presence of atypical forms of *P. s.* pv. *morsprunorum* in the wild cherry group suggests that these forms do not truly belong to this pathovar and they should be considered intermediate forms. Actually, they seem a very wide group with several differences from pv. *morsprunorum* and should be better included in the *P. s.* pv. *syringae* group if in any. One of the wild cherry cultures (R8), isolated in 1994, should even be identified as *P. s.* pv. *syringae*.

Almost all the cultures showed pathogenicity in the tobacco hypersensitivity test. Only four isolates from the wild cherry group were not HR-positive, but further tests in the true host should be made to clarify the pathogenicity of the cultures. Of the acquired collection of isolates seven were HR-negative, which could mean that these cultures had lost their pathogenicity while in culture.

The possibility of doing cross infections in other hosts could clarify the pathogenic position of the isolates. If, for instance, the *Prunus laurocerasus* isolate (Ps73), identified as *Pseudomonas syringae* pv. *syringae*, could cause bacterial canker in wild cherry trees, the epidemiology of this disease would have to be very conscientiously studied. The possibility of *Prunus laurocerasus* and other *Prunus* species susceptible to *Pseudomonas syringae* pv. *syringae* being a reservoir for bacterial canker in wild cherry sites would have important epidemiological implications.

CHAPTER 5
NUTRITIONAL PROFILE

5.1 Introduction

Bacteria have traditionally been identified by analysing a few tests, primarily morphological and nutritional. Because a small number of subjective characters, even if important, was used to classify and identify the organisms, the classification was based on little information and lacked stability and predictability (PRIEST & AUSTIN, 1993). An increase in the number of tests has been greatly limited because the tools for performing these analyses are cumbersome, slow, and laborious. STEAD (1992) points to a minimum of one week to accomplish these analyses. Nonetheless, a study, using the same approach, on *Pseudomonas syringae* strains isolated from deciduous fruit trees examined 215 diagnostic characters (ROOS & HATTINGH, 1987b). The classical nutritional tests for characterisation of *P. syringae* pvs. *morsprunorum* and *syringae* based upon the ability of the pathogen to utilise specific compounds (described in section 4.2.1), though few, are very time consuming and somewhat subjective to interpret.

The identification of an organism according to its catabolic enzymes, which can be examined by profiling what chemicals the organism uses as a carbon and energy source, has been developed into several commercial diagnostic kits (LELLIOTT & STEAD, 1987; STEAD, 1992). These nutritional profiles are based on the classical cultural methods but they tend to be in a form which confers greater reproducibility of results with a much wider range of compounds.

In 1989, Biolog, Inc. (Hayward, California, USA) made the approach much more manageable by developing a technology that can simply and efficiently characterise the carbon utilisation profiles of microbial isolates. They introduced a panel containing 95 tests for the identification of Gram-negative species. As the tests can be either positive or negative there are 2^{95} (approximately 4×10^{28}) possible patterns that theoretically

could result, therefore providing an extremely fine resolution. Additional resolution can be obtained by following the changes kinetically instead of reading them as positive or negative (BOCHNER, 1989). This approach shows great promise in speeding, simplifying, and standardising the process of metabolic testing.

All the necessary selected nutrients and chemical substrates are preloaded and dried in the wells of a microplate which are easily inoculated with a cell suspension by the user. Tetrazolium violet is used as a redox dye to colorimetrically indicate the utilisation of the carbon source. Utilisation of a carbon source is detected as an increase in the respiration of cells in the well, leading to a reduction of the tetrazolium dye. Tetrazolium violet is reduced at one or more sites in the bacterial electron transport chain. Regardless of its structure, virtually any chemical substrate that is oxidised by a cell will result in the formation of NADH, which donates electrons to the electron transport chain. Tetrazolium violet is reduced by electrons tapped from the electron transport chain to produce a deep purple colour. Cells capable of catabolising the test substrate reduce the dye and produce the colour, whereas cells failing to catabolise the substrate will remain colourless. Those with intermediate rates of catabolism differ in rate and/or extent of colour formation. In all cases the colour is stable because the reduction is essentially irreversible (BOCHNER & SAVAGEAU, 1977). Because this chemistry detects the cell's metabolic rate, it can be used to test not only the utilisation of traditional carbohydrates, but the utilisation of virtually any biodegradable chemical. The vast array of potential chemical substrates includes carbohydrates, carboxylic acids, amides, esters, amino acids, peptides, amines, alcohols, aromatic chemicals, halogenated chemicals, phosphorus- and sulphur-containing chemicals, and polymeric chemicals (BOCHNER, 1989).

The positive use of a carbon source, indicated by colour reaction, can be assessed visually or by measuring the optical density with a spectrophotometer at an appropriate wavelength. Computerised matching of the metabolic fingerprint of the isolate to a library of culture collection strains can identify the microorganism under study. It is a very practical approach for testing and identifying large numbers of diverse microbial isolates, and facilitates large-scale surveys that were previously inconceivable.

The testing process can also aid in the development of growth media or selective enrichment media for the microorganism of interest. As a research tool, this system can be used in combination with DNA techniques and serological assays to greatly improve the existing taxonomic structure of many of the plant pathogenic bacteria and to place newly described pathogens in the existing structure (JONES *et al.*, 1993).

The results of the Biolog system can be used as the characters of the operational taxonomic units (OTU's) needed for numerical classification (PRIEST & AUSTIN, 1993). OTU's are the lowest ranking taxa employed in a given study (SNEATH & SOKAL, 1973), e.g. isolates in this work.

The simplicity and efficiency of this technology provide a very practicable approach for testing and identifying large numbers of microbial strains. It became an important tool, in this research, for the identification of the isolates, as well as of some important saprophytes which were to be found in the plant material examined.

5.2 Materials and methods

Nutritional profiles

Nutritional profiles were obtained using Biolog's GN MicroPlate and the recommended test procedures were followed. The pure cultures were grown on Tryptic Soy Agar (Difco), for 16-18 hours at 25°C, and with a sterile cotton swab or 1 µl plastic loop the cells were harvested and suspended in capped tubes filled with 18 ml of sterile saline (0.85% NaCl), prewarmed at 25°C. A few slow growing isolates were allowed to grow for 24 hours.

The inoculum cell density of approximately 3×10^8 cells.ml^{-1} was achieved by adjusting it within an acceptable turbidity range on a turbidimeter (Biolog). The density was raised by adding more cells and lowered by adding more saline until a transmittance level of 53% to 59% was obtained. These values corresponded to an A_{600} between 0.145 and 0.124 in a spectrophotometer (SP8-400, Pye Unicam), using the high and low turbidity standards of Biolog.

After establishing the acceptable inoculum density, the wells of the GN MicroPlate (Fig. 5.1), prewarmed at 25°C, were filled with 150 µl of the cell suspension, using a 12-channel pipette (Titerman 4908, Eppendorf), and incubated at 25°C in an incubator with humidification (Gassed Incubator BB6060, Heraeus). The plate was read, after 24 hours, with a microplate reader (MKII, Titertek Multiskan Plus) set at a wavelength of 590 nm and confirmed visually. The recommended practice of taking a 4-6 hour reading was unsuitable, because virtually no colour formation was visible by then. The reading was zeroed on well A1 with the Titersoft software, and a threshold of 0.3 in absorbance was set to distinguish which wells were positive.

	1	2	3	4	5	6	7	8	9	10	11	12
A	water	α-cyclodextrin	dextrin	glycogen	tween 40	tween 80	N-acetyl-D-galactosamine	N-acetyl-D-glucosamine	adonitol	L-arabinose	D-arabitol	cellobiose
B	i-erythritol	D-fructose	L-fucose	D-galactose	gentiobiose	α-D-glucose	m-inositol	α-D-lactose	lactulose	maltose	D-manitol	D-mannose
C	D-melibiose	β-methyl D-glucoside	D-psicose	D-raffinose	L-rhamnose	D-sorbitol	sucrose	D-trehalose	turanose	xylitol	methyl pyruvate	mono-methyl succinate
D	acetic acid	cis-aconitic acid	citric acid	formic acid	D-galactonic acid lactone	D-galacturonic acid	D-gluconic acid	D-glucosaminic acid	D-glucuronic acid	α-hydroxybutyric acid	β-hydroxybutyric acid	γ-hydroxybutyric acid
E	p-hydroxy phenylacetic acid	itaconic acid	α-keto butyric acid	α-keto glutaric acid	α-keto valeric acid	D,L-lactic acid	malonic acid	propionic acid	quinic acid	D-saccharic acid	sebacic acid	succinic acid
F	bromo succinic acid	succinamic acid	glucuronamide	alaninamide	D-alanine	L-alanine	L-alanyl-glycine	L-asparagine	L-aspartic acid	L-glutamic acid	glycyl-L-aspartic acid	glycyl-L-glutamic acid
G	L-histidine	hydroxy L-proline	L-leucine	L-ornithine	L-phenylalanine	L-proline	L-pyroglutamic acid	D-serine	L-serine	L-threonine	D,L-carnitine	γ-amino butyric acid
H	urocanic acid	inosine	uridine	thymidine	phenyl ethylamine	putrescine	2-amino ethanol	2,3-butanediol	glycerol	D,L-α-glycerol phosphate	glucose-1-phopahte	glucose-6-phosphate

Figure 5.1
Metabolic test panel used in GN MicroPlate.

The wells with A_{590} inferior to 0.3 but still with a faint colour, or with small purple flecks or clumps were scored as borderlines.

The results were then manually entered in the MicroLog 1 computer software (Biolog) to access the MicroLog GN database (version 3.7 – 566 species). An identification is then displayed based on the goodness of match between the given pattern and reference patterns in the database.

Almost all the bacteria isolates were tested at least twice for their ability to oxidise carbon sources using the Biolog identification system. Because of subsequent loss of metabolic properties or contamination, seven isolates were tested only once.

Numerical analysis of the nutritional profiles

The bacteria identified as *P. syringae*, a total of 75 cultures, were subjected to cluster and principal coordinate analyses using the NTSYS-pc computer program (ROHLF, 1992) to identify strain relationships. The reference strains used were Psm1462 for *P. s.* pv. *morsprunorum* and Pss2942 for *P. s.* pv. *syringae*.

The normalised absorbance values from the microplate reader were used as quantitative characters for the numerical analysis data matrix. The normalisation was performed by dividing each value by the mean of the five highest characters (L-arabinose; α-D-glucose; cis-aconitic acid; D-saccharic acid; L-aspartic acid). These characters were consistently positive in all the isolate's replicates except in one of the replicates of Psm5252, where the absorbance for cis-aconitic acid was below 0.3. In this case, the values were divided by the mean of the other four characters. There were no missing values and the average value of the two replicates was calculated.

Symmetric matrices were obtained using both the average taxonomic distance and the Pearson product-moment correlation coefficient. The average distance is commonly used as measure of dissimilarity. The distance computed between OTU's j and k is

$$d_{jk} = \sqrt{\frac{1}{n}\sum_{i=1}^{n}\left(X_{ij} - X_{ik}\right)^2}$$

where X_{ij} stands for the character value of character i in OTU j and n is the number of characters sampled (SNEATH & SOKAL, 1973; ROHLF, 1992).

The Pearson coefficient is among the most frequently employed coefficients of similarity in numerical taxonomy and when interpreted taxonomic structure on the basis of phenograms is usually the most suitable measure. This coefficient is defined as

$$r_{ik} = \frac{\sum_{i=1}^{n}\left(X_{ij} - \overline{X}_j\right)\left(X_{ik} - \overline{X}_k\right)}{\sqrt{\sum_{i=1}^{n}\left(X_{ij} - \overline{X}_j\right)^2 \sum_{i=1}^{n}\left(X_{ik} - \overline{X}_k\right)^2}}$$

where $\overline{X}_j$ is the mean of all values for OTU j (SNEATH & SOKAL, 1973).

The isolates were clustered and phenograms were obtained by unweighted pair-group method using arithmetic averages (UPGMA). This method works well for both dissimilarity and similarity coefficients (SNEATH & SOKAL, 1973).

The taxonomic distance between the replicates for each isolate was also calculated, except for the seven isolates not replicated.

The matrices of cophenetic values were computed and used to test the goodness of fit of the clusters to the sets of data. The actual dissimilarity or similarity values implied by a given furcation in the phenogram were generated and used as cophenetic values. A product-moment correlation coefficient was then computed between the elements of the original dissimilarity/similarity matrix and the cophenetic values of the new matrix. This cophenetic correlation coefficient (r_{cs}) is a measure of the agreement between the similarity values implied by the phenogram and those of the original similarity matrix.

The principal coordinate analysis was obtained by transforming the distance matrix to scalar product form so that its eigenvectors could be computed. The off-diagonal element, d_{jk}, is first replaced with $-\frac{1}{2}d_{jk}^2$. Each value of the new matrix is then corrected by subtracting from it the row and column means and adding the grand mean. The next step was to compute the eigenvectors of the transformed matrix. The resulting vectors were the principal coordinate axes. As there were no missing values and distance coefficients were used, the results are identical to principal component analysis (ROHLF, 1992).

5.3 Results

All the strains of *P. syringae* from HRI and NCPPB, were accurately identified with the Biolog library at the species level, but none were correctly identified at the pathovar level. Twenty-three cultures isolated from wild cherry trees, during this work in 1994 and 1995, were also identified as *P. syringae*.

A typical *P. s.* pv. *morsprunorum* pattern obtained with the Biolog GN MicroPlate can be seen on Figure 5.2.

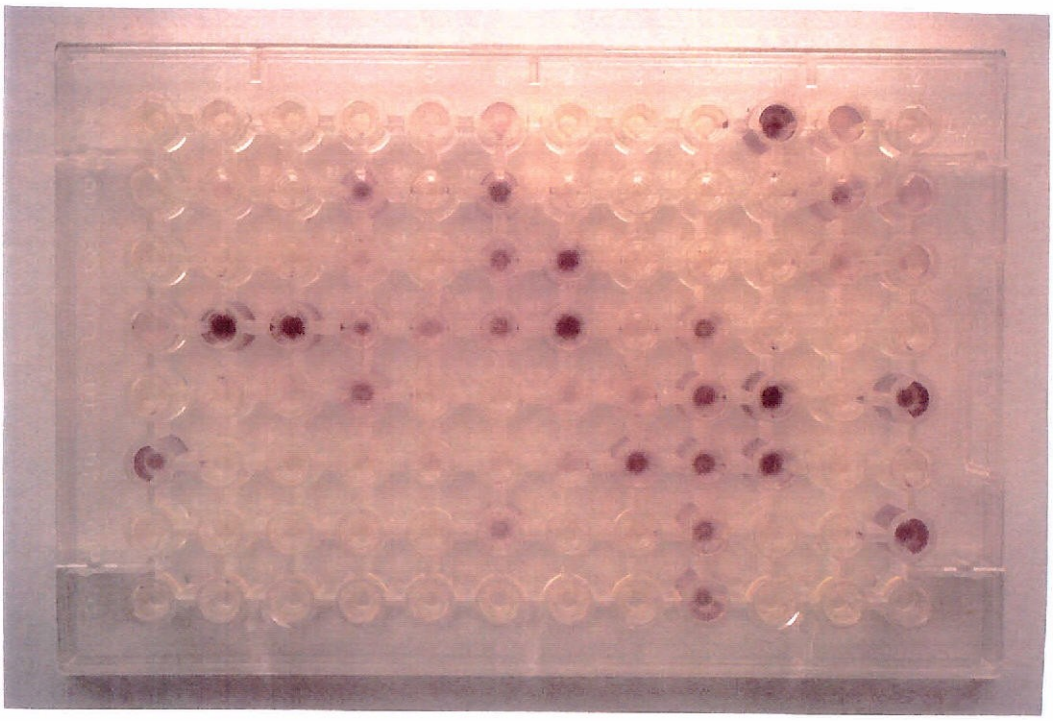

Figure 5.2
Nutritional profile pattern generated by *P. s.* pv. *morsprunorum* in Biolog's GN MicroPlate.

Among the *P. syringae* isolates, there were five carbon sources (mentioned in section 5.2) invariably utilised and 32 which were always negative. The carbon sources never metabolised by any of the isolates were: α-cyclodextrin; dextrin; glycogen; tween 80; N-acetyl-D-galactosamine; N-acetyl-D-glucosamine; cellobiose; L-fucose; gentiobiose; α-D-lactose; lactulose; D-melibiose; β-methyl-D-glucoside; L-rhamnose; D-trehalose; turanose; α-hydroxybutyric acid; p-hidroxyphenilacetic acid; itaconic acid; α-ketovaleric acid; sebacic acid; glycyl-L-aspartic acid; hydroxy L-proline; L-

ornithine; L-phenylalanine; thymidine; phenylethylamine; putrescine; 2-aminoethanol; 2,3-butanediol; glucose-1-phosphate; and glucose-6-phosphate.

The differences in carbon source utilisation profiles, among the 75 isolates, are illustrated in a phenogram using average taxonomic distances (Fig. 5.3). The Pearson product-moment correlation coefficient generated a very similar clustering (Fig. 5.4).

The cophenetic correlations, r_{cs}, showed a good fit for UPGMA clustering with both resemblance coefficients. The phenogram obtained with average taxonomic distances showed a $r_{cs} = 0.868$, whereas with Pearson correlation coefficient the cophenetic correlation was $r_{cs} = 0.828$.

Three major clusters could easily be recognised. They were denominated cluster I comprising 26 isolates, and clusters II and III both with 20 isolates (Figs. 5.3 and 5.4). The only difference within clusters, between the two resemblance coefficients, was the replacement of Psm617 by 11L(f3) in cluster I of the phenogram obtained with Pearson correlation coefficients. In this phenogram, Psm617 formed a separate cluster with Pss281, whereas with taxonomic distance the isolate 11L(f3) was considered an outlier.

The reference strain for *P. s.* pv. *syringae* (Pss2942) appeared in cluster I, as well as almost all *P. s.* pv. *syringae* isolates, except for Pss5357 and Pss281, which did not fit in any cluster. The majority of the isolates previously identified as *P. s.* pv. *morsprunorum* formed cluster III, including the reference strain Psm1462 which fell into the inner core of the cluster. Only seven isolates (Psm881, Psm889, Psm5239, 12AF, Psm680, Psm5271, and Pss281) were considered outliers, not being included in any grouping or forming one themselves. The strain Pss5357, while not being exactly an outlier, did not cluster significantly close to any of the described phena.

The presence of the three groupings could also be visualised by a perspective of the three-dimensional OTU's projection onto the first three principal coordinates, and the same outliers were confirmed (Fig. 5.5).

The taxonomic distances between the two replicates for each isolate (Table 5.1.) show quite large variation between replicates.

The distribution of test responses defined by UPGMA clusters on the average taxonomic distance, expressed in terms of the percentage of the isolates in each group that utilise each specific carbon source, are presented in Table 5.2.

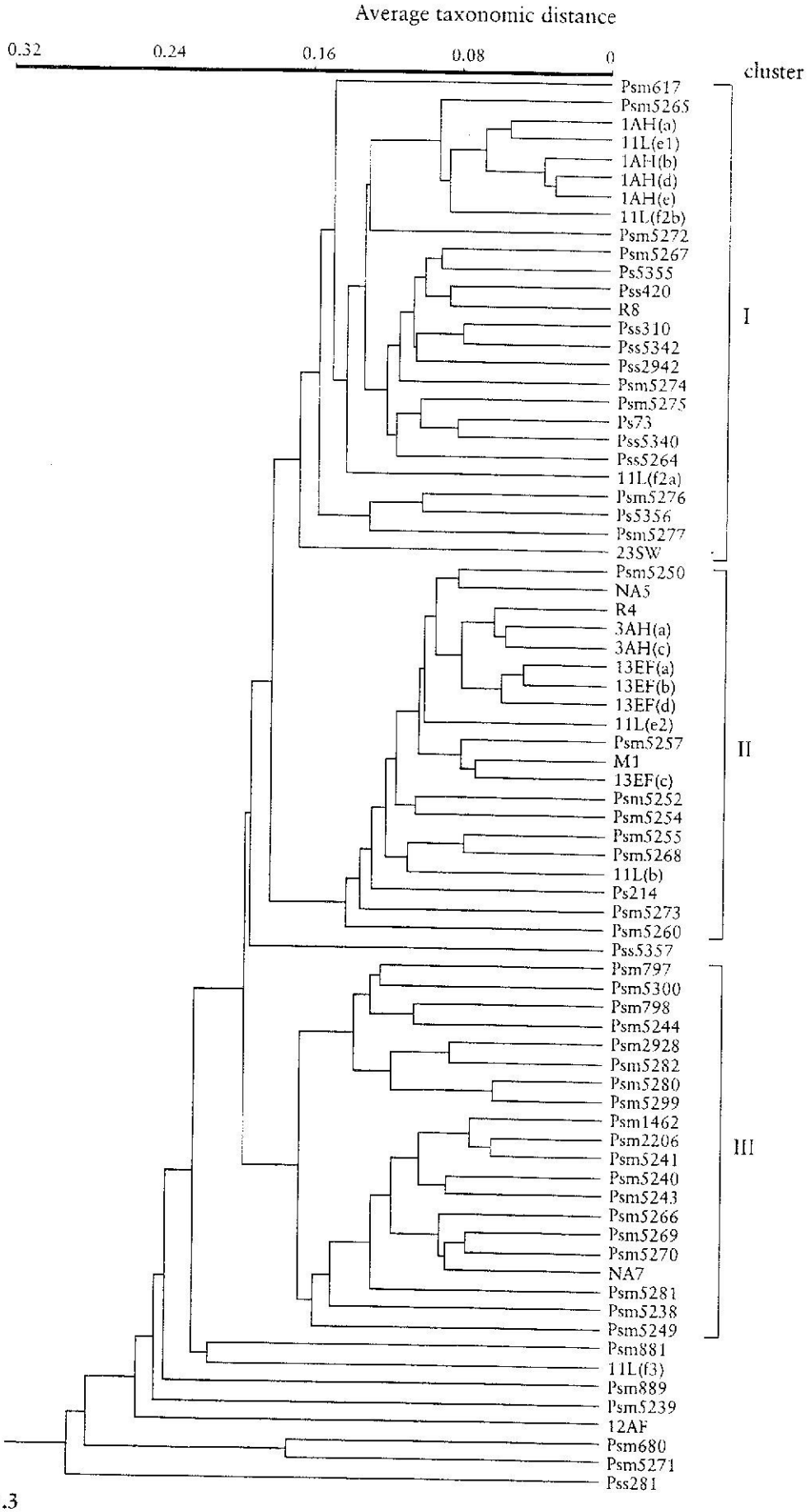

Figure 5.3

Phenogram of the average taxonomic distance, *d*, grouped by UPGMA cluster analysis, showing the relationships of 75 *P. syringae* isolates based on Biolog's nutritional profiles.

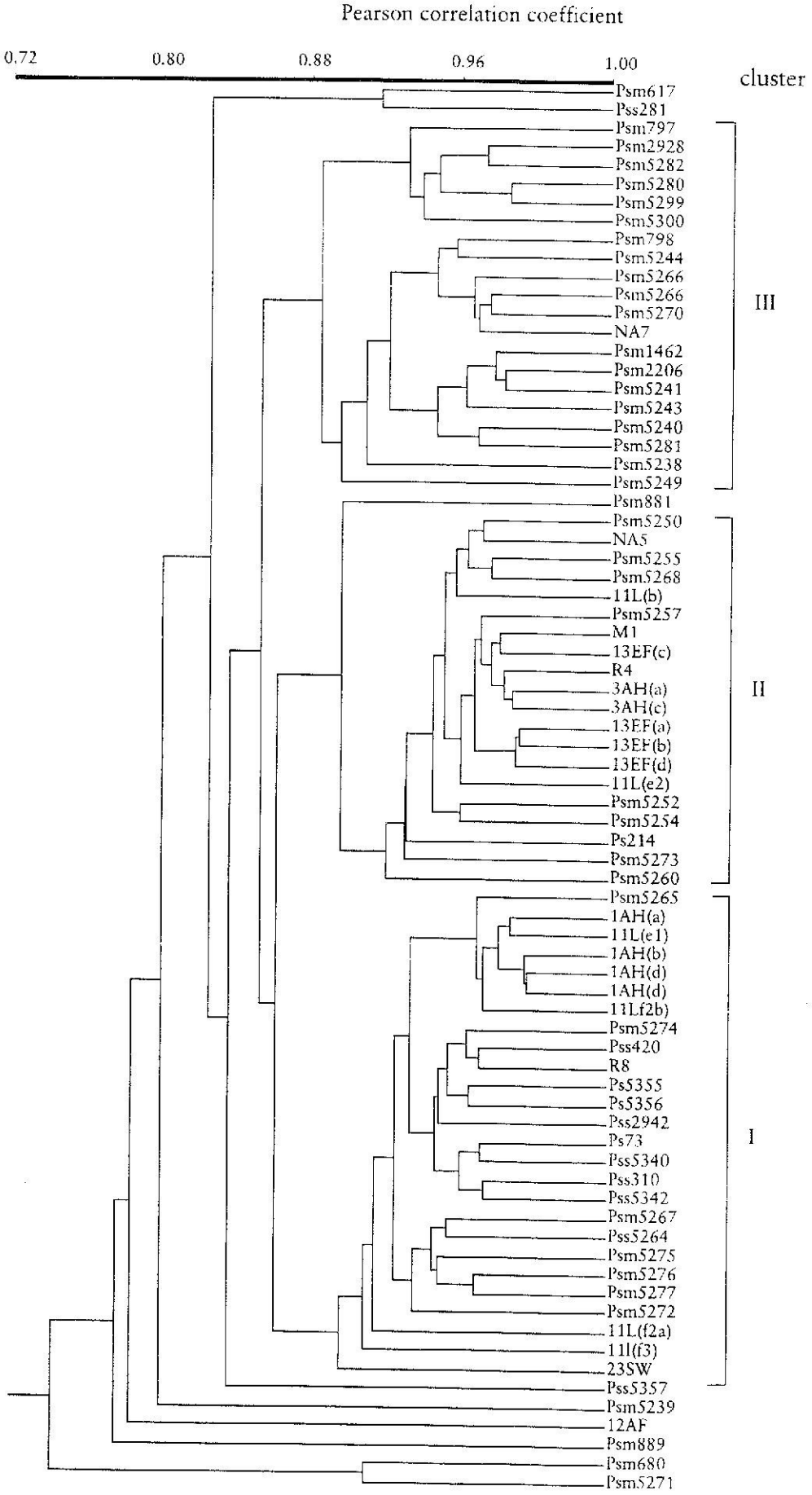

Figure 5.4

Phenogram of the Pearson correlation coefficient, *r*, grouped by UPGMA cluster analysis, showing the relationships of 75 *P. syringae* isolates based on Biolog's nutritional profiles.

In addition to the five carbon sources referred to previously as being utilised by all the strains, another five were metabolised by all isolates in clusters I-III, but not by the eight outliers. Those carbon sources were D-fructose, sucrose, D-gluconic acid, L-asparagine, and L-serine. D-fructose and sucrose were not metabolised by the isolate 12AF, D-gluconic acid was not utilised by Psm5271 and Psm680, L-asparagine not by Pss281, and L-serine not by Psm680. Other sources of carbon were utilised by most of the isolates in clusters I-III, with small discrepancies in one or two isolates per cluster. These were the cases of: D-galactose not utilised by Psm5276 and Psm5277 (cluster I); D-manitol not by Psm5240 (cluster III); D-mannose not by 23SW (cluster I); D-sorbitol not by Psm5254 (cluster II) and Psm5240 (cluster III); methyl pyruvate not by Psm5254 (cluster II) and Psm5249 (cluster III); mono-methyl succinate not by Psm617 (cluster I) and Psm5249 (cluster III); succinic acid not by Psm5254 (cluster II); bromo succinic acid not by Psm5254 (cluster II) and Psm5249 (cluster III); L-glutamic acid not by Psm5249 (cluster III); and glycerol not by Psm5252 and Psm5254 (cluster II).

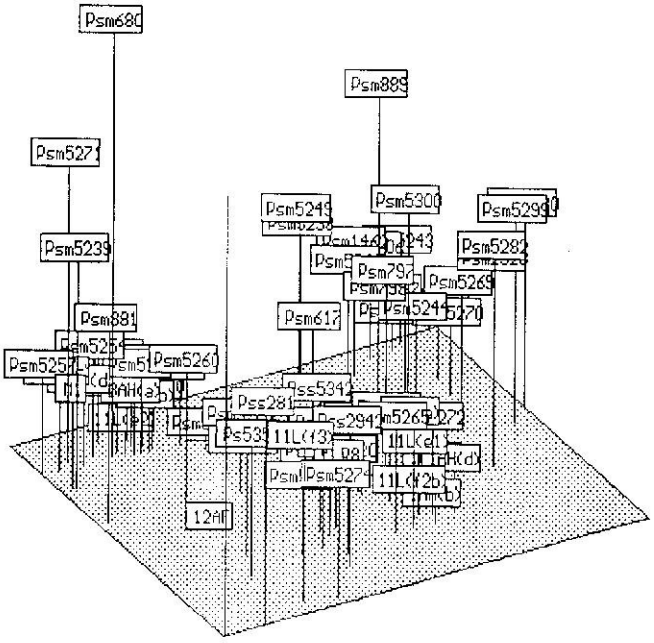

Figure 5.5

Three dimensional projection of the 75 *P. syringae* isolates, based on Biolog's nutritional profiles, onto the first three principal coordinates.

In addition to the 32 carbon sources referred to previously as not being used by any of the cultures, another five were metabolised by none or very few isolates in clusters I-III. These carbon sources were maltose, xylitol, β-hydroxybutyric acid, γ-hydroxybutyric acid, and α-ketobutyric acid. Maltose was only metabolised by Ps5356 (cluster I); xylitol only by 3AH(c) and Ps214 (cluster II); β-hydroxybutyric acid by Ps5356 (cluster I); α-ketobutyric acid by Psm5274 and Ps5356 (cluster I).

Table 5.1

Average taxonomic distance, *d*, calculated between the two replicates of each isolate.

Isolate	Distance	Isolate	Distance	Isolate	Distance
Psm617	0.326	Psm5267	0.186	Pss5264	0.204
Psm680	0.092	Psm5268	0.140	Pss5340	0.266
Psm797	0.193	Psm5269	0.188	Pss5342	0.312
Psm798	0.146	Psm5270	0.222	Pss5357	0.052
Psm881	0.099	Psm5271	0.064	M1	0.159
Psm1462	0.082	Psm5272	0.169	NA5	0.174
Psm2206	0.105	Psm5273	0.123	NA7	0.196
Psm2928	0.106	Psm5274	0.154	R4	0.253
Psm5238	0.116	Psm5275	0.180	R8	0.204
Psm5239	0.128	Psm5276	0.157	1AH(a)	0.117
Psm5240	0.102	Psm5277	0.113	1AH(b)	0.140
Psm5241	0.128	Psm5280	0.158	1AH(d)	0.144
Psm5243	0.209	Psm5281	0.101	1AH(e)	0.135
Psm5244	0.195	Psm5282	0.134	11L(e1)	0.165
Psm5249	0.071	Psm5299	0.159	11L(e2)	0.123
Psm5250	0.104	Psm5300	0.142	3AH(a)	0.168
Psm5252	0.232	Ps214	0.102	3AH(c)	0.133
Psm5254	0.084	Ps73	0.171	12AF	0.160
Psm5255	0.184	Ps5355	0.166	13EF(a)	0.145
Psm5257	0.120	Ps5356	0.144	13EF(b)	0.187
Psm5260	0.161	Pss310	0.348	13EF(c)	0.098
Psm5265	0.149	Pss420	0.448	13EF(d)	0.136
Psm5266	0.143	Pss2942	0.201	Mean	0.160

Tween 40, i-erythritol, D,L-lactic acid, glucuronamide, and D,L-α-glycerol phosphate were not metabolised by any cluster III isolates but were metabolised by

approximately half (42% to 69%) of the cluster I isolates. The isolates in cluster II had an intermediate behaviour for tween 40 and i-erythritol but used D,L-lactic acid, glucuronamide, and D,L-α-glycerol phosphate like cluster III. D-raffinose was not metabolised by cluster I isolates, and by only one isolate in cluster II, but it was by 65% of cluster III isolates. Adonitol was different again: while none of the isolates in clusters I and III used it, 45 % of the cluster II isolates did.

The most important carbon sources to discriminate the clusters were: m-inositol, D-galacturonic acid, succinamic acid, and L-pyroglutamic acid. M-inositol was metabolised by 92% and 90% of the isolates in cluster I and II, respectively, but only by 20% of cluster III isolates; D-galacturonic acid was utilised by all the strains of cluster I except Ps73 and by 60% of cluster III isolates, but only by one isolate of cluster II (Psm5260); succinamic acid was used by all the strains in cluster II except by 11L(e2) and by 62% of cluster I isolates, but only by two isolates in cluster III (Psm798 and Psm5238); and L-pyroglutamic acid was metabolised by all isolates in cluster II except 13EF(c) and Psm5254, but by none in cluster III and only by Psm5265 in cluster I.

Inosine and uridine were less discriminative but could give an impression of the clustering. Most of cluster I and II isolates were able to use these compounds but only approximately 30% of cluster III isolates.

5.4 Discussion

The nil percentage of correct identification at the pathovar level for *P. s.* pvs. *morsprunorum* and *syringae* with the MicroLog database appears to have resulted from the lack of accurate differentiation of *P. syringae* pathovars by carbon utilisation profiles, but most probably because of a low number of strains in the library. Other researchers have documented the difficulty of discriminating between *P. syringae* pathovars by biochemical tests, although nonrandom variation in phenotypic characteristics has been alluded to with the fluorescent pseudomonads (JONES *et al.*, 1993). STEAD (1992) affirms that the nutritional profiles systems rarely, if ever, allow accurate identification at an infraspecific level, even if the library used contains a wide range of plant pathogenic bacteria. He even remarks that it is difficult to see why the Biolog system should be any better than the previous biochemical identification systems.

Table 5.2

Characteristics of major groups in terms of percentage positive responses, defined by UPGMA on the average taxonomic distance.

Carbon source	Cluster I	Cluster II	Cluster III
α-cyclodextrin	0	0	0
dextrin	0	0	0
glycogen	0	0	0
tween40	50	30	0
tween 80	0	0	0
N-acetyl-D-galactosamine	0	0	0
N-acetyl-D-glucosamine	0	0	0
adonitol	0	45	0
L-arabinose	100	100	100
D-arabitol	100	90	85
cellobiose	0	0	0
i-erythritol	69	30	0
D-fructose	100	100	100
L-fucose	0	0	0
D-galactose	92	100	100
gentiobiose	0	0	0
α-D-glucose	100	100	100
m-inositol	92	90	20
α-D-lactose	0	0	0
lactulose	0	0	0
maltose	4	0	0
D-mannitol	100	100	95
D-mannose	96	100	100
D-melibiose	0	0	0
β-methyl-D-glucoside	0	0	0
D-psicose	19	50	5
D-raffinose	0	5	65
L-rhamnose	0	0	0
D-sorbitol	100	95	95
sucrose	100	100	100
D-trehalose	0	0	0
turanose	0	0	0
xylitol	0	10	0
methyl pyruvate	100	95	95
mono-methyl succinate	96	100	95
acetic acid	35	10	5
cis-aconitic acid	100	100	100
citric acid	100	100	75
formic acid	69	35	40
D-galactonic acid lactone	88	70	90
D-galacturonic acid	96	5	60
D-gluconic acid	100	100	100
D-glucosaminic acid	77	10	15
D-glucuronic acid	100	65	65
α-hydroxybutyric acid	0	0	0
β-hydroxybutyric acid	4	0	0
γ-hydroxybutyric acid	0	0	0
p-hydroxyphenylacetic acid	0	0	0
itaconic acid	0	0	0
α-ketobutyric acid	8	0	0
α-ketoglutaric acid	92	45	50
α-ketovaleric acid	0	0	0
D,L-lactic acid	58	0	0
malonic acid	46	5	30
propionic acid	23	5	10
quinic acid	100	85	90
D-saccharic acid	100	100	100
sebacic acid	0	0	0
succinic acid	100	95	100
bromo succinic acid	100	95	95
succinamic acid	62	95	10
glucuronamide	42	0	0
alaninamide	12	5	5
D-alanine	88	70	35
L-alanine	96	65	35
L-alanyl-glycine	65	70	40
L-asparagine	100	100	100
L-aspartic acid	100	100	100
L-glutamic acid	100	100	95
glycyl-L-aspartic acid	0	0	0
glycyl-L-glutamic acid	65	85	45
L-histidine	27	0	5
Hydroxy L-proline	0	0	0
L-leucine	12	0	0
L-ornithine	0	0	0
L-phenylalanine	0	0	0
L-proline	96	75	65
L-pyroglutamic acid	4	90	0
D-serine	8	0	5
L-serine	100	100	100
L-threonine	15	5	10
D,L-carnitine	27	0	0
γ-aminobutyric acid	100	85	100
urocanic acid	19	15	25
inosine	96	90	30
uridine	96	85	35
thymidine	0	0	0
phenylethylamine	0	0	0
putrescine	0	0	0
2-aminoethanol	0	0	0
2,3-butanediol	0	0	0
glycerol	100	90	100
D,L-α-glycerol phosphate	65	5	0
glucose-1-phosphate	0	0	0
glucose-6-phosphate	0	0	0

Definitive phenotypic differentiation of many of the *P. syringae* pathovars may require the use of more than the 95 tests available in the Biolog system. Some authors using other systems have included up to 141 biochemical characters (ROOS & HATTINGH, 1987b). Others suggested that a minimum of 30-40 tests is required to obtain a high level of confidence in positive identification of all strains including atypical strains (LAPAGE *et al.*, 1970). The possibility of using a different choice of carbon sources for these pathovars should also be considered since there was a great similarity between the two pathovars in the compounds tested. Another way of improving the identification could be through the construction of a user's own library. This facility can also be provided by the Biolog system.

The taxonomy of pseudomonads and their interrelationships is not yet fully resolved. Consequently, practical difficulties are frequently encountered in their routine classification and identification. Solid bacteriological criteria for the differentiation of *Pseudomonas* pathovars from one another and from saprophytic forms are still needed (VAN ZYL & STEYN, 1990; PALLERONI, 1994).

Although the Biolog system could not identify *P. syringae* accurately at the infraspecific level, identification at specific level was completely accurate for all the 52 named strains obtained from NCPPB and HRI. Therefore Biolog is an excellent identification method at the species level. This view is also shared by other researchers working with several strains of *Agrobacterium*, *Clavibacter*, *Erwinia*, *Pseudomonas*, and *Xanthomonas* (JONES *et al.*, 1993). Furthermore, the Biolog system was used in this work to identify all the bacteria isolated from the woodlands and it proved to be very useful in the identification of several saprophytes, mainly of the complex *Erwinia herbicola – Pantoea agglomerans*.

These biochemical tests, designed primarily for identification purposes, can also be used to gather large amounts of taxonomic data (PRIEST & AUSTIN, 1993). Hence, the Biolog tests were used as characters for a study in numerical taxonomy that included the strains identified as *Pseudomonas syringae* obtained from the NCPPB and HRI, and the ones isolated from wild cherry trees in 1994 and 1995. Other numerical taxonomic studies using Biolog tests have been quite successful and in the case of *Xanthomonas vesicatoria* (Doidge) Vauterin *et al.*, this system was even used to distinguish a new tomato race (JONES *et al.*, 1995).

The validation of similarity measures has so far been primarily empirical. Nonmorphological similarities, such as are computed from biochemical data matrices,

are far more difficult to verify empirically. Studies to date have employed a variety of dissimilarity/similarity coefficients (SNEATH & SOKAL, 1973).

Although the ordination method used, principal coordinate analysis, revealed the major structure, the finer divisions, that might be ecologically significant, were less evident than in the clustering techniques. This agrees with the observation of SNEATH & SOKAL (1973) that with many OTU's ordination methods do not give simple low-dimensional results. There could be clusters that overlap in 2- or 3-space, though they are quite distinct in hyperspace. Therefore the chosen method to investigate the relationships among strains was cluster analysis. Two resemblance coefficients were used, one, the average taxonomic distance, a dissimilarity coefficient, and other, the Pearson correlation coefficient, a similarity coefficient much used with continuous variables. The most commonly applied method to test the fit of a phenogram to the original similarity matrix among OTU's is that of cophenetic correlations, developed by SOKAL & ROHLF (1962). Although correlation coefficients are usually the most suitable measure, when the interpretation of taxonomic structure was made on the basis of phenograms (SNEATH & SOKAL, 1973), a slightly higher cophenetic correlation was obtained with the average taxonomic distance. Nevertheless, if the adequacy of a phenogram in representing taxonomic structure is indicated by a high cophenetic correlation, where a r_{cs} over 0.8 is likely to be quite good (SNEATH & SOKAL, 1973), then both trees are fairly satisfactory. As pair-group clustering, in particular UPGMA, will always maximise this cophenetic coefficient, this technique was chosen to cluster the OTU's with both resemblance coefficients.

The representation of the taxonomic structure either by phenogram (Figs. 5.3 and 5.4) or by principal coordinate analysis (Fig. 5.5) clearly indicated the presence of three phena to which most isolates could be assigned. The presence of eight outliers was also confirmed with both methods. These three phena were designated as clusters I, II, and III. The three clusters were joined at 83.7% similarity (Fig. 5.4). Cluster III was separated from cluster I and II at 85.4% similarity, and cluster I from II at 86.3%.

To test the robustness of the clusters a small experiment was done where the three isolates forming the inner cores of each cluster were submitted to a random noise of the same extent of the one predicted by the mean of replicates' variation. This was achieved by generating nine sets of 95 random numbers, with mean zero and standard deviation 0.16 (Table 5.1) and adding those numbers to the original absorbance values used for the phenograms. A mean was then calculated between the original values and the ones submitted to the alteration with random variation. A new phenogram was obtained with only those nine isolates and compared with the original

one (Fig. 5.6). Even after adding the noise the same clusters, now with higher distances, remained visible which clearly indicates the robustness of those groupings.

Cluster I grouped all *P. s.* pv. *syringae* strains, even the ones from different hosts like pear (*Pyrus communis*) and cherry-laurel (*Prunus laurocerasus*), with the exception of the two outliers Pss281 and Pss5357. Cluster III is only composed by *P. s.* pv. *morsprunorum* strains with the exception of NA7 isolate and includes the reference strain Psm1462. This cluster seems to reproduce the typical pathovar *morsprunorum* isolated from sweet cherry and plum trees. There was even a small subgroup constituted almost entirely by plum strains (Psm797, Psm5300, Psm2928, Psm5282, and Psm5299) that clustered tightly together at 93.2% (Pearson correlation coefficient) inside cluster III, while the rest of the cluster did it at 89.6% of similarity. Seven *P. s.* pv. *morsprunorum* strains joined cluster I, but these had all been isolated from wild cherry trees, and eight joined cluster II, six from sweet cherry and two from wild cherry. The wild cherry cultures isolated during this work, in 1994 and 1995, were scattered between cluster I (9 isolates) and II (11 isolates). Cluster I was formed at a level of 89.7% of similarity while cluster II was formed at 92.1%. By the observation of the phenograms it seems that cluster II is constituted by intermediate forms between *P. s.* pv. *syringae* and *P. s.* pv. *morsprunorum*, that are actually closer to the former pathovar. Therefore, the isolates from wild cherry trees seem to be taxonomically much closer to *P. s.* pv. *syringae* than to *P. s.* pv. *morsprunorum*. This appears to be in contradiction with the work of GARRETT (unpublished data) where all the isolates from wild cherry trees were assigned to *P. s.* pv. *morsprunorum*. However, because the distances among the three clusters are not very large, one can ponder if the existence of an intermediate cluster is not part of a continuous variation among these isolates, where the two pathovars represent the extremes of a continuum.

Overall cultural differences between the three clusters were minor. Only 53 tests out of the 95 tested showed differences in a positive/negative type of response, and in most of the cases these were small variations. The most discriminatory compounds were m-inositol, D-galacturonic acid, succinamic acid, and L-pyroglutamic acid. Lactic acid has been used to distinguish the two pathovars since the work of GARRETT *et al.* (1966), and although in our study this carbon source was not metabolised by any isolate of clusters II and III, it was only utilised by 58% of the cluster I isolates, which is not sufficient to discriminate among the clusters.

The phenotypic heterogeneity of *P. s.* pv. *syringae*, and to a lesser extent of *P. syringae* pv. *morsprunorum*, appears to be quite extensive but other authors have also

commented on this heterogeneity of *P. syringae* pv. *syringae* (ROOS & HATTINGH, 1987b).

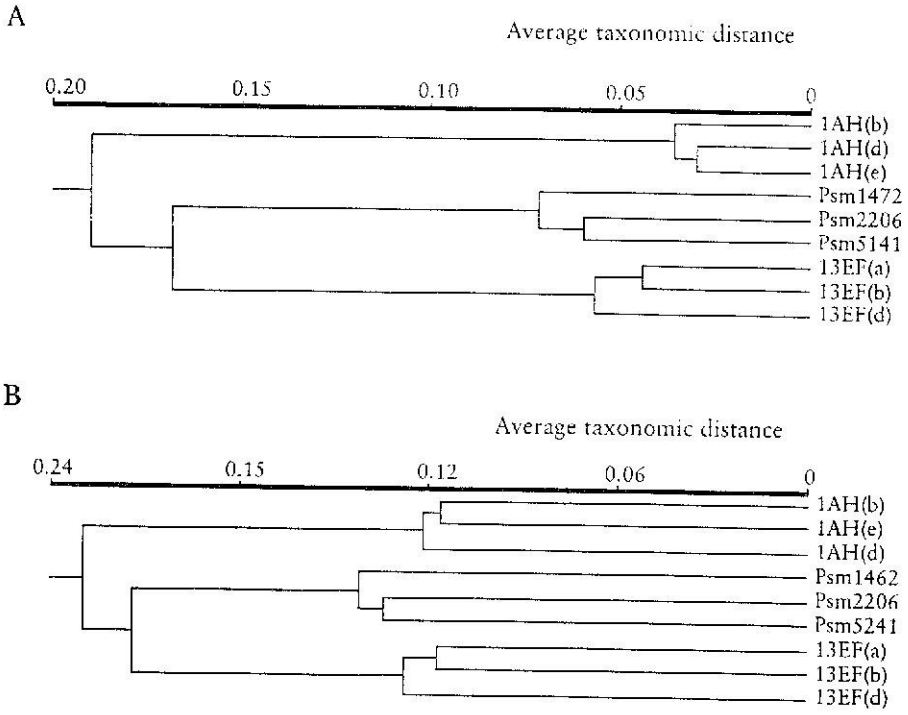

Figure 5.6

A part of the phenogram in Figure 5.4 of the three inner cores of each cluster showing, A, the original arrangement and, B, the arrangement after adding a random noise.

5.5 Conclusions

Usually, bacteria have been identified by classical methods which, in some cases, are poorly defined and subject to error because of their subjectivity. Identification systems, like Biolog, with such a wide range of nutritional tests allow the use of a standardised method to produce useful fingerprints. These fingerprints can be very useful for identification of bacteria, if matched against a library of strains, and for comparing individual strains.

Identification at the pathovar level, using the library available from Biolog, was not possible, but it was possible at the species level. Hence, the Biolog system allowed or confirmed the identification of all the isolates at the species level used throughout this work. In addition, several saprophytic bacteria used as negative controls in the nucleic acid and immunological techniques were also identified with this system.

The span of time in which the Biolog test can be accomplished, around two days, makes it very fast compared with some of the traditional cultural tests which have to be extended up to 7-14 days, like gelatin liquefaction. The rapidity of the test and the ease with which positive and negative reactions can be distinguished makes it one of the most useful ways to identify bacteria at the species level. However, the price (US$5.47–7.87, 1.05.95) and the relatively short shelf life of each plate makes this diagnostic technique rather unsuitable for large scale surveys and for routine use in epidemiological studies.

One of the major advantages of the Biolog system is the possibility to quantify the use of a determined carbohydrate. This quantification permitted a taxonomical study through the use of numerical classification. With cluster analysis eight outliers could be easily distinguished, some of them confirmed later with other techniques.

Although the overall cultural differences were small, the taxonomic structure represented either by phenograms or by principal coordinate analysis revealed the presence of three groups or major clusters. One cluster grouped all *P. s.* pv. *syringae* isolates, another cluster was solely composed by *P. s.* pv. *morsprunorum* isolates, and the last cluster seems to be constituted by intermediate forms between *P. s.* pvs. *morsprunorum* and *syringae*. Although it can be argued that the variation existing is just part of a continuous variation, where each pathovar represents an extreme, it seems probable that the latter cluster represents a sub-group. As this group is mainly formed by the *P. syringae* cultures isolated from wild cherry and is more similar to *P. s.* pv. *syringae* cluster than to the *P. s.* pv. *morsprunorum* cluster, hence, this cluster is probably a diverse group of *P. s.* pv. *syringae* strains attacking mainly wild cherry trees. Whether this group can be elevated to the pathovar level or other taxon depends on the results of taxonomical work that has yet to be done.

CHAPTER 6
NUCLEIC ACID TECHNOLOGY – REP-PCR

6.1 Introduction

Several workers consider that phenotypic characters do not provide an adequate basis for classifying strains of the phytopathogenic pseudomonads (SCHROTH *et al.*, 1981; PALLERONI, 1984). Even if some pathovars of *P. syringae* can be distinguished by phenotypic characters, in the absence of genetic analyses the taxonomic status of the pathovars remains problematical (DENNY *et al.*, 1988).

The polymerase chain reaction (PCR) for amplification of specific nucleic acid sequences was introduced by SAIKI *et al.* (1985). PCR is an enzymatic method for amplifying exponentially a specific pre-selected fragment of DNA. The amplification is achieved with a DNA polymerase, two synthetic oligonucleotide primers, and the four standard deoxyribonucleoside triphosphates that are incorporated into synthesised DNA. Each PCR amplification is subdivided into three steps which are repeated in cycles: the denaturation of the template DNA; the annealing of the two primers to their complementary sites; and the extension of the primers by polymerase-mediated nucleotide additions to produce two copies of the original sequence (TOWNER & COCKAYNE, 1993). The process has been improved greatly by the use of a thermostable DNA polymerase (*Taq*) isolated from *Thermus aquaticus* (SAIKI *et al.*, 1988).

Various strategies have been devised by which the power of PCR can be used for the typing of microorganisms. A method of generating fingerprints directly, i. e. without the use of restriction endonucleases, has been termed repetitive element sequence-based PCR (rep-PCR). The term rep-PCR refers to the general methodology involving the use of oligonucleotide primers, designed by VERSALOVIC *et al.* (1991), based on short repetitive sequence elements that are dispersed throughout the

prokaryotic kingdom. These short repeated sequences were first found in enteric bacteria (STERN *et al.*, 1984).

The PCR products using these primers, with chromosomal DNA of different bacterial strains as template, generate very characteristic patterns when separated on agarose gels. Differences in band sizes resolved using electrophoresis on agarose gels represent polymorphisms in the distances between repetitive sequence elements in different genomes. Thus, it was proposed that rep-PCR may constitute a useful method to fingerprint bacterial genomes (DE BRUIJN, 1992).

Although the actual function of these highly repeated and conserved elements remains an enigma (DE BRUIJN, 1992), it seems that REP (repetitive extragenic palindromic) and ERIC (enterobacterial repetitive intergenic consensus) sequences have the potential to form stem-loop structures and may play an important role in the organisation of the bacterial genome. Genome organisation is thought to be shaped by selection, and thus the dispersion of these sequences may be indicative of the structure and evolution of the bacterial genome. On the basis of this assumption and knowledge about the clonal nature and population dynamics of pathogenic bacteria, LOUWS *et al.* (1994) hypothesised that each evolutionary specialised line, or pathovar, of the pathogen might have a unique distribution or arrangement of repetitive sequences throughout the genome and it should be possible to generate genomic fingerprints that correlate with a specific lineage or pathovar.

Rep-PCR has been successfully employed to identify a variety of plant-associated bacteria including *Rhizobium* sp. and *Agrobacterium* spp. (DE BRUIJN, 1992); *Pseudomonas* spp. and *Xanthomonas* spp. (LOUWS *et al.*, 1994; LOUWS *et al.*, 1995; OPGENORTH *et al.*, 1996); and *Erwinia amylovora* (Burrill) Winslow *et al.* (MCMANUS & JONES, 1995). REP- and ERIC-like DNA sequences are also widely distributed in *P. syringae* strains (LOUWS *et al.*, 1995).

Genotypes of *Xanthomonas vesicatoria* (Doidge) Vauterin *et al.*, as defined by rep-PCR, correlated with selected phenotypic characteristics, such as amylolytic and pectolytic activity. This suggests that rep-PCR may distinguish phytopathogenic bacteria at the pathovar level (LOUWS *et al.*, 1995).

In this chapter, the use of the rep-PCR technique to generate unique genomic fingerprints to facilitate the rapid and reliable identification of *P. s.* pvs. *morsprunorum* and *syringae* is tested. I also wished to determine the genetic diversity among the isolates.

As REP- and ERIC-PCR-generated fingerprints of specific *P. syringae* strains were found to yield similar conclusions with regard to the identity of and relationship among these strains (LOUWS *et al.*, 1994), it was decided to use only the REP primer in the present study.

6.2 Material and methods

Direct REP-PCR of colonies was carried out but three DNA isolations from different isolates were made to confirm the band patterns.

DNA isolation

One isolate of *P. s.* pv. *morsprunorum* (Psm5260), one isolate of *P. s.* pv. *syringae* (Pss2942), and one isolate of *P. syringae* (Ps214) had the DNA extracted as follows (CHEN & KUO, 1993): 1.5 ml of a 10 ml 24 h culture at 25 °C in LB (see section 3.1) broth was harvested by centrifugation for 3 min at 13,000 rpm. The cell pellet was resuspended and lysed in 200 µl of lysis buffer (40 mM Tris–acetate, pH 7.8; 20 mM sodium acetate; 1 mM EDTA; and 1% SDS) by vigorous pipetting. To precipitate most proteins and cell debris, 66 µl of 5 M NaCl solution was added and mixed well. The solution was inverted and centrifuged at 13,000 rpm for 10 min at 4°C. After transferring the clear supernatant into a new microfuge tube, an equal volume of chloroform was added, and the tube was gently inverted at least 50 times until a milky suspension was completely formed. Following centrifugation at 13,000 rpm for 3 min, the supernatant containing the DNA was transferred to another microfuge tube and the DNA was precipitated with 100% ethanol and centrifuged at 13,000 rpm for 3 min. The ethanol was decanted and the pellet washed twice with 70% ethanol. After a last centrifugation at 13,000 rpm for 3 min, the ethanol was decanted and the pellet allowed to dry at room temperature. The pellet was then resuspended in 50 µl of TE buffer (10 mM Tris-HCl and 1 mM EDTA).

The DNA concentration was determined using a DyNA Quant 200 fluorimeter with the DNA-specific dye Hoechst 33258, as specified by the manufacturer.

REP-PCR

Primer sequences corresponding to REP [REP1R-I (5'-IIIICGICGICATCIGGC-3') and REP2-I (5'-ICGICTTATCIGGCCTAC-3')] were synthesised by Gibco BRL Custom Primers (Palo Alto, California, USA).

The PCR reactions were carried out essentially as described by DE BRUIJN (1992). PCR reactions were performed in a total volume of 25 µl. Working concentrations were: 1X NH_4^+ buffer (Gibco BRL, Gaithersburg, Maryland, USA), 2.0 mM $MgCl_2$, 0.3125 mM each of dATP, dCTP, dGTP, and dTTP, 2 µM (50 pmol) each of opposing primers, and 2.0 units of *Taq* DNA polymerase (Gibco BRL). Colony PCR was performed on a 2 µl aliquot from a 1:1,000 dilution of a 2 days old colony on LB agar (see section 3.1). The negative control was made with 2 µl of molecular biology grade water (Purite, Purite, Oxford, UK). A 3.5 µl aliquot of isolated DNA (approximately 50 ng), 1 µl loopful of the whole colony, and two dilutions of the colony (1:50 and 1:500) were tested to determine the best method. Samples were overlaid with 25µl of mineral oil (Sigma).

PCR amplification was performed using a thermal cycler (Model PTC–100, MJ Research, Watertown, Massachusetts, USA). The cycling conditions were as follows: one initial cycle of 95°C for 7 min; 30 cycles of denaturation at 94°C for 1 min, annealing at 44°C, and extension at 65°C for 8 min; a single final extension at 65°C for 15 min and a final soak at 4°C.

A 7 µl aliquot of amplified PCR product was separated by gel electrophoresis on 1.5% agarose gels containing 0.35µg.ml^{-1} ethidium bromide. Electrophoresis was performed in TAE buffer (40 mM Tris-acetate and 1 mM EDTA) and the fragments, separated during 4 hours at 4 V.cm^{-1}, were photographed on a UV transilluminator with Polaroid type 667 film. Differences in fingerprint patterns among genotypes were assessed visually.

All 75 isolates described in section 4.2 were analysed by REP-PCR. In addition, eight cultures of *Erwinia herbicola* isolated from wild cherry trees, originally misidentified as *P. syringae*, and one *P. syringae* isolate (Ps5400) from *Prunus cerasifera* Ehrh., obtained in HRI, were analysed. Each PCR experiment was done at least twice for each bacterial isolate and included a control lacking template DNA.

6.3 Results and discussion

DNA fingerprints were generated by REP-PCR from all the 75 *P. syringae* isolates and the eight *E. herbicola* isolates tested. Therefore, the REP-like sequence elements are present in the genomes of all the bacteria examined, which extends the range of taxa presented by LOUWS *et al.* (1994).

Dispensing cells directly into the PCR tubes from solid media yielded very weak band patterns, possibly because of the common presence of polysaccharides (Fig. 6.1). However, once the colonies were diluted 50 or 500 times the resolution of the band patterns was greatly enhanced. No major differences were observed between the two colony dilutions and the extracted DNA in all the cultures tested. Therefore, the same REP-PCR genomic fingerprints were generated from purified DNA and from cells suspended in water, derived from cultures on solid media. In subsequent work the diluted colonies method was used.

The REP-PCR produced five to more than 20 distinct PCR products, ranging in size from approximately 200 bp to 5Kb (Figs 6.2 to 6.8).

The *P. s.* pv. *morsprunorum* patterns, from sweet cherry and plum (Figs. 6.2 and 6.6), were found to be very similar, with very minor differences. The REP-PCR analysis revealed clearly distinct patterns for all the *P. s.* pv. *morsprunorum* isolates from sweet cherry and plum.

The patterns generated were found to be very different among the *P. syringae* and *P. s.* pv. *syringae* isolates examined (Figs. 6.4 to 6.8). In addition, the *P. s.* pv. *morsprunorum* cultures isolated from wild cherry (Figs. 6.3, 6.4, and 6.6 to 6.8) were also very diverse. These results suggest that the diversity within *P. syringae* and *P. s.* pv. *syringae* is rather large and the REP-PCR experiments, while yielding distinct patterns, did not allow direct identification of these isolates. The same applies to the *P. s.* pv. *morsprunorum* isolates from wild cherry.

The almost unique genomic fingerprint of *P. s.* pv. *morsprunorum* detected in sweet cherry and plum, could easily distinguish the atypical forms Psm5250 and Psm5252 (Fig. 6.2, lanes 12 and 13, respectively). The similarity of *P. s.* pv. *morsprunorum* isolates from sweet cherry (lanes 1 to 4) and the dissimilarity of the *P. s.* pv. *morsprunorum* isolates from wild cherry are also clear from Figure 6.3, although Psm5269 and Psm5270 (lanes 9 and 10, respectively) were quite similar to each other. From Figure 6.4, it is still possible to observe the high genetic variability of the *P. s.* pv. *morsprunorum* isolates from wild cherry (lanes 1 to 6). Although the isolates Psm5275, Psm5276, and Psm5277 had the same fingerprint, they were always different from the typical *P. s.* pv. *morsprunorum*. In the same Figure, the last four lanes are from *P. s.* pv. *morsprunorum* isolated from plum and the lane before those is from sweet cherry. It is possible to observe again the great similarity between fingerprints from plum isolates and the one from typical *P. s.* pv. *morsprunorum*.

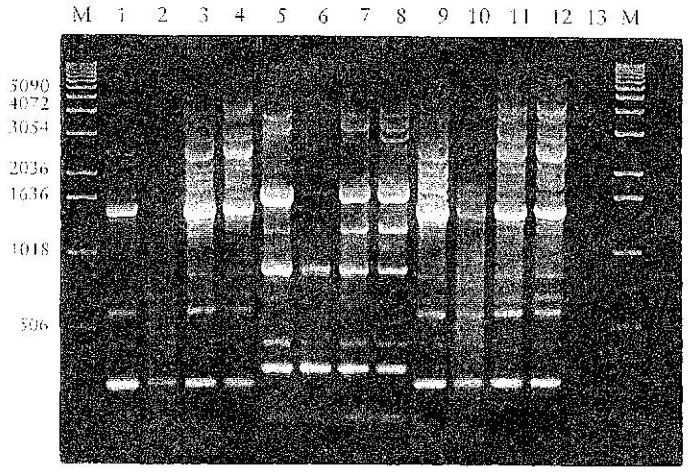

Figure 6.1

Genomic fingerprint of *P. syringae* isolates generated from REP-PCR. Polymerase chain reaction products were electrophoresed in a 1.5% agarose gel and stained with ethidium bromide. Three sets of lanes: lanes 1 to 4, *P. s.* pv. *morsprunorum* (Psm5260); lanes 5 to 8, *P. s.* pv. *syringae* (Pss2942); lanes 9 to 12, *P. syringae* (Ps214). In each set the first lane is isolated DNA, the second lane is whole-colony, the third lane is 1:50 colony dilution, and the fourth lane is a 1:500 colony dilution. The left and right lanes (M) contain DNA molecular size marker (1 Kb ladder); the sizes are indicated in base pairs; and lane 13 is the water control.

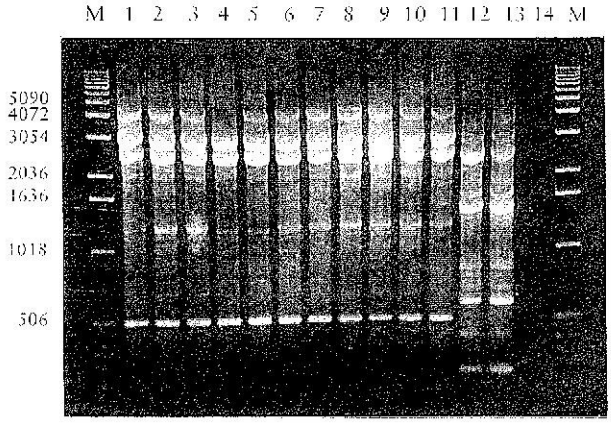

Figure 6.2

Genomic fingerprint of *P. s.* pv. *morsprunorum* isolates from sweet cherry and plum trees generated by REP-PCR. Polymerase chain reaction products were electrophoresed in a 1.5% agarose gel and stained with ethidium bromide. Lane 1, Psm797 (plum); lane 2, Psm798; lane 3, Psm2206; lane 4, Psm2928 (plum); lane 5, Psm5238; lane 6, Psm5239; lane 7, Psm5240; lane 8, Psm5241; lane 9, Psm5243; lane 10, Psm5244; lane 11, Psm5249; lane 12, Psm5250; lane 13, Psm5252; and lane 14, water control. The left and right lanes (M) contain DNA molecular size marker (1 Kb ladder); the sizes are indicated in base pairs.

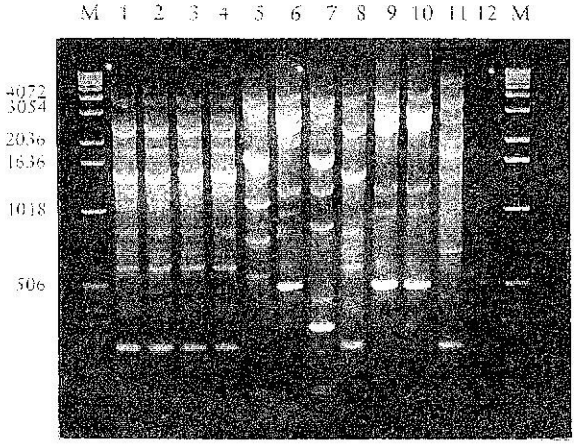

Figure 6.3

Genomic fingerprint of *P. s.* pv. *morsprunorum* isolates from sweet and wild cherry trees generated by REP-PCR. Polymerase chain reaction products were electrophoresed in a 1.5% agarose gel and stained with ethidium bromide. Lanes 1 to 4 are from sweet cherry, and lanes 5 to 11 are from wild cherry. Lane 1, Psm5254; lane 2, Psm5255; lane 3, Psm5257; lane 4, Psm5260; lane 5, Psm5265; lane 6, Psm5266; lane 7, Psm5267; lane 8, Psm5268; lane 9, Psm5269; lane 10, Psm5270; lane 11, Psm5271; and lane 12, water control. The left and right lanes (M) contain DNA molecular size marker (1 Kb ladder); the sizes are indicated in base pairs.

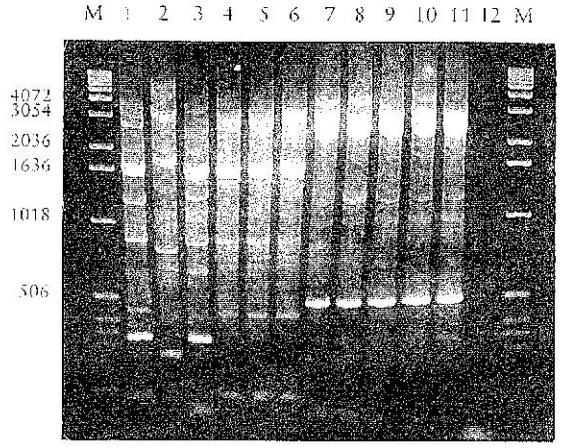

Figure 6.4

Genomic fingerprint of *P. syringae* isolates from wild cherry (lanes 1 to 6), sweet cherry (lane 7), and plum trees (lanes 8 to 11) generated by REP-PCR. Polymerase chain reaction products were electrophoresed in a 1.5% agarose gel and stained with ethidium bromide. Lane 1, Psm5272; lane 2, Psm5273; lane 3, Psm5274; lane 4, Psm5275; lane 5, Psm5276; lane 6, Psm5277; lane 7, Psm5280; lane 8, Psm5281; lane 9, Psm5282; lane 10, Psm5299; lane 11, Psm5300; and lane 12, water control. The left and right lanes (M) contain DNA molecular size marker (1 Kb ladder); the sizes are indicated in base pairs.

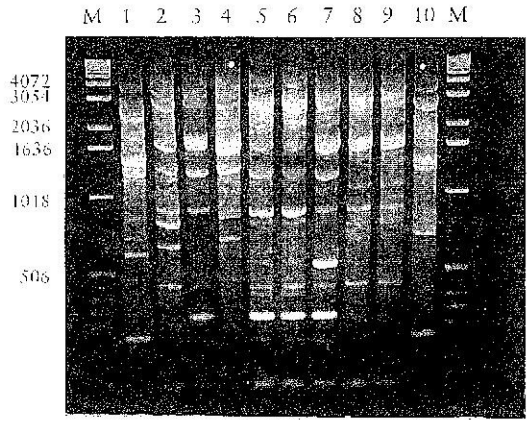

Figure 6.5

Genomic fingerprint of *P. syringae* isolates from sweet cherry trees (lanes 1, 7, and 8), cherry laurel (lane 2), and *Prunus cerasifera* (lane 10); and *P. s.* pv. *syringae* isolates from sweet cherry (lanes 3 and 9), wild cherry (lane 4), and pear (lanes 5 and 6) trees generated by REP-PCR. Polymerase chain reaction products were electrophoresed in a 1.5% agarose gel and stained with ethidium bromide. Lane 1, Ps214; lane 2, Ps73; lane 3, Pss2942; lane 4, Pss5264; lane 5, Pss5340; lane 6, Pss5342; lane 7, Ps5355; lane 8, Ps5356; lane 9, Pss5357; lane 10, Ps5400. The left and right lanes (M) contain DNA molecular size marker (1 Kb ladder); the sizes are indicated in base pairs.

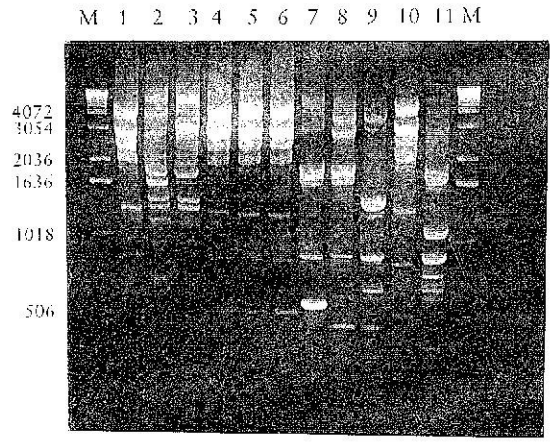

Figure 6.6

Genomic fingerprint of *P. s.* pv. *morsprunorum* isolates from sweet cherry (lanes 1, 3-6) and wild cherry (lane2); *P. s.* pv. *syringae* isolates from lilac (lane7), sweet cherry (lane 8) and Portuguese laurel (lane 9); and *P. syringae* isolates from wild cherry (lanes 10 and 11) trees generated by REP-PCR. Polymerase chain reaction products were electrophoresed in a 1.5% agarose gel and stained with ethidium bromide. Lane 1, Psm617; lane 2, Psm680; lane 3, Psm881; lane 4, Psm889; lane 5, Psm1462; lane 6, Psm5243; lane 7, Pss281; lane 8, Pss310; lane 9, Pss420; lane 10, NA7; lane 11, 1AH(a). The left and right lanes (M) contain DNA molecular size marker (1 Kb ladder); the sizes are indicated in base pairs.

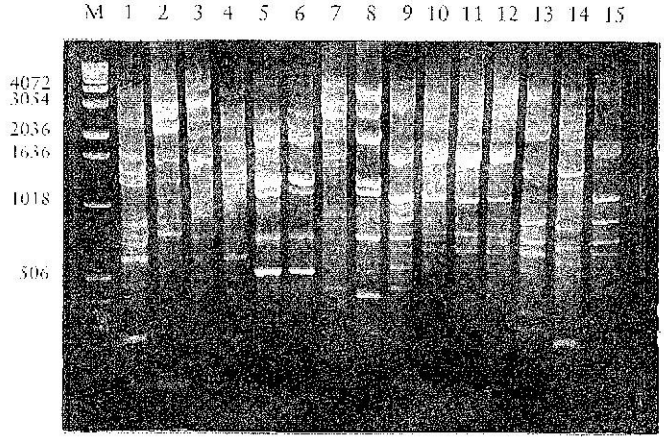

Figure 6.7

Genomic fingerprint of *P. syringae* and *Erwinia herbicola* isolates from wild cherry trees generated by REP-PCR. Polymerase chain reaction products were electrophoresed in a 1.5% agarose gel and stained with ethidium bromide. Lane 1, M1; lanes 2 and 3, *E. herbicola* isolates; lane 4, R4; lanes 5 and 6, *E. herbicola* isolates; lane 7, R8; lane 8, *E. herbicola* isolate; lane 9, 1AH(a); lane 10, 1AH(b); lane 11, 1AH(d); lane 12, 1AH(e); lane 13, *E. herbicola* isolate; lane14, 11L(b); and lane 15, 11L(e1). The left lane (M) contain DNA molecular size marker (1 Kb ladder); the sizes are indicated in base pairs.

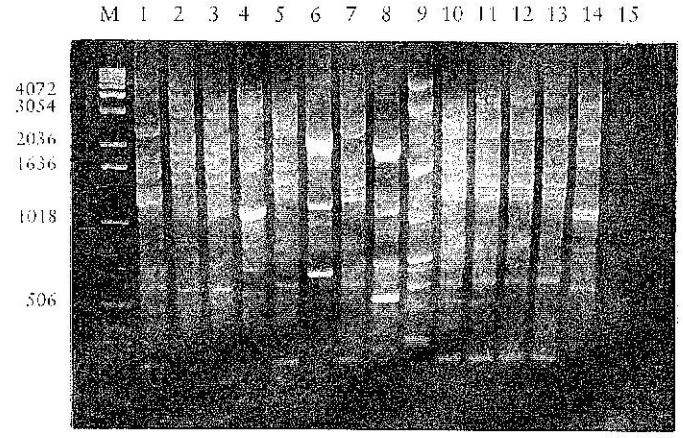

Figure 6.8

Genomic fingerprint of *P. syringae* and *Erwinia herbicola* isolates from wild cherry trees generated by REP-PCR. Polymerase chain reaction products were electrophoresed in a 1.5% agarose gel and stained with ethidium bromide. Lane 1, 11L(e2); lane 2, 11L(f2a); lane 3, 11L(f2b); lane 4, 11L(f3); lane 5, 3AH(a); lane 6, *E. herbicola* isolate; lane 7, 3AH(c); lane 8, *E. herbicola* isolate; lane 9, 12AF; lane 10, 13EF(a); lane 11, 13EF(b); lane 12, 13EF(c); lane 13, 13EF(d); lane14, 23SW; and lane 15, water control. The left lane (M) contain DNA molecular size marker (1 Kb ladder); the sizes are indicated in base pairs.

The difference between the genomic fingerprints of *P. s.* pvs. *morsprunorum* and *syringae* isolates is well displayed in Figure 6.6. Typical forms of *P. s.* pv. *morsprunorum* (Psm617 – lane 1, Psm889 – lane 4, Psm1462 – lane 5, and Psm5243 – lane 6) had the typical *P. s.* pv. *morsprunorum* fingerprint, while the *P. s.* pv. *syringae* isolates were very different from each other and from *P. s.* pv. *morsprunorum*. However, one culture from wild cherry (NA7) had the typical *P. s.* pv. *morsprunorum* band pattern (Fig.6.6, lane 10), although it did not show the characteristic phenotypic features (see section 4.2).

The great genetic variability of the *P. syringae* and *P. s.* pv. *syringae* isolates, analysed by REP-PCR, is very well revealed in Figure 6.5. Nevertheless, some isolates have similar patterns, like Pss5340 and Pss5342 (lanes 5 and 6, respectively) both from pear, and Ps5356 and Pss5357 (lanes 8 and 9) both from sweet cherry. The same wide genotypic variability was obtained with the *P. syringae* cultures isolated from wild cherry trees (Figs. 6.7 and 6.8) in the present work. However, some isolates have a very similar pattern, like 1AH(b), 1AH(d), and 1AH(e), denoting that they could be the same isolate (Fig. 6.7, lanes 10 to 12), especially as they were isolated from the same site. Likewise, the isolates 11L(f2a) and 11L(f2b); and 13EF (a), 13EF(b), 13EF (c), and 13EF(d) had very similar band patterns (Fig. 6.8, lanes 2 and 3; and lanes 10 to 13, respectively).

The *E. herbicola* cultures also presented very different patterns (Fig. 6.7, lanes 2, 3, 5, 6, 8, and 13; Fig.6.8, lanes 6 and 8), which made it impossible to distinguish them by REP-PCR.

The ability to measure genetic diversity with the REP-PCR assay was limited by the excessive variability in the patterns produced. Little can be concluded regarding the extent of diversity within the *P. s.* pv. *syringae* and the *P. s.* pv. *morsprunorum* isolates from wild cherry. Because the fingerprinting patterns were so dissimilar in the *P. s.* pv. *syringae* and wild cherry isolates, and so similar in the *P. s.* pv. *morsprunorum* isolates, cluster analyses were not performed.

Adaptation to a narrow ecological niche (the sweet cherry host), could explain the low genetic diversity observed within *P. s.* pv. *morsprunorum*. The relatively high genetic diversity of *P. s.* pv. *syringae* may reflect the diversity of hosts. The *P. syringae* cultures isolated from wild cherry were not homogenous, because they seem to include a range of varying forms from different host genotypes.

6.4 Conclusions

Dispensing cells directly into the PCR tubes from solid media cultures yielded fingerprinting patterns identical to patterns generated from isolated DNA. Analogous rep-PCR-derived genomic fingerprints were generated from purified genomic DNA and colonies on agar plates. It was possible to avoid the DNA isolation step in this PCR technique which improves the method tremendously. The possibility of using cells collected from plant lesions for *P. s.* pv. *morsprunorum* should be further investigated. Positive diagnosis could be achieved within 24 hours if a profile could be generated directly from infected plant tissue, or after two to four days if bacteria had to be first cultured on a solid medium. However, it seems very difficult to distinguish *P. s.* pv. *morsprunorum* from other bacteria amplified with the same technique.

The REP-PCR showed clearly that the isolates from wild cherry, even the ones formerly identified as *P. s.* pv. *morsprunorum*, should not be included in this taxonomic group.

Furthermore, although the sample size is still too small to warrant firm conclusions, the rather large diversity among strains of *P. s.* pv. *syringae* suggested that further study may well lead to the taxonomic subdivision of this pathovar. The level of genetic diversity in *P. s.* pv. *syringae* appeared to support the hypothesis that variation was higher among strains from diverse habitats than among those from a uniform habitat.

The high degree of uniformity observed in *P. s.* pv. *morsprunorum* from sweet cherry further suggests that we are dealing with forms stabilised during a long association with the host.

CHAPTER 7
NUCLEIC ACID TECHNOLOGY – DNA HYBRIDISATION PROBE

7.1 Introduction

Nucleic acid technology enables rapid and sensitive diagnosis. A range of molecular methodologies is now currently available for the identification of plant pathogenic bacteria (VIVIAN, 1992). Among those methods, hybridisation of DNA probes have especially proven their usefulness in detecting bacterial pathogens (DENNY, 1988).

Hybridisation involves the annealing of single stranded labelled oligonucleotide probes to complementary, denatured DNA sequences immobilised on membranes. A DNA hybridisation probe (PST-DNA) was developed for differentiating *P. s.* pv. *tomato* (Okabe) Young *et al.* from *P. s.* pv. *syringae* (DENNY, 1988), but it also reacted positively with other pathovars, including pv. *morsprunorum*. This PST-DNA probe consisted of cloned 3.5- and 3.6-Kb *Eco*RI fragments of unknown sequence. The potential of this probe as a diagnostic tool to differentiate among 20 strains of *P. s.* pvs. *morsprunorum* and *syringae* was well established by PATERSON & JONES (1991). Although the probe was not specific for *P. s.* pv. *morsprunorum*, its reaction with DNA from other pathovars or from saprophytic pseudomonads was not a serious defect. PATERSON & JONES (1991) conclude that the probe works well for detecting colonies of *P. s.* pv. *morsprunorum*, obtained from lesions or isolated as epiphytes, and would allow one to study the epidemiology of this organism in the presence of *P. s.* pv. *syringae*.

The essential simplicity of colony hybridisation offers one of the most rapid and reliable methods at present available for routine analysis of hybridisation (VIVIAN, 1992). The colony blotting technique involves transferring colonies of bacteria, grown on an agar plate, to a nylon membrane. The membrane is then treated so as to lyse the bacterial cells, denature the released DNA and attach it to the filter. The labelled

probe is then added in solution following a prehybridisation step to prevent non-specific binding of labelled probe to the filter. The labelled bound probe is then detected with an appropriate detection system (TOWNER & COCKAYNE, 1993). Colony hybridisation techniques can allow *in situ* application of the probes to identify colonies without prior DNA isolation and purification. In addition, the versatility offered by the improvements in labelling methods may allow widespread and routine use (ROTH & JOHNSON, 1987).

The objective of this study was to evaluate the PST-DNA probe for differentiating *P. s.* pv. *morsprunorum* from *P. s.* pv. *syringae* and clarifying the taxonomic position of *P. syringae* cultures from wild cherry trees. As the colony blotting technique is the most rapid hybridisation format to obtain a sample of total genomic DNA ready for probing (VIVIAN, 1992), it was preferred in the present study.

7.2 Material and methods

Preparation of the DNA probe

The DNA probe, obtained from Dr. Timothy Denny (Department of Plant Pathology, University of Georgia, Athens, GA 30602, USA) on 18 May, 1994, was prepared from plasmids pJCA2 and pJCA11, which contained 3.6- and 3.5-Kb *Eco*RI restriction fragments of DNA, respectively, from *P. s.* pv. *tomato*. These two plasmids were transformed into competent cells of *Escherichia coli* strain DH5α, using the method in use at the Molecular Biology Research Service of the School of Plant Sciences (The University of Reading). The following method was used: 375 µl of an overnight culture of *E. coli* DH5α on LB broth (see section 3.1) was added to 10 ml of LB broth and put at 37°C in a shaker for 1 h and 30 min; after centrifugation at 2,000 rpm for 5 min, the cells were washed and resuspended with 5 ml ice cold Tm1 (10 mM $CaCl_2$ and 10 mM MOPS pH 6.8); after being centrifuged again at 2,000 rpm for 5 min the cells were resuspended in 500 µl of Tm2 (75 mM $CaCl_2$ and 10 mM MOPS); the transformation was performed in 90 µl Tm3 (75 mM $CaCl_2$; 50 mM MOPS; 5% (w/v) glycerol; 1 mM EDTA; and 10 mM KCl) with 1 µl (≈50 ng) of the DNA to be transformed and 100 µl of the competent cells; the control was set without the DNA but with a known plasmid; incubation on ice for 45 min was followed by another incubation at 42°C for 10 min; after 3 min on ice the tubes were incubated at 37°C for one hour and the recombinants were selected on LB agar (see section 3.1) containing 50 $\mu g.ml^{-1}$ of ampicillin, and subsequently grown in LB broth containing 25 $\mu g.ml^{-1}$ of ampicillin. The transformed

cells were stored in 15% glycerol at -70°C after being snap-frozen in liquid nitrogen for 4 min.

The plasmid DNA was extracted from the cells with a commercial DNA purification kit (Wizard Minipreps, Promega, Madison, Wisconsin, USA), digested with *Eco*RI, and electrophoresed on agarose (0.9%) gel using λ*Hind* III as a molecular weight marker. The relevant fragment was selected, cut out of the gel, and recovered from the agarose using a DNA extraction kit (Kristal Gelex DNA Extraction Kit, Cambridge Molecular Technologies, Cambridge, UK). The final DNA concentration was 25 ng.μl^{-1}, determined using an ethidium bromide plate with serial dilutions of salmon sperm DNA as standards.

Hybridisation and detection method

The hybridisation and detection method used was the DIG system (Boehringer Mannheim, Lewes, UK). This system uses digoxigenin, a steroid hapten, to label the DNA probe for hybridisation and subsequent detection. The probe was labelled with DIG-11-dUTP via random primed labelling. The DIG-labelled probes are hybridised to a membrane-bound colony lift. These hybridised probes are immuno-detected with an alkaline phosphatase-conjugated anti-digoxigenin antibody and then visualised with a chemiluminescent substrate.

Probe labelling

The PST-DNA probe was labelled with digoxigenin-11-dUTP using a random primed method. After being heat-denatured in a boiling water bath for 10 min and immediately chilled on ice for 30 sec, the probe (400 ng – 4 μg.ml^{-1}) was mixed with 10 μl (1X) of DIG-hexanucleotide mixture (random hexanucleotides, 500 mM Tris-HCl, 100 mM $MgCl_2$, 1 mM dithioerythritol, and 2 mg.ml^{-1} BSA; pH 7.2), 10 μl (1X) of DIG-dNTP labelling mixture (1mM dATP, 1mM dCTP, 1mM dGTP, 0.65 mM dTTP, and 0.35 mM DIG-11-dUTP; pH 7.5), 59 μl of pure water (molecular biology grade), and 5 μl (100 units.ml^{-1}) of DNA polymerase I (Klenow enzyme, large fragment) from *E. coli* in a sterile microfuge tube (on ice). The reaction, in a total volume of 100 μl, was incubated overnight at 37°C and terminated by adding 4 μl of 0.5 M EDTA. The labelled DNA was then precipitated with 10 μl of 4 M LiCl solution and 3.0 volumes of chilled 100% ethanol, mixed well, and incubated at -20°C for 1 h. The labelled probe was then centrifuged at 13,000 rpm for 15 min. After decanting the ethanol, the pellet was washed with 100 μl of chilled 70% ethanol, dried at room temperature, resuspended in 50 μl of TE buffer (10 mM Tris-HCl and

1mM EDTA, pH 8.0), and stored at -20°C until required. The yield of the Dig labelled probe, determined by a direct detect assay, was estimated to be around 20 $ng.\mu l^{-1}$.

Colony hybridisation

One day cultures on LB agar, pre-cooled for 30 min at 4°C, were transferred to uncharged nylon membrane for colony hybridisation (Boehringer Mannheim) by carefully placing a 5.5 × 5.5 cm membrane piece onto the agar surface. After allowing the transfer to take place, approximately one minute, the membrane was removed carefully with tweezers and blotted briefly on dry Whatman 3MM paper. The membrane was then placed on filter paper soaked with denaturation solution (0.5 N NaOH; 1.5 M NaCl; and 0.1% SDS) for 15 min, followed by a soak on neutralisation solution (1.0 M Tris-HCl, pH 7.5, and 1.5 M NaCl) for 15 min, and a final soak on 2X SSC (0.3 mM NaCl and 30 mM sodium citrate, pH 7.0) for 10 min. Between each soaking step the membrane was blotted briefly on Whatman 3MM paper. The DNA was then crosslinked to the nylon membrane with UV-light. Both sides of each membrane were irradiated using a UV Crosslinker (Stratalinker, Stratagene Ltd., Cambridge, UK). The membranes were then placed on a clean piece of aluminium foil and 0.5 ml of proteinase K (2 $mg.ml^{-1}$) was pipetted on each membrane piece. The solution was distributed evenly and incubated for one hour at 37°C. The cellular and agar debris were removed by blotting the membranes between filter paper saturated with distilled water and by applying pressure over the area with a bottle. The debris was stuck to the upper filter paper when it was gently pulled off. This step had to be repeated a few times, with fresh pieces of filter paper each time, until no cellular debris was visible.

The prehybridisation and hybridisation steps were carried out in a roller tube inside a rotisserie oven (Hybridiser HB-1D, Techne, Cambridge, UK). The membranes were prehybridised in 50 ml of prehybridisation solution (5X SSC; 50% formamide, deionised; 0.1% sodium lauroylsarcosine; 0.02% SDS; and 2% Dig-system blocking reagent) for one hour at 42°C. After denaturation by boiling for 5 min at 95-100°C, and rapid cooling on ice, the labelled probe was mixed with 10 ml of prehybridisation solution to give a final concentration of 15 $ng.ml^{-1}$. After decanting off the prehybridisation solution, the hybridisation solution was added to the roller tube and left to incubate overnight at 42°C. At the end of the hybridisation, the hybridisation solution was poured into a tube and stored at -20°C for reuse. When reused the mix was heated at 68°C for 10 min, before hybridisation.

Afterwards, the membranes were washed twice for 5 min in ample 2X SSC, 0.1% SDS at room temperature with gentle agitation. They were then transferred to 0.5X SSC, 0.1% SDS and washed twice for 15 min at 68°C with gentle agitation.

After the post-hybridisation washes, the membranes were equilibrated in washing buffer (100 mM maleic acid; 150 mM NaCl, pH 7.5; and 0.3% Tween 20) for 1 min, followed by a blocking step consisting of gently agitating them in blocking solution [(1% blocking reagent (Dig system) dissolved in maleic acid buffer (100 mM maleic acid and 150 mM NaCl, pH 7.5)] for 30-60 min. After pouring off the blocking solution, the membranes were incubated in antibody solution (diluted anti-digoxigenin-AP 1:10,000 in blocking solution) for 30 min. After discarding this solution the membrane was washed twice, 15 min per wash, in washing buffer, and equilibrated in detection buffer (100 mM Tris-HCl and 100 mM NaCl, pH 9.5) for 2 min. For the detection step, the CSPD® {25 mM disodium 3-[4-methoxyspiro(1,2-dioxetane-3,2'-5'-chlorotricyclodecan)-4-yl] phenyl phosphate} substrate was diluted (1:100) in detection buffer and 200 µl were carefully applied onto each membrane piece. After an incubation for 5 min at room temperature, and another for 15 min at 37°C, the X-ray film (X-OMAT, Eastman Kodak Company, Rochester, New York, USA) was exposed to the membranes for approximately 7-8 min.

The bacteria cultures used to test the DNA hybridisation probe with the colony blotting method were the ones described in Tables 4.1 (except Psm680) and 4.2 (except NA7). Fifteen cultures, isolated from cherry leafwashings, were used as negative controls to test the specificity of the method: four cultures of *Pseudomonas fluorescens* (Trevisan) Migula; one culture of *Pseudomonas tolaasii* Paine; three cultures of *Pseudomonas viridiflava* (Burkholder) Dowson; one culture of *Pseudomonas cichorii* (Swingle) Stapp; one culture of *Pseudomonas corrugata* (Scarlett *et al.*) Roberts & Scarlett; and five cultures of the group *Erwinia herbicola – Pantoea agglomerans*. In addition, other five cultures were used as negative controls: two cultures of *Erwinia carotovora* subsp. *atroseptica* (van Hall) Dye; two cultures of *Erwinia carotovora* subsp. *carotovora* (Jones) Bergey *et al.*; and one culture of *Xanthomonas campestris* (Pammel) Dowson. All the cultures were tested against the PST-DNA hybridisation probe at least twice.

7.3 Results and discussion

The transformation of the competent cells of *E. coli* strain DH5α was effective with the plasmid pJCA2, but unsuccessful with pJCA11. The reason the bacteria with this

plasmid grew on agar with ampicillin, but not afterwards in LB broth with ampicillin, was obscure. Nevertheless, the plasmid was apparently hampering the bacteria growth in the broth. The hybridisations had to proceed only with the 3.6-Kb fragment.

The probe reactions of the various *Pseudomonas syringae* isolates after colony hybridisation are presented in Table 7.1 and Figures 7.1 to 7.4. The probe hybridised strongly with almost all the *P. s.* pv. *morsprunorum* cultures from sweet cherry except with Psm881 (Fig. 7.2, cell c4), which had already been confirmed by other tests to be possibly not well identified, and with Psm2206 (Fig. 7.1, cell a3), Psm 5239 and Psm5249 (Fig. 7.1, cell b5), which showed only a weak hybridisation response. All the *P. s.* pv. *morsprunorum* cultures from plum showed a very strong response to the probe.

The *P. s.* pv. *syringae* cultures did not hybridise with the PST-DNA probe or if they did the reaction was very weak. The probe also failed to hybridise with *P. fluorescens* (Fig.7.3, cell b3), *P. tolaasii* (Fig. 7.3, cell d2), *P. viridiflava* (Fig.7.3, cells d4 and d5), *P. cichorii*, *P. corrugata*, and *E. herbicola – P. agglomerans* (Fig. 7.3, cells a4, b2, b5, and c1). In addition, the probe did not hybridise with *E. carotovora* subsps. *atroseptica* and *carotovora*, or *X. campestris*. Therefore, the PST-DNA probe can be considered quite specific and useful to distinguish *P. s.* pv. *morsprunorum* colonies from different contaminants in primary isolation plates from leafwashings or bacterial canker lesions. The data of DENNY (1988) and PATERSON & JONES (1991) were confirmed and extended in this work.

The *P. s.* pv. *morsprunorum* cultures from wild cherry showed again their intermediate behaviour with some cultures reacting strongly with the probe, some weakly, and others with no hybridisation at all. The *P. syringae* cultures isolated by me from wild cherry (Figs. 7.3 and 7.4) also showed very strong hybridisation with the probe in some cultures, weak or none in others.

Table 7.1

Probe reactions of *P. syringae* isolates after colony hybridisation with the Dig-labelled PST-DNA probe.

Isolate	Hybridisation response
P. syringae	
Sweet cherry	
Ps214	–
Ps5355	–
Ps5356	–
Wild cherry	
1AH(a)	–
1AH(b)	–
1AH(d)	–
1AH(e)	–
3AH(a)	++
3AH(c)	++
11L(b)	–
11L(e1)	++
11L(e2)	–
11L(f2a)	+
11L(f2b)	+
11L(f3)	–
12AF	–
13EF(a)	++
13EF(b)	++
13EF(c)	++
13EF(d)	++
23SW	–
M1	+
NA5	++
R4	++
R8	–
Cherry laurel	
Ps73	–
P. s. pv. *morsprunorum*	
Sweet cherry	
Psm617	++
Psm798	++
Psm881	–
Psm889	++
Psm1462	++
Psm2206	+
Psm5238	++
Psm5239	+
Psm5240	++
Psm5241	++
Psm5243	++
Psm5244	++
Psm5249	+
Psm5250	++
Psm5252	++
Psm5254	++
Psm5255	++
Psm5257	++
Psm5260	++
Psm5280	++
Wild cherry	
Psm5265	–
Psm5266	++
Psm5267	–
Psm5268	++
Psm5269	++
Psm5270	++
Psm5271	++
Psm5272	–
Psm5273	–
Psm5274	–
Psm5275	+
Psm5276	+
Psm5277	+
Plum	
Psm797	++
Psm2928	++
Psm5281	++
Psm5282	++
Psm5299	++
Psm5300	++
P. s. pv. *syringae*	
Lilac	
Pss281	+
Pear	
Pss5340	–
Pss5342	–
Sweet cherry	
Pss310	+
Pss2942	–
Pss5357	+
Wild cherry	
Pss5264	–
Portuguese laurel	
Pss420	–

– = no hybridisation
+ = weak hybridisation
++ = strong hybridisation

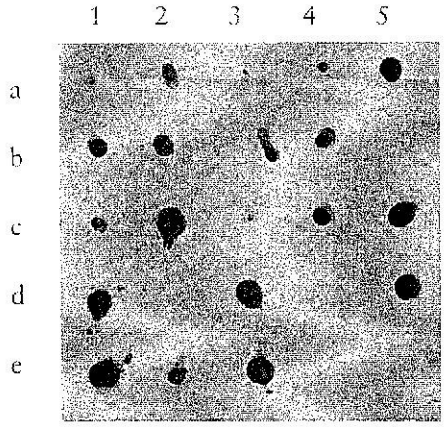

Figure 7.1

Colony hybridisation of *P. syringae* pv. *morsprunorum* cultures with the PST-DNA probe. The bacterial cultures were: Psm797 (a1); Psm798 (a2); Psm2206 (a3); Psm2928 (a4); Psm5238 (a5); Psm5240 (b1); Psm5241 (b2); Psm5243 (b3); Psm5244 (b4); Psm5249 (b5); Psm5250 (c1); Psm5252 (c2); Psm5254 (c3); Psm5255 (c4); Psm5257 (c5); Psm5260 (d1); Psm5265 (d2); Psm5266 (d3); Psm5267 (d4); Psm5268 (d5); Psm5269 (e1); Psm5270 (e2); Psm5271 (e3); Psm5272 (e4); and Psm5274 (e5).

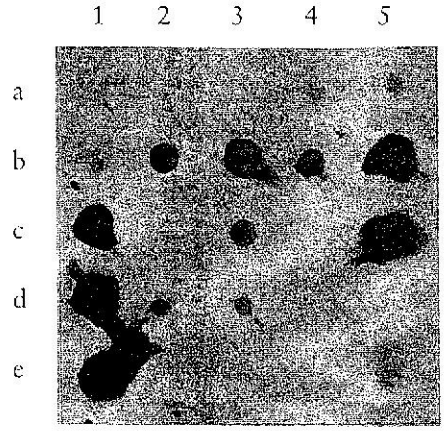

Figure 7.2

Colony hybridisation of *P. syringae* pv. *morsprunorum*, *P. s.* pv. *syringae*, and *P. syringae* cultures with the PST-DNA probe. The bacterial cultures were: Psm5272 (a1); Psm5273 (a2); Psm5274 (a3); Psm5275 (a4); Psm5276 (a5); Psm5277 (b1); Psm5280 (b2); Psm5281 (3); Psm5282 (b4); Psm5299 (b5); Psm5300 (c1); Ps73 (c2); Psm617 (c3); Psm881 (c4); Psm889 (c5); Psm1462 (d1 and e1); Pss281 (d2); Pss310 (d3); Pss420 (d4); Pss2942 (d5); Pss5342 (e2); Ps5355 (e3); Ps5356 (e4); and Pss5357 (e5).

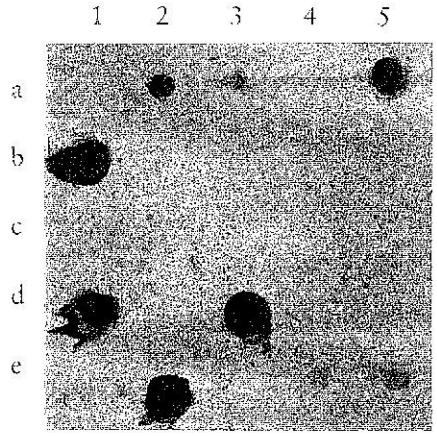

Figure 7.3

Colony hybridisation of *P. syringae* cultures from wild cherry, *P. s.* pv. *syringae*, and negative controls with the PST-DNA probe. The bacterial cultures were: Pss5340 (a1); M1 (a3); *E. herbicola* (a4, b2, b5, and c1); NA5 (a5); R4 (b1); *P. fluorescens* (b3); R8 (b4); 1AH(a) (c2); 1AH(b) (c3); 1AH(d) (c4); 1AH(e) (c5); 3AH(a) (d1); *P. tolaasii* (d2); 3AH(c) (d3); *P. viridiflava* (d4 and d5); 11L(b) (e1); 11L(e1) (e2); 11L(e2) (e3); 11L(f2a) (e4); and 11L(f2b) (e5).

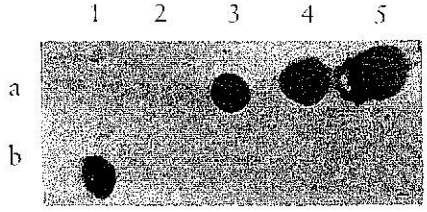

Figure 7.4

Colony hybridisation of *P. syringae* cultures from wild cherry with the PST-DNA probe. The bacterial cultures were: 11L(f3) (a1); 12AF (a2); 13EF(a) (a3); 13EF(b) (a4); 13EF(c) (a5); 13EF(d) (b1); and 23SW (b2).

There were differences in the intensity of the hybridisation reaction between the first and second time the colonies were examined. These differences were clearly connected with the amount of colony transferred to the membrane. Some of the weak positive reactions could also be explained by that. This feature seemed a problem for this technique because it would compel the worker to repeat the test considering the possible presence of false negatives.

In some cultures the cellular debris were very difficult to remove even after the Proteinase K treatment. A few membranes had to be wiped with a clean wet tissue until no more debris could be visualised. This process, that could damage the membrane, dragged the DNA from the initial spot and increased the background in some areas of the membrane (Fig. 7.2).

7.4 Conclusions

It is confirmed that the PST-DNA probe hybridises with DNA from typical *P. s.* pv. *morsprunorum* and generally fails to hybridise with typical *P. s.* pv. *syringae*. Hence, the probe seems an effective tool for detecting typical *P. s.* pv. *morsprunorum* in the presence of *P. s.* pv. *syringae*. The same conclusions were reached by PATERSON & JONES (1991). However, the probe failed to clearly detect the atypical forms of *P. s.* pv. *morsprunorum* isolated from wild cherry trees. Therefore, the PST-DNA probe could not be used to identify the *P. syringae* cultures isolated during the present work.

The failure of the probe to hybridise with a few *P. s.* pv. *morsprunorum* cultures and with the cultures isolated during this work from wild cherry could also be connected with the lack of one fragment from the original probe. Hence, a probe constituted by both fragments could wider the detection to other *P. s.* pv. *morsprunorum* cultures (T.P. DENNY, personal communication 1994).

The presence of false positives, due to poor transfer to the membranes, would be a disadvantage of this technique unless the bacteria were allowed to grow in the membrane. But then, removal of the cellular debris, which is already difficult, would be even more difficult or impossible. A second possibility would be to use dot-blots which involves the blotting of isolated DNA instead of the whole colony. But then, because of the isolation step, this would be much more time-consuming.

CHAPTER 8
IMMUNODIAGNOSIS

8.1 Introduction

The advantages of using serological tests for diagnosis of plant pathogenic bacteria have been thoroughly discussed by several workers (LELLIOTT & STEAD, 1987; STEAD, 1992). The main advantage is speed, and in some cases, diagnosis may be achieved within a few seconds. There is also the potential for direct use on diseased tissue without the need to isolate the organisms, which can be extremely useful for fastidious organisms. The full potential of serological methods for bacterial disease diagnosis has not been obtained, primarily because although antisera are not particularly difficult to produce, the required specificity is rarely attainable and their development and production are laborious (LELLIOTT & STEAD, 1987). Nevertheless, STEAD (1992) assumes that the serological techniques offer the best potential for detection of bacteria in plant material, in subclinical infections, in plant debris or for detection of epiphytic bacteria.

Polyclonal antibodies are raised in mammals following injections with the antigen. With time, the mammalian antibody-producing cells (β-lymphocytes) secrete antibody into the blood, which may be removed, allowed to clot and thus give rise to antiserum. If the antibody associates with the antigen against which it was prepared, then the response is useful for diagnosis (PRIEST & AUSTIN, 1993). However, the antibody can cross react with different antigens and misdiagnosis may result. The variety of the antigenic determinants in a bacterial cell will often result in common antigens, which are shared by more than one type of bacterium (LELLIOTT & STEAD, 1987).

STEAD (1992) believed that antisera produced to whole cells are not suitable in agglutination tests to identify bacteria such as the *Pseudomonas syringae* pathovars, since these contain many common antigens. Nevertheless, SAMSON & SAUNIER (1987)

had already shown that not all *P. syringae* isolates share the same antigens in immunodiffusion assays with antibodies raised against whole cells. These studies even demonstrated that *P. s.* pv. *morsprunorum* belongs to a particular serogroup, solely comprised of this pathovar.

Because polyclonal antisera consist of heterologous populations of antibodies with variable specificities, different segments of the antibody population may be reactive when using different serological tests (BALL *et al.*, 1990). DE BOER & SCHAAD (1990) believe that for immunofluorescence assays untreated whole cells are best as an antigen.

It was decided in this research firstly to use the indirect immunofluorescence staining technique, as this is probably the most widely used for detection of bacteria, without requiring isolation methods (STEAD, 1992). The second assay used was the conjugated *Staphylococcus aureus* agglutination test, as this is probably the fastest way of obtaining an identification.

8.2 Immunofluorescence

The immunofluorescence procedure allows the bacterial cells to actually be seen with a fluorescent halo around the cell wall. Rather than simply observing an enzyme reaction, bacterial cell morphology and the size of the cell population can be seen. However, this test detects dead as well as live cells. The immunofluorescence is based on the ability of a fluorescent compound to bind to antibody. When this conjugated antibody binds to bacteria, its presence can be seen in a microscope using an ultraviolet light source.

The technique can be carried out in an indirect mode in which the fluorescent dye is conjugated to a secondary antibody which is specific for the primary antibody. This procedure involves fixing an appropriately diluted sample onto a microscope slide, treating the preparation with an antibody specific for the bacterium of interest, and then reacting the first antibody with a secondary antibody to which the fluorescent dye has been conjugated. The most used fluorescent agent is fluorescein isothiocyanate (FITC) which, under ultraviolet light, fluoresces bright green against a dark background (DE BOER, 1990).

The immunofluorescence test is one of the most sensitive, permitting detection in the range of 10^3 cells.ml^{-1} (DE BOER, 1990), and the indirect method gives clearer differentiation of positive and negative results.

For any serological reaction it is important to have antibodies and antigens in optimal proportions, and to obtain this dilutions of both must be tested. Titres of the antisera and working dilutions should be determined. Titre of the antiserum is the reciprocal of the highest dilution at which maximum fluorescence is observed, and working dilution is the highest dilution which would give a clear positive result with all known strains. The working dilution is usually about two or three doubling dilutions below the homologous titre (STEAD, 1992).

8.2.1 Material and methods

Antiserum production

The antiserum was produced against live cells of *P. s.* pv. *morsprunorum* in a pool of the isolates Psm617, Psm680, Psm889, and Psm1462. Before the animal immunisation, the cells were grown in glycerol agar, washed five times in 10 mM phosphate buffered saline (PBS), pH 7.2 (2.7 g $Na_2HPO_4.12H_2O$; 0.4 g $NaH_2PO_4.2H_2O$; 8.0 g NaCl; and 1 l distilled water), by centrifuging at 13,000 rpm for 10 minutes, and the pellet resuspended in the same solution.

The antibodies were developed in two New Zealand White rabbits by Dr. Philip Knight (School of Animal and Microbial Sciences, The University of Reading, UK). Pre-immune serum (normal serum) was removed just before the beginning of the immunisation for later use as a control. The immunisation schedule started on 16 May 1994, and four inoculations were made until September. The injections were monthly spaced, but no booster was done in August. The bleeds were taken on 7 and 21 June, 12 July, and a final one on 5 October 1994. The serum of all the bleeds was stored at -20°C until required.

To test for the presence of antibodies a range of doubling dilutions (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, and 1:128) of the antisera, of both rabbits, from the 12 July bleed and from pre-immune serum were evaluated against a bacterial suspension at 10^9 cells.ml^{-1} (LELLIOTT & STEAD, 1987) by a preliminary slide agglutination test. The isolates of *P. s.* pv. *morsprunorum* to which the antisera were produced, *P. s.* pv. *morsprunorum* Psm881, and three isolates of *P. s.* pv. *syringae* (Pss281, Pss310, and Pss420) were used to preliminarily evaluate the specificity of the sera. In addition, three isolates of *Erwinia herbicola – Pantoea agglomerans* were tested. A positive reaction was given by agglutination of bacterial cells due to multiple bridging between cells by specific antibodies attached to antigens on the cell wall. The agglutination was seen, under a microscope, as a clumping of cells in a drop of the bacterial suspension.

Immunofluorescence test method

The procedure described by DE BOER (1990) was generally followed. One loopful (1 µl) of a colony grown in NSA was transferred to a vial containing 10 ml of sterile distilled water and three 10-fold dilutions in sterile distilled water were made. To different wells of a 12-well multitest slide (ICN, Aurora, Ohio, USA) were applied 20 µl of the undiluted and the three diluted samples. The preparations were air dried, under a hair dryer, and fixed by soaking the slide in acetone for 10 min followed by air drying. The diluted (1:500) primary antibody in PBS plus 20 $\mu l.ml^{-1}$ of blocking agent (10% skim milk powder and 0.2% sodium azide) was applied in an amount of 20 µl to each well. The slide was incubated for 30-60 min in a moist chamber at 37°C. After that, it was rinsed with a gentle stream of distilled water, avoiding directing the stream directly on the preparation, and gently blot dried with Whatman 3 MM paper. Then, 20 µl of the diluted (1:40) secondary antibody (goat anti-rabbit immunoglobulin-FITC conjugate) mixed with 20 $\mu l.ml^{-1}$ of blocking agent was pipetted into each well and the slide incubated for another 30-60 min in a dark, moist chamber at 37°C. The anti-rabbit immunoglobulin (developed in goat) FITC conjugate was commercially obtained (Product no. F-6005, Sigma Immunochemicals, St. Louis, Missouri, USA) and used at a working dilution of 1:40 as suggested by the manufacturer and also determined in the present work on a known positive sample (Psm1462). The slide was again rinsed and blot dried as before. The coverslip was mounted with mounting fluid (18 ml glycerol; 2 ml PBS; and 20 mg p-phenylenediamine) that had been kept in the dark at -20°C.

The working dilution of the primary antibody (1:500) was determined by using a similar procedure to that described above but with only one concentration of the bacteria (10^6 $cells.ml^{-1}$) against a range of eleven antiserum dilutions in a two-fold series from 1:10 to 1:5,120 in PBS. The bacteria tested to determine the working dilution were the ones to which the antiserum was produced. The titres varied from 1:640 to 1:1,280.

The dilutions were repeated twice in each slide and two negative controls, instead of the primary antibody, were used, one the normal (pre-immune) serum at a dilution of 1:40, and the other only PBS.

If the cells of test bacterium fluoresce brightly at the working dilution then a presumptive diagnosis may be made. The results were considered positive when all the three antigen dilutions were positive in at least one of the repetitions.

All the *Pseudomonas syringae* isolates presented in Tables 4.1 and 4.2 were tested by the immunofluorescence assay. In addition, 27 isolates of different bacterial species were tested as negative controls to test the specificity of the serum and the reliability of the test. These negative controls included six isolates of *P. fluorescens*, three isolates of *P. tolaasii*, four isolates of *P. viridiflava*, two isolates of *P. cichorii*, one isolate of *P. corrugata*, two isolates of *E. c.* subsp. *carotovora*, two isolates of *E. c.* subsp. *atroseptica*, one isolate of *Xanthomonas campestris*, and six isolates of *E. herbicola – Pantoea agglomerans*.

8.2.2 Results and discussion

Antiserum production

In the preliminary slide agglutination test, after two minutes, there were already quite big lumps of cells, completely immobilised, with all the strains of *Pseudomonas syringae* pv. *morsprunorum* tested, except with Psm881, which probably does not belong to this pathovar, as is indicated by the results in the nutritional tests (chapters 4 and 5) and molecular biology (chapters 6 and 7). With this strain there was low clumping of cells, and only after 10 min were they immobilised. The agglutination behaviour of this isolate was very similar to *P. s.* pv. *syringae* Pss281 and Pss420. The latter have shown some slight clumping but only after one hour and four hours, respectively, and always with several free mobile cells. The culture Pss310 (*P. s.* pv. *syringae*) gave the same result as the *P. s.* pv. *morsprunorum* isolates. The three *E. herbicola – Pantoea agglomerans* isolates from cherry leaf washings failed to clump the bacteria and almost all the cells were very mobile even after 4 hours of exposure to the antiserum. The pre-immune serum failed to cluster the cells with all the cultures tested.

The results mentioned above were encouraging enough to proceed to testing field isolates to confirm the specificity and reliability of the antisera and to select the quickest and most economical assay for routine identification.

Immunofluorescence test method

The immunofluorescence test results for *Pseudomonas syringae* isolates are presented in Table 8.1. In Figure 8.1 is possible to see one positive result as it was observed through the microscope with UV light.

Table 8.1

Immunofluorescence test results for *P. syringae* isolates.

Isolate	Fluorescent response
P. syringae	
Sweet cherry	
Ps214	+
Ps5355	–
Ps5356	+
Wild cherry	
1AH(a)	+
1AH(b)	+
1AH(d)	+
1AH(e)	+
3AH(a)	+
3AH(c)	+
11L(b)	+
11L(e1)	+
11L(e2)	+
11L(f2a)	+
11L(f2b)	+
11L(f3)	+
12AF	(+)
13EF(a)	–
13EF(b)	+
13EF(c)	+
13EF(d)	+
23SW	(+)
M1	+
NA5	+
R4	+
R8	+
Cherry laurel	
Ps73	+
P. s. pv. *morsprunorum*	
Sweet cherry	
Psm617	+
Psm798	+
Psm881	(+)
Psm889	+
Psm1462	+
Psm2206	+
Psm5238	+
Psm5239	+
Psm5240	+
Psm5241	+
Psm5243	+
Psm5244	+
Psm5249	(+)
Psm5250	+
Psm5252	+
Psm5254	(+)
Psm5255	+
Psm5257	+
Psm5260	+
Psm5280	+
Wild cherry	
Psm5265	(+)
Psm5266	+
Psm5267	–
Psm5268	+
Psm5269	+
Psm5270	+
Psm5271	+
Psm5272	(+)
Psm5273	(+)
Psm5274	+
Psm5275	+
Psm5276	(+)
Psm5277	+
Plum	
Psm797	+
Psm2928	+
Psm5281	+
Psm5282	+
Psm5299	+
Psm5300	+
P. s. pv. *syringae*	
Lilac	
Pss281	+
Pear	
Pss5340	+
Pss5342	+
Sweet cherry	
Pss310	+
Pss2942	–
Pss5357	+
Wild cherry	
Pss5264	+
Portuguese laurel	
Pss420	(+)

– = no fluorescence
(+) = weak fluorescence
+ = fluorescence in all the dilutions

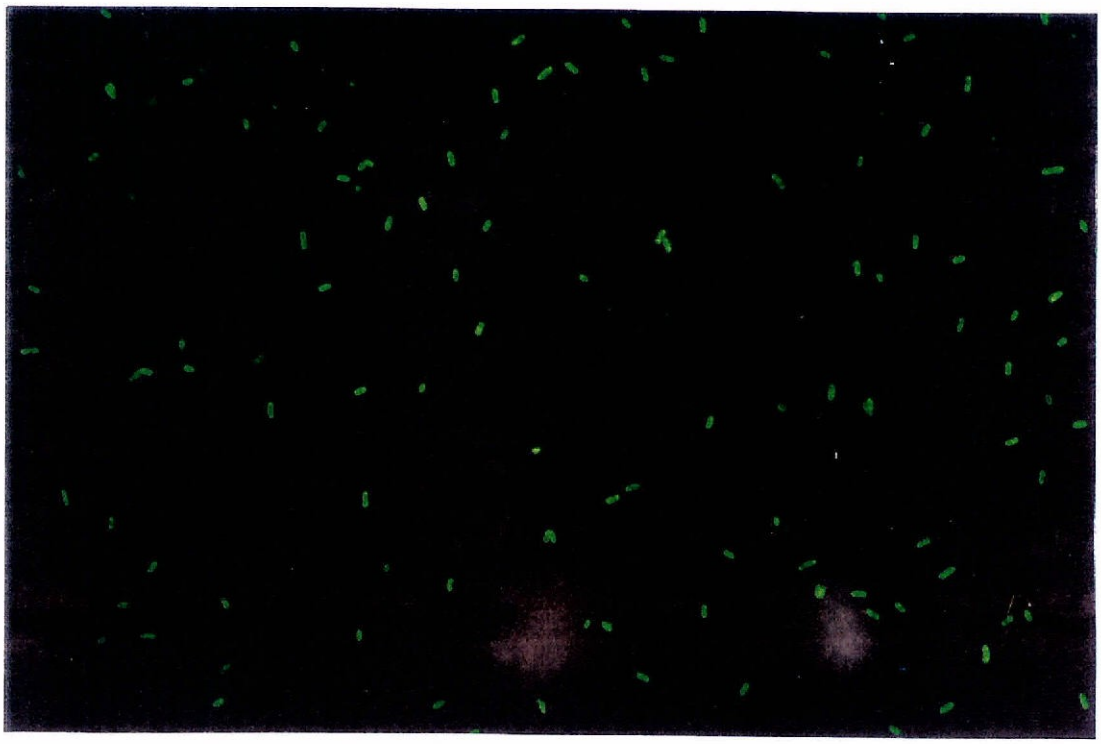

Figure 8.1
Fluorescent antibody staining of *Pseudomonas syringae* cells on a microscopic slide.

The specificity of the antiserum with this test was very low since 10 of the 27 bacteria isolates used as negative controls showed fluorescence. The negative bacteria which cross-reacted were one isolate of *P. fluorescens*, one isolate of *P. tolaasii*, three isolates of *P. viridiflava*, both isolates of *P. cichorii*, and three isolates of *E. herbicola* – *Pantoea agglomerans*. Moreover, all the *Pseudomonas syringae* pv. *syringae* isolates, except Pss2942, had a positive response while they had not in the preliminary agglutination test. However, one *P. syringae* pv. *morsprunorum* isolate from wild cherry (Psm5267) and one *P. syringae* isolate from sweet cherry (Ps5355) had a negative response.

The negative controls used in every slide always showed a negative response. There was no fluorescence with normal (pre-immune) serum or with PBS, which shows that the conjugated secondary antibody did not react directly with the antigens and that the normal serum was also not reacting with the antigens.

The low specificity of the test is probably connected with the difficulty of distinguishing clearly positive from negative results. It was very difficult in some cases to evaluate if the response was negative, with several isolates showing a dimmed or blurred fluorescence. These responses were considered positive or weak positive

because it was not possible to discard them completely as negatives. The fluorescence determination was quite subjective, even with the availability of positive controls.

Consequently, the polyclonal antibody obtained could not be considered suitable for immunofluorescence slide tests.

8.3 Conjugated *Staphylococcus aureus* agglutination

Direct agglutination, the simplest form of the serological techniques, has been in use for many years. Greater sensitivity of this method has been achieved by attachment of an inert carrier to the antibody, including latex beads, but broad variation in the adsorption conditions is frequently found and relatively high titre antiserum is required. The need for such high titre was overcome by latex sensitised with protein A derived from *Staphylococcus aureus*. An alternative is to conjugate the antiserum directly to protein A on the surface of *S. aureus* cells, thus using the bacteria as a carrier (LYONS & TAYLOR, 1990). The first report of the use of whole staphylococcal cells for serotyping bacteria was for *Streptococcus pneumoniae* (KRONVALL, 1973).

Antiserum is conjugated directly to protein A which occurs in high concentrations on the cell wall of many *S. aureus* strains (WALKEY *et al.*, 1992). Protein A of *S. aureus* is known to combine with an immunoglobulin fragment and this property was used for the immunodiagnosis of plant viruses (CHIRKOV *et al.*, 1984; WALKEY *et al.*, 1992) and plant pathogenic bacteria (LYONS & TAYLOR, 1990).

The use of antiserum conjugated to *S. aureus* eliminates many of the restrictions imposed by the normal agglutination method. The turbidity of the cell suspension, which is often critical to the accurate observation of agglutination is determined by the concentration of *S. aureus* in the conjugate which is pre-set for maximum visibility of agglutination. The concentration of antigen is also much less important than in the normal agglutination method. This test can be used in practical applications to the detection of specific bacteria in plant tissues, even where it is difficult to isolate the target organism (LYONS & TAYLOR, 1990).

Recently, several detection kits (Express Bacterial Detection Kits, Adgen Diagnostics Systems, Auchincruive, UK) based on this technique have been developed for the diagnosis of plant pathogenic bacteria.

This piece of work presents the use of whole-cell staphylococcal preparation conjugated to the polyclonal antiserum, obtained as described in section 8.2.1, to

identify the *P. syringae* isolates described in chapter 4, and to access the specificity of the antisera.

8.3.1 Material and methods

The *S. aureus* working reagent was obtained from Dr. Steve Roberts (HRI, Wellesbourne, Warwick CV35 9EF, UK). The preparation of this reagent is thoroughly described in LYONS & TAYLOR (1990).

The batch of antiserum utilised was titred for optimal dilution for conjugation and the following ranges of antiserum dilution were used: 1:8, 1:24, 1:60, and 1:120. The conjugated working reagent was then prepared *ex tempore* by mixing 1 volume of 1:1 antiserum/glycerol mixture, 24 volumes of PBS (2.9 g $Na_2HPO_4.12\ H_20$; 0.2 g KH_2PO_4; 8.0 g NaCl; 0.2 g KCl; 0.2 g NaN_3; and 1 l distilled water; pH 7.2), 5 volumes of *S. aureus* working reagent, and 0.5 volumes of filtered saturated alcoholic basic fuchsin. When the conjugate was stored, in a refrigerator at 4°C, it had to be well shaken on a Vortex stirrer to eliminate clumping and checked for any auto-agglutination before use.

To the wells of a 12-well multitest slide were applied 7 µl of the *S. aureus* conjugated working reagent and enough bacteria, from a pure culture of the test organism, to create a thin suspension. These were mixed together for several seconds by using a sterile wooden toothpick. A positive agglutination reaction was characterised by granular clamping of the stained *S. aureus* conjugated suspension (Fig. 8.2) which was formed within 30-45 sec of mixing with the test organism. A drop of test reagent alone was examined as a negative control.

All the *P. syringae* isolates listed in Tables 4.1 and 4.2 were tested, at least twice, by the *S. aureus* agglutination slide assay. In addition, the same 27 isolates of different bacterial species, presented in section 8.2.1, were tested as negative controls to test the specificity of the serum and the reliability of the test.

8.3.2 Results and discussion

Since the *S. aureus* conjugate using the 1:24 antiserum dilution gave the best agglutination and relatively quickly, this working dilution was subsequently used in the conjugated *S. aureus* agglutination test to detect the bacteria.

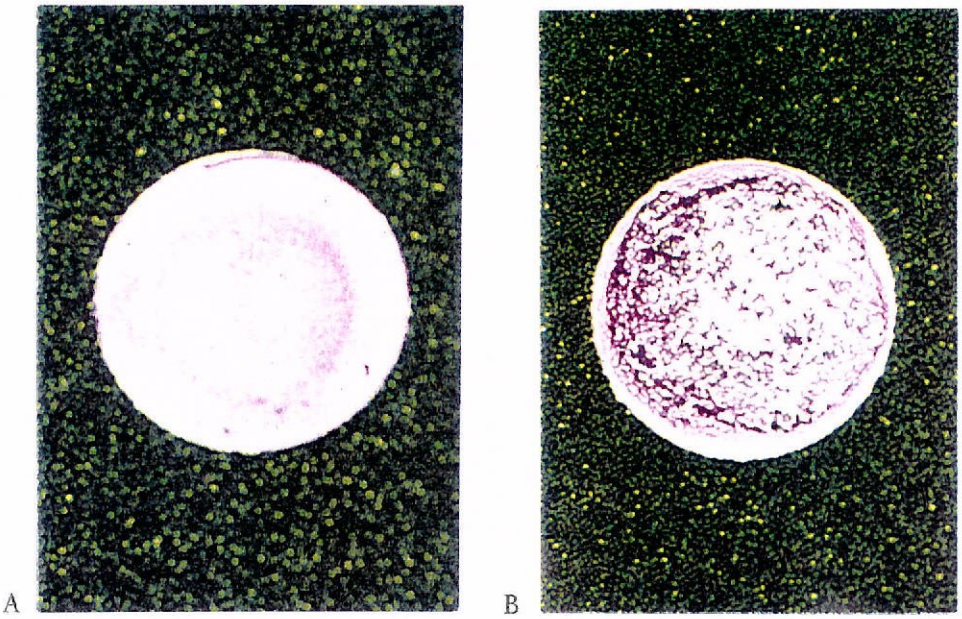

Figure 8.2
Agglutination with *Staphylococcus aureus* conjugated antiserum. A. Negative agglutination. B. Positive agglutination.

The results of the conjugated *S. aureus* slide agglutination test screening each of the isolates, listed in Tables 4.1 and 4.2, are shown in Table 8.2. The results of the bacteria used as negative controls to test the specificity of the antiserum with this method are presented in Table 8.3.

Occasionally a loose, incomplete reaction occurred between heterologous antiserum/antigen combinations. This appeared to be related to an excess of antigen, was markedly different from normal agglutination and was considered to be a negative result (LYONS & TAYLOR, 1990).

The specificity of the polyclonal antiserum was much higher when used with this test. Only 15% of the bacteria isolates used as negative controls to test the specificity of the antiserum showed agglutination: one isolate of *P. tolaasii*, two isolates of *P. viridiflava*, and one isolate of *E. herbicola – Pantoea agglomerans*.

None of the *Pseudomonas syringae* pv. *syringae* isolates, except weakly Pss5342 and Pss5264, agglutinated the working reagent. The *P. syringae* isolates from sweet cherry were also all negative. On the other hand, almost all the *P. s.* pv. *morsprunorum* isolates from sweet cherry were positive, except Psm798, Psm5241, and Psm5252.

Table 8.2

Agglutination reaction for *P. syringae* isolates by conjugated *Staphylococcus aureus* slide agglutination test.

Isolate	Agglutination reaction
P. syringae	
Sweet cherry	
Ps214	–
Ps5355	–
Ps5356	–
Wild cherry	
1AH(a)	+
1AH(b)	–
1AH(d)	–
1AH(e)	–
3AH(a)	+
3AH(c)	–
11L(b)	(+)
11L(e1)	(+)
11L(e2)	(+)
11L(f2a)	–
11L(f2b)	–
11L(f3)	–
12AF	–
13EF(a)	+
13EF(b)	+
13EF(c)	(+)
13EF(d)	+
23SW	–
M1	+
NA5	(+)
R4	(+)
R8	(+)
Cherry laurel	
Ps73	(+)
P. s. pv. *morsprunorum*	
Sweet cherry	
Psm617	+
Psm798	–
Psm881	(+)
Psm889	+
Psm1462	+
Psm2206	+
Psm5238	(+)
Psm5239	+
Psm5240	(+)
Psm5241	–
Psm5243	+
Psm5244	+
Psm5249	(+)
Psm5250	+
Psm5252	–
Psm5254	+
Psm5255	+
Psm5257	+
Psm5260	+
Psm5280	+
Wild cherry	
Psm680	+
Psm5265	–
Psm5266	+
Psm5267	–
Psm5268	–
Psm5269	+
Psm5270	+
Psm5271	(+)
Psm5272	–
Psm5273	(+)
Psm5274	(+)
Psm5275	–
Psm5276	+
Psm5277	+
Plum	
Psm797	+
Psm2928	+
Psm5281	–
Psm5282	(+)
Psm5299	(+)
Psm5300	(+)
P. s. pv. *syringae*	
Lilac	
Pss281	–
Pear	
Pss5340	–
Pss5342	(+)
Sweet cherry	
Pss310	–
Pss2942	–
Pss5357	–
Wild cherry	
Pss5264	(+)
Portuguese laurel	
Pss420	–

– = no agglutination
(+) = weak agglutination
+ = strong agglutination

The *P. syringae* pv. *morsprunorum* isolates from wild cherry showed an intermediate behaviour, six isolates being clearly positive, five negative, and three showing a weak agglutination. The same happened with the *P. syringae* cultures isolated from wild cherry during the present work. This intermediate reaction of the wild cherry isolates again confirms their intermediate position between *P. syringae* pvs. *morsprunorum* and *syringae*.

Because of the *P. syringae* colonies mucoid consistency and the procedure used, it was difficult with some isolates to mix and incorporate the antigen with the conjugated working reagent. The use of only 7µl of working reagent also caused difficulties in mixing the reagent with so much antigen.

Table 8.3

Agglutination reaction for 27 negative control bacteria to test the specificity of the antiserum in conjugated *Staphylococcus aureus* slide agglutination test.

Bacteria (no. of isolates)	No. of negative isolates
Pseudomonas fluorescens (6)	6[a]
Pseudomonas tolaasii (3)	2
Pseudomonas viridiflava (4)	2[a]
Pseudomonas cichorii (2)	2[b]
Pseudomonas corrugata (1)	1
Erwinia carotovora subsp. *carotovora* (2)	2
Erwinia carotovora subsp. *atroseptica* (2)	2
Xanthomonas campestris (1)	1
Erwinia herbicola – Pantoea agglomerans (6)	5[c]

[a] one isolate showed weak agglutination.
[b] both isolates showed weak agglutination.
[c] two isolates showed weak agglutination.

It may be possible to achieve more specific polyclonal antisera for use in the this test by immunisation with antigen prepared from a somatic extract of the whole bacterial cell. Antiserum produced by this method from different *P. syringae* pathovars did not cross-react with other pathovars as did the antisera prepared from whole-cell antigen (LYONS & TAYLOR, 1990). Antiserum obtained by this method could help in clarifying the taxonomic position of the wild cherry isolates.

Agglutination with conjugated antiserum would appear to have many advantages over other serological methods but it is less sensitive than enzyme-linked immunosorbent assay (ELISA) and immunofluorescence. It was possibly this lack of sensitivity compared with the immunofluorescence that permitted the *S. aureus* agglutination technique to differentiate between *P. syringae* pvs. *morsprunorum* and *syringae* isolates. The sensitivity of the *S. aureus* conjugated agglutination test with plant viruses (VBA- virobacterial agglutination test) is also considerably lower than that of the ELISA test (CLARK & ADAMS, 1977) but it can be increased with high-titred antisera if desired.

8.4 Conclusions

The polyclonal antiserum obtained from whole-cell of four isolates *P. syringae* pv. *morsprunorum* from sweet cherry, although it showed a good specificity in a preliminary slide agglutination test was not specific when using the immunofluorescence slide test. However, the specificity was much increased and considered suitable for presumptive identification, when the conjugated *S. aureus* slide agglutination test was used.

There are several advantages with the *S. aureus* agglutination test, which have been explored for use in rapid commercial diagnostic kits. It is very rapid and in less than one minute it is normally possible to obtain a quite clear response; very simple to perform and no special equipment is required; the costs per test are quite low and it is economical in the use of test reagents; and it is possible to use in direct testing of diseased plants which allows the test to be performed in the field.

Although not as sensitive as the direct ELISA test, the simplicity and rapidity of the conjugated *S. aureus* agglutination test should make it a viable alternative for some purposes. From the data presented we suggest that the conjugated *S. aureus* agglutination slide test can be suitable for mass immunodiagnosis of plant pathogenic bacteria.

Cross-reactions were seen within the species and pathovars of the pseudomonads. Spurious, weak cross-reactions also occurred with other species, but the simplicity of the technique makes it extremely useful and the search for antisera with higher specificity very desirable.

Nevertheless, the specificity achieved with this test with the antiserum obtained in this work was considered adequate for a putative identification of *P. syringae* pv.

morsprunorum, and it was used in chapter 9 to identify the bacteria in an epidemiology study.

CHAPTER 9
SPATIAL AND TEMPORAL DISTRIBUTION

9.1 Introduction

The first report demonstrating that phytopathogenic bacteria are a component of leaf surface microbial communities was made by CROSSE (1959). His findings that *Pseudomonas syringae* pv. *morsprunorum* could be readily isolated in large numbers from the surface of healthy cherry leaves provided new insight into the epidemiology of foliar bacterial diseases (HIRANO & UPPER, 1983). He also pointed out the existence of a permanent epiphytic microflora on the leaves. LEBEN (1965) formed a new concept that phytopathogenic bacteria are capable of a resident epiphytic phase of growth on the healthy host. According to this hypothesis, the pathogen may multiply on the surfaces of the healthy host, having a resident as well as a pathogenic phase of growth.

CROSSE (1959 and 1963) suggested that the leaf surface populations of *P. s.* pv. *morsprunorum* are the main source of inoculum for infection of cherry leaf scars in autumn, and thus provide inoculum in the absence of disease. This was later confirmed by FREIGOUN (1974) and formed a milestone in the study of the epidemiology of foliar diseases caused by bacterial pathogens, particularly *P. syringae* (HIRANO & UPPER, 1990 and 1994). Direct relationships between epiphytic population size and disease were not established for *P. s.* pv. *morsprunorum* in relation to leaf spot on cherry, although the numbers of bacteria recovered from healthy leaves in autumn were related to the incidence of canker infections measured the following May; and *P. s.* pv. *syringae* numbers were related to leaf spot on cherry (HIRANO & UPPER, 1983).

Each epiphytic phytopathogenic bacterium in a given leaf ecosystem has a finite but extremely small probability of causing disease. From ROUSE *et al.* (1985) this

probability, in the *P. s.* pv. *syringae*-brown spot of snap bean system, can be inferred to be about 3×10^{-6}. This being a very small number would support the suggestion that *P. s.* pv. *syringae* is a very weak pathogen (HIRANO & UPPER, 1990). The probability that disease will occur on a given leaf depends on the number of pathogenic bacteria on that leaf. The disease hazard to all the leaves is the sum, over all leaves, of the number of pathogenic bacteria on each leaf times the probability of disease given that population size. The amount of disease, then, is directly related to pathogen population sizes on individual leaves within a canopy (HIRANO & UPPER, 1983 and 1990).

P. syringae may be viewed as a group of organisms that is primarily adapted to reside on leaf surfaces and is widely distributed as an epiphyte on many plants. Phytopathogenic bacteria frequently reside epiphytically on nonhost plant species, that is, on plants that are not known to be susceptible to diseases incited by these pathogens. *P. s.* pv. *syringae* and, less frequently, *P. s.* pv. *morsprunorum* were isolated from grasses and broad-leaf herbaceous plants from under sour cherry orchards (LATORRE & JONES, 1979b). BACA & MOORE (1987) even suggest that the rapid increase of *P. syringae* populations, after a near absence from the woody tissues during winter and in the hot days of summer, may be due to transfer from the adjacent grasses to the trees.

P. syringae epidemics are thought to occur when inoculum is dispersed by rain-splash during wind-driven rain. Driving rain also facilitates the entrance of bacteria into leaves, petioles, and other organs bearing stomata (CROSSE, 1956). For HIRANO & UPPER (1983), rain-splash can only account for short distance travel – leaf to leaf of the same plant or nearby plants. Rain-generated aerosols may have greater potential for transporting bacteria to modest distances, but even aerosols tend to be scrubbed from the air during rain.

Leaf inhabitants are exposed to extreme fluctuations in temperature, to ultraviolet and visible radiation, and to periods of desiccation. Seasonal variation and rainfall causes cyclical patterns of multiplication and survival of epiphytic bacteria. In the spring, developing and mature flowers are rapidly colonised, after which resident bacterial populations decline to low or undetectable levels during the warm, dry summer. In autumn, numbers of bacteria increase gradually until leaf abscission. During winter dormancy of the fruit tree, bacterial numbers decline slowly until new vegetative growth begins in the spring. Thus, an overall cyclical pattern can be observed (KNUDSEN, 1991). CAMERON (1970) and FREIGOUN (1974) reported a cyclical pattern of sweet cherry tree colonisation by *P. s.* pv. *morsprunorum*.

Environmental factors severely restrict the growth of microorganisms on leaf surfaces. Physical factors, of which solar radiation, moisture, and temperature are most important, vary on a large scale with climate, on a smaller scale within the plant canopy, and on a microscopic scale over the leaf surface. Water is frequently limiting on plant surfaces under temperate conditions, and growth of epiphytic microbes may only occur following rain, periods of dew, or at least high humidity. Water affects survival, multiplication, and dispersal of plant surface bacteria, and free water is probably necessary (KNUDSEN, 1991).

Epiphytic populations of pathogenic *P. syringae* may provide a source of inoculum and larger *P. syringae* populations are found during rainy compared with dry weather. HIRANO & UPPER (1983) adopted a rather loose functional definition of epiphytic bacteria as those that can be removed from above ground plant parts by washing. This definition does not distinguish between residents and casuals [in the sense of LEBEN (1965): residents if they multiply on plant surfaces and casuals if they are present accidentally or growing only on foreign debris on plant surface], nor does it count all the bacteria. However, it provides a relatively rapid, quantitative result that can be used as an estimate of total population size.

As suggested by HIRANO & UPPER (1983), if only a rough estimate of the mean population size of the microbial community of interest is required, then, bulked samples would be more time-efficient than would dilution plating from individual leaves. For some purposes, estimates of epiphytic bacterial populations from bulked samples are adequate. Rates of changes in epiphytic population size estimated from data points taken even a few days apart represent trends, or long-term rates. The few generalisations that can be made on the population dynamics of epiphytic bacteria are based, at best, on relatively long-term trends (HIRANO & UPPER, 1983).

Spread of bacterial canker between nurseries and movement of the bacterium from nurseries to mature plantings on contaminated replant trees are two epidemiological aspects for considerable concern. No studies have been conducted on the development of disease or spread of the pathogen in cherry plantations or even in nurseries. The spatial and temporal dynamics of bacterial canker originating from a known source of inoculum within a uniform planting of a single species have never been investigated. This kind of research has been carried out on citrus canker [*Xanthomonas axonopodis* pv. *citri* (Hasse) Vauterin *et al.*] by GOTTWALD *et al.* (1988a, 1988b, 1989 and 1992).

The purpose of this study was to investigate the dynamics of the *P. s.* pv. *morsprunorum* epiphytic populations on the phylloplane and to quantify the spatial and temporal progress of *P. s.* pv. *morsprunorum* in simulated new cherry plantations with a known, focal source of inoculum.

9.2 Materials and methods

Field trials

Three field plots were located at the Shinfield Unit of the School of Plant Sciences and one at the Sonning Farm, The University of Reading. Two of the plots at Shinfield (S-A and S-B) and the plot at Sonning (SON) were formed by 49 trees of sweet cherry cultivar Stella, grafted on Colt rootstock. S-A was established on 27 May 1994, S-B on 31 March 1995, and SON on 6 April 1995, by planting maiden trees in a 7 × 7 square with 2 m between trees and between rows. Another plot of the same trees was planted on 14 June 1994, at Shinfield (S-C), in a 5 × 5 square with the same spacing. This last plot served as a noninoculated control. S-A and S-B were separated from S-C by a tall barrier of oak and elm trees, and all of them were protected with a 2.5m high wire fence to avoid deer damage. The trees were irrigated manually with a hosepipe one to two days after the plantation, once in the midsummer of 1994, and twice in midsummer of 1995.

Inoculation of central focal trees

In 1994, the inoculum was prepared by flooding 24 hours old colonies on NSA plates, containing the *P. s.* pv. *morsprunorum* cultures Psm617, Psm889, and Psm1462 (Table 4.1), with sterile distilled water. The bacteria were brought into suspension with a bent glass rod and were all collected in a common bottle. The bottle was shaken to suspend the cells evenly and the suspension was adjusted with a spectrophotometer to a concentration of 10^8 cfu.ml^{-1} (A_{600}=0.17). In 1995, the procedure was the same but it was only used the culture Psm1462.

The bacterial suspension was applied onto adaxial and abaxial leaf surfaces with a spray gun at a constant pressure of 250 KPa until run off. The nozzle was held about 4 cm from the leaf surface so that the inoculum was forced into the intercellular spaces of the leaf through the stomata. The inoculations were made once in 1994, 8 July, and twice in 1995, 12 July and 11 October. The inoculations were made on cloudy misty

days. In 1994, the inoculated branches were covered with a plastic bag during three days to retain foliar moisture.

Sampling design and procedure

Epiphytic bacteria, were quantified on the inoculated tree, on the 8 trees surrounding the central focal tree and on each tree of the block diagonals. Ten leaves were taken from all parts of the designated trees, from 17 trees on the inoculated plots (Fig. 9.1) and from 13 trees on the control plot (Fig. 9.2). The assessment dates are presented on Table 9.1. Additional samplings were made on the focal tree, five and seven days after the inoculation in 1994 and in 1995, respectively, to confirm the inoculation effectiveness.

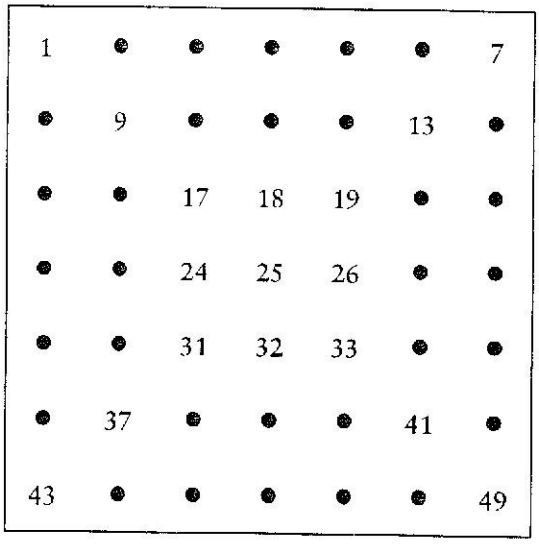

Figure 9.1
Diagram of the inoculated plots illustrating the number and position of the trees sampled.

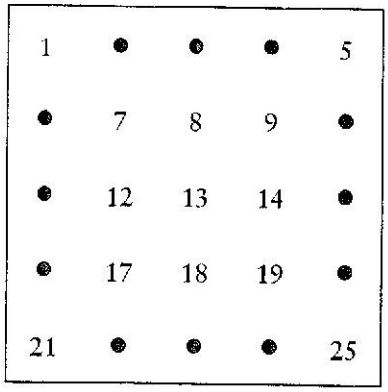

Figure 9.2
Diagram of the control plot illustrating the number and position of the trees sampled.

On each assessment day, the leaves were brought to the laboratory on ice and 10 disks of 1 cm diameter were excised, with a no.6 cork borer, from each leaf sampled. The disks pooled by tree (100 disks) were placed in a 100 ml Erlenmeyer flask containing 40 ml of sterile distilled water and shaken at 140 rpm for two hours on an orbital shaker. Both the wash water and serial dilutions were plated, following the procedures in section 3.2, on NSA amended with 50 ppm of actidione and 2 ppm of crystal violet (see section 3.1). All the translucent levan type colonies were counted and the readings adjusted to cfu.cm^{-2} of leaf surface area.

Table 9.1

Assessment dates of *P. syringae* population in the four experimental plots during 1994, 1995 and 1996. All the assessments were done on leaves except the ones done in April.

	Date of assessment						
Plot	1994		1995				1996
S-A	7 July	3 October	18 April	3 July	18 September	13 November	11 April
S-B	—	—	—	7 July	—	13 November	13 April
SON	—	—	—	7 July	21 September	8 November	10 April
S-C	7 July	3 October	19 April	5 July	18 September	14 November	9 April

In early spring, 20 dormant buds per tree, were crushed coarsely, with a mortar and a pestle, to obtain interior as well as exterior bacterial colonisers. The samples were soaked and shaken using thereafter the same method as in the leaf samples. The bud samples were taken in April 1995 and April 1996 on the dates shown in Table 9.1.

Identification of the bacteria

After the use of the semi-selective media for the detection of levan type colonies, two more assays were used for the characterisation of the representative colonies of *P. s.* pvs. *morsprunorum* and *syringae*: conjugated *Staphylococcus aureus* slide immunoagglutination (method described in section 8.3.1) for the putative bacterial identification, and whole colony REP-PCR (method described in section 6.2) to determine the relationship among isolates.

For the whole colony REP-PCR, one purified culture of the representative colonies from each tree and from each assessment date was used.

Meteorological data

Weather parameters were recorded throughout the growing season. The parameters of the physical environment that were recorded included daily amount of rain; maximum and minimum air temperature; and wind speed and direction at 2.0 m high. The 1994 data were available from the Meteorological Office located at the Whiteknights campus (The University of Reading), distanced 2.9 km from the Shinfield site. The 1995 data were available from the respective sites (Shinfield and Sonning) from automated weather stations (Campbell Scientific).

9.3 Results

P. syringae pvs. *morsprunorum* or *syringae* were not found before the inoculation of the focal tree, on the new simulated plantations. Neither on the 7 July 1994, in S-A and S-C, nor on the 7 July 1995, in S-B and SON, were epiphytic *P. syringae* detected on the leaf surfaces sampled. Therefore, it was assumed that the leaves of the maiden trees were free of *P. syringae* or had such minute numbers that they could not be detected by the technique and sampling procedure used.

On 3 October 1994, the number of bacteria had greatly increased to almost 10^5 cfu.cm^{-2} of leaf area, on the focal inoculated tree, but they were detected at much smaller numbers on all the other trees (Fig. 9.3). With the exception of tree number 17, with 1.6×10^3 cfu.cm^{-2}, and 33, with 8.5×10^2 cfu.cm^{-2}, the levels on the other trees were quite low or impossible to quantify. The presence of many *Erwinia*-like organisms affected the detection and quantification of small numbers of *P. syringae* colonies on some plates.

No epiphytic populations of *P. syringae* were detected on the noninoculated (S-C) plot on 3 October 1994. The spread of bacteria predominantly in the westerly direction from the focus was probably not caused by the direction of the prevailing winds. Analysing the average windrun on wet days (rainfall >1 mm) after the inoculation (Fig. 9.4), we can notice that the predominant winds when the rainfall was higher than 1mm were from the directions south, south-west, and west.

In the following year (1995), on the first leaf assessment date (3 July) there were no bacteria present in the control plot, but there were already a few resident *P. syringae* on the south and east quadrants of the S-A plot (Fig. 9.5). However, no bacteria could be detected on the focal tree and no cankers or leaf spots were present in any of the assessed trees. These findings suggest that after one year *P. syringae*

populations were resident in the cherry trees, and possibly they were different strains from the one inoculated. Nevertheless, the control plot after one year remained free of *P. syringae*.

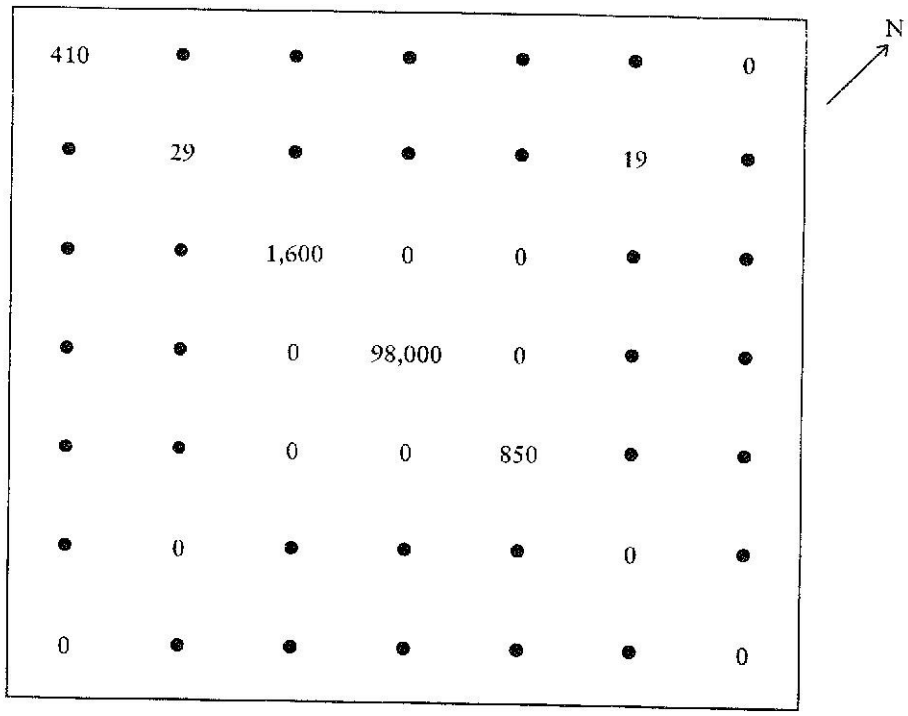

Figure 9.3

Diagram of S-A plot illustrating the leaf surface population of *P. syringae* on 3 October 1994. The numbers are expressed in cfu.cm^{-2}.

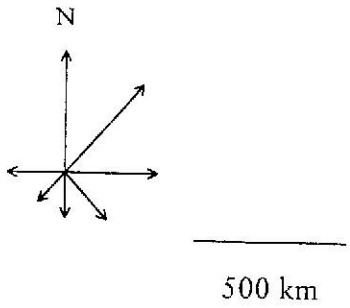

Fig. 9.4

Windrun direction, at Shinfield Unit, on days with precipitation higher than 1 mm, for the period between 8 July and 3 October 1994.

The number of bacteria decreased rapidly on the focal trees after the inoculation. In 1994, five days after the inoculation, there were only 24 cfu.cm^{-2}, and in 1995, seven days after the inoculation, they were only detected at SON at a level of 1.3 ×

10^3 cfu.cm^{-2}. After an extraordinarily hot and dry summer in 1995 (with a total amount of rainfall at Shinfield Unit, from 12 July to 17 September, of 73 mm), the numbers of bacteria at S-A on 18 September (Fig. 9.6), and at SON on 21 September (Fig. 9.7), were very small. In the S-B plot it was impossible to make the assessment at the end of summer because of very severe defoliation. Several trees did not survive the hot and dry summer even with three copious irrigations. At S-A it was not possible to detect *P. syringae* even on the inoculated tree and only 5 cfu.cm^{-2} of leaf were detected on trees 1 and 17. At SON, although numbers were very small it was possible to detect bacteria in several trees with maxima of 74 cfu.cm^{-2} on tree 33, and 43 cfu.cm^{-2} on the focal inoculated tree.

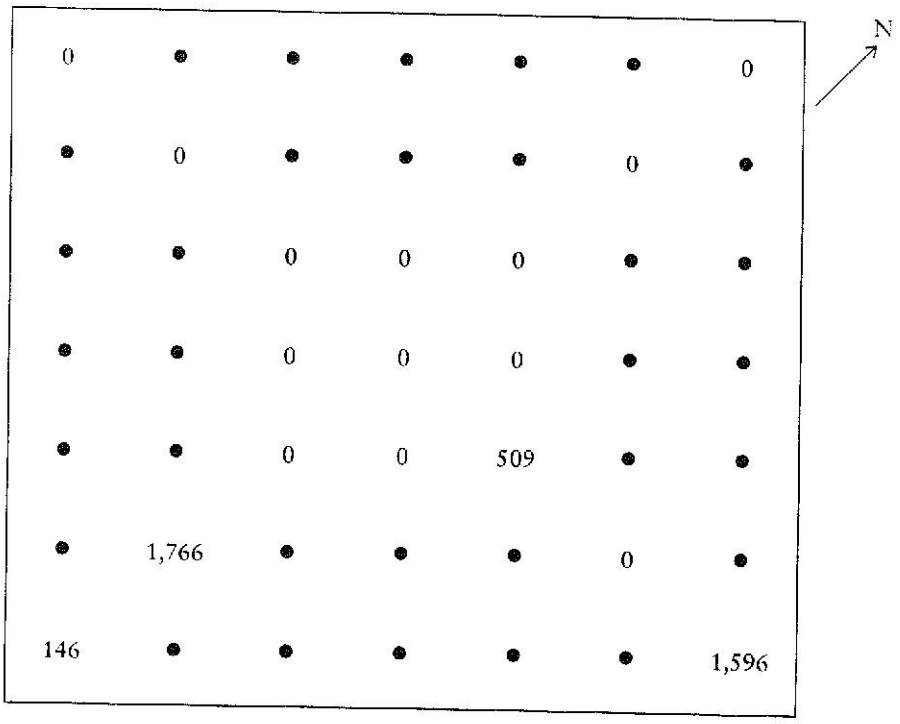

Figure 9.5

Diagram of S-A plot illustrating the leaf surface population of *P. syringae* on 3 July 1995. The numbers are expressed in cfu.cm^{-2}.

One month after the second inoculation made in 11 October 1995, the numbers of bacteria exploded (Figs. 9.8, 9.9, and 9.11). The inner core of trees in S-A and SON harboured many more bacteria than the trees on the edges. The windrun directions, in 1995, for Shinfield Unit can be seen on Figure 9.10, and for Sonning Farm on Figure 9.12.

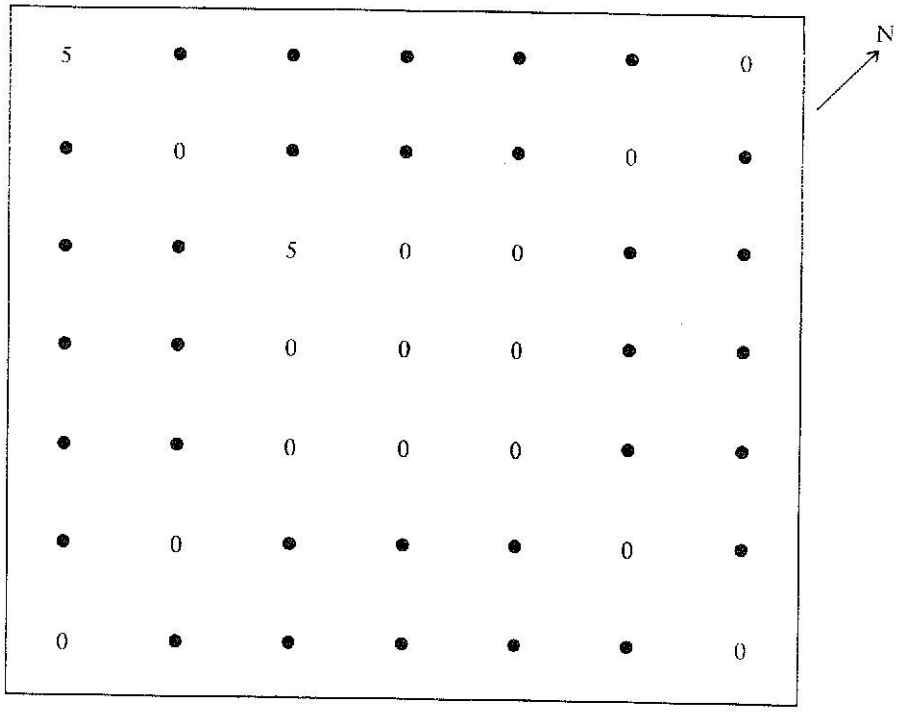

Figure 9.6

Diagram of S-A plot illustrating the leaf surface population of *P. syringae* on 18 September 1995. The numbers are expressed in cfu.cm^{-2} .

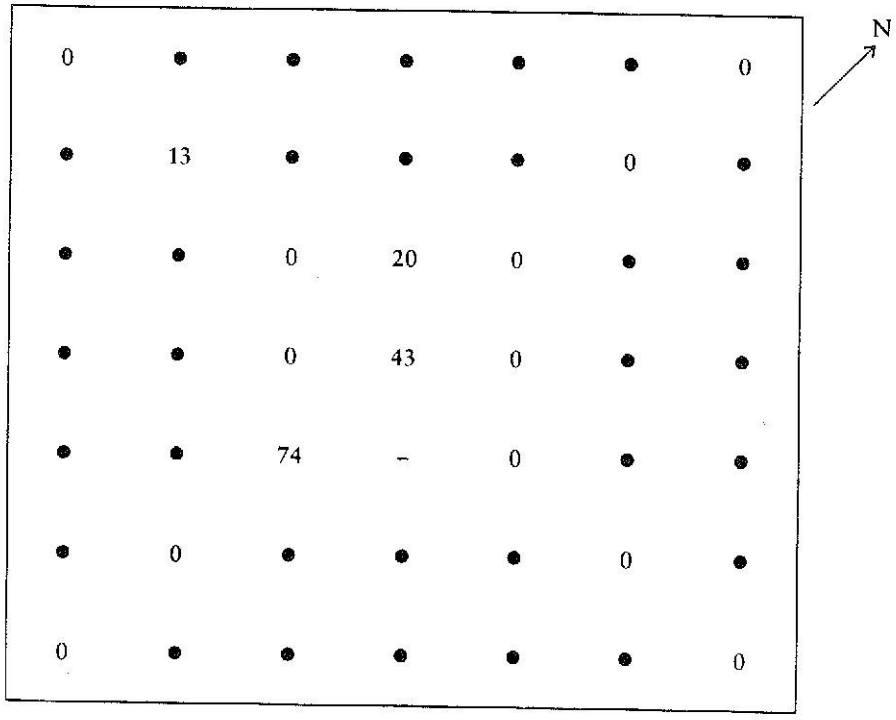

Figure 9.7

Diagram of SON plot illustrating the leaf surface population of *P. syringae* on 21 September 1995. The numbers are expressed in cfu.cm^{-2} .

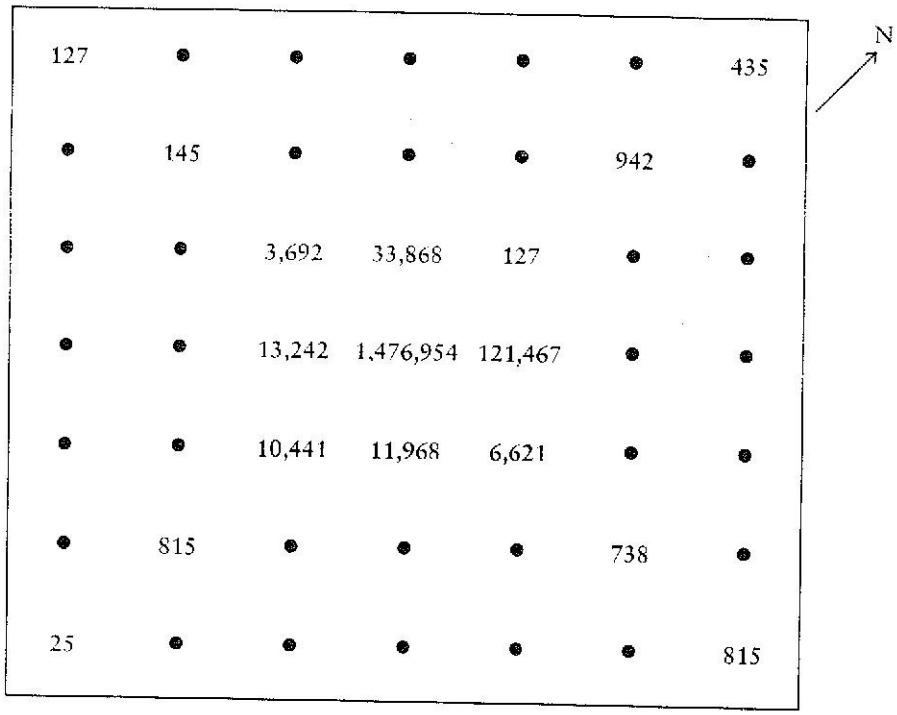

Figure 9.8

Diagram of S-A plot illustrating the leaf surface population of *P. syringae* on 13 November 1995. The numbers are expressed in cfu.cm^{-2}.

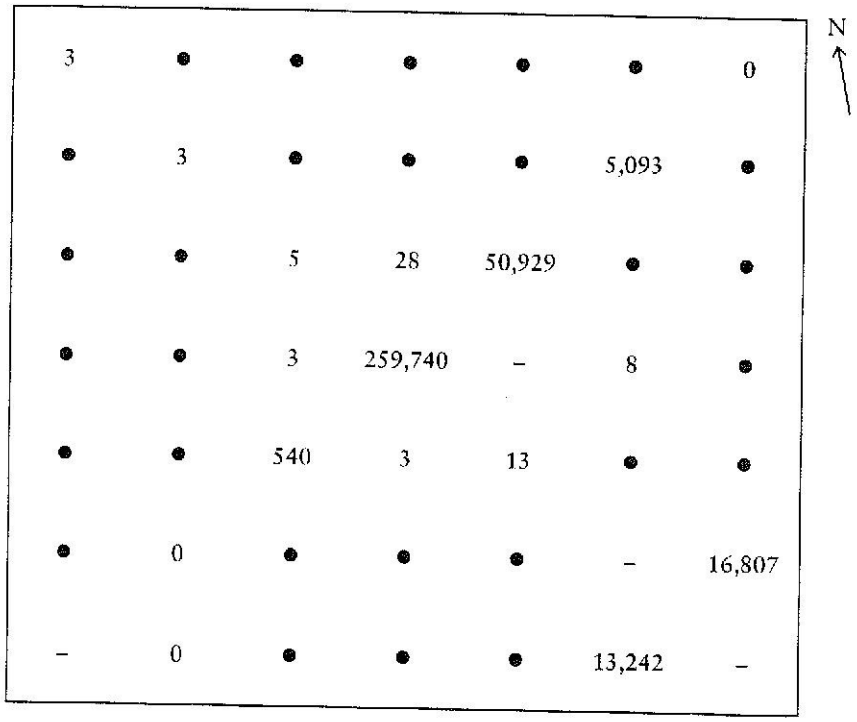

Figure 9.9

Diagram of S-B plot illustrating the leaf surface population of *P. syringae* on 13 November 1995. The numbers are expressed in cfu.cm^{-2}.

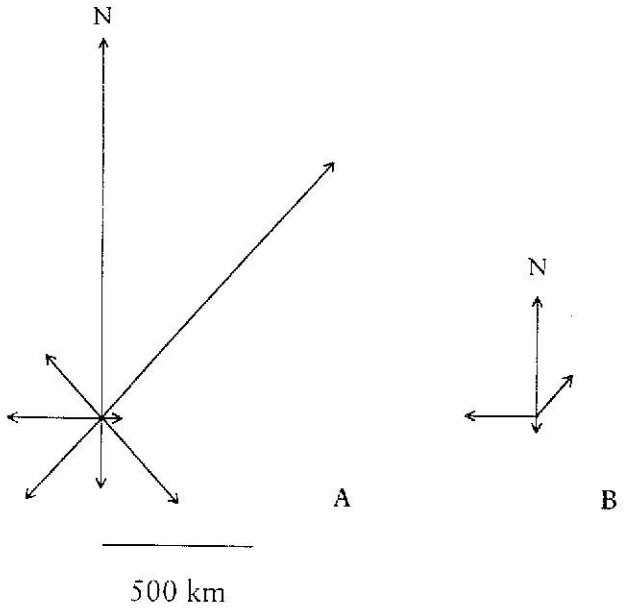

Fig. 9.10
Windrun direction, at Shinfield Unit, on days with precipitation higher than 1 mm, for the period between 12 July and 13 November 1995 (A) and between 11 October and 13 November 1995 (B).

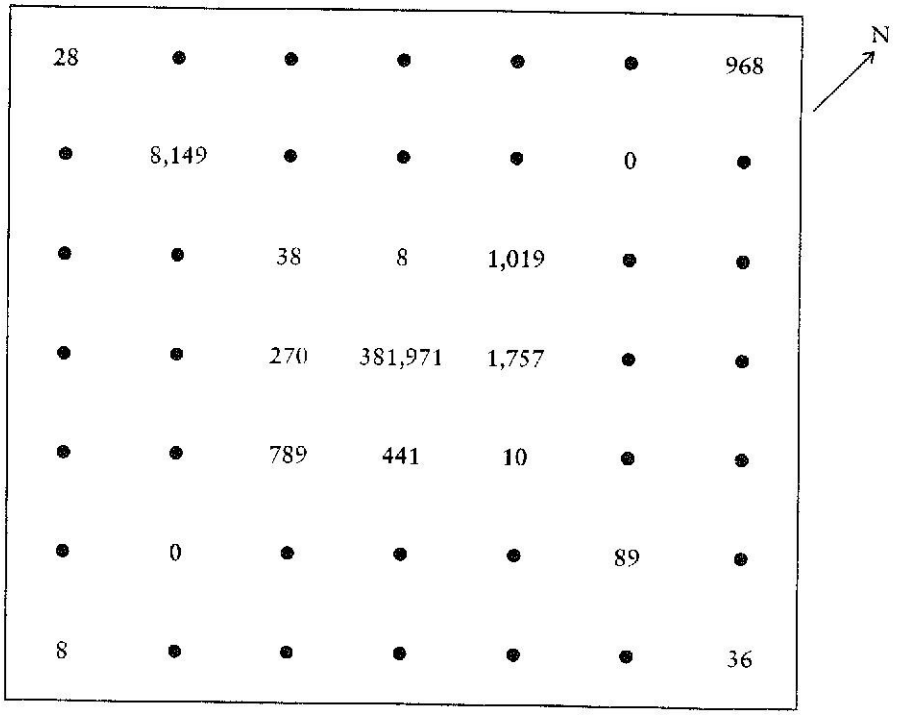

Figure 9.11
Diagram of SON plot illustrating the leaf surface population of *P. syringae* on 8 November 1995. The numbers are expressed in cfu.cm^{-2}.

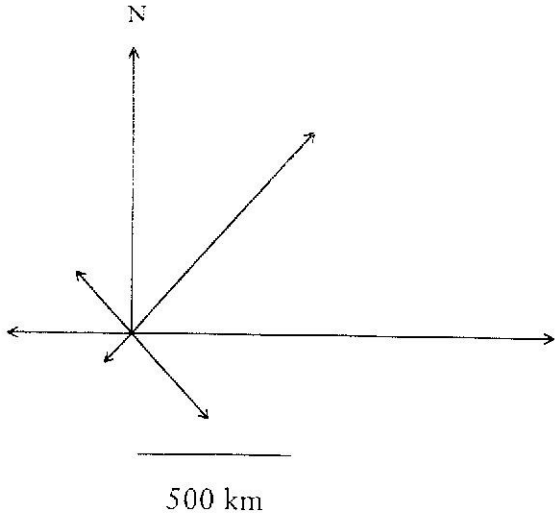

Fig. 9.12
Windrun direction, at Sonning Farm, on days with precipitation higher than 1 mm, for the period between 12 July and 8 November 1995.

The prevailing winds during the wet days, at Shinfield Unit, had south and south-west directions, and, at Sonning Farm, west, south, and south-west directions.

The control plot (S-C) showed the first bacteria in September 1995, more than one year after planting (Fig. 9.13). *P. syringae* was detected in relatively small numbers on the two trees located at the south (20 cfu.cm^{-2}) and east (94 cfu.cm^{-2}) extremes of the plot. In November 1995 (Fig.9.14), *P. syringae* could also be detected in the central tree of the plot and the numbers of bacteria were then very large at the south and east boundaries of the plot.

The maximum and minimum temperatures, and precipitation recorded at Whiteknights Campus, in 1994, and Shinfield Unit, in 1995, during the period between the inoculation and the last assessment, can be observed in Figures 9.15 and 9.16, respectively. The temperature and precipitation data for Sonning, in 1995, are not presented because they were very similar to the ones of Shinfield Unit.

The bud analysis, in April 1995, showed the presence of *P. syringae* in S-A while the control plot (S-C) was still free from the bacteria. Even on S-A, the bacteria were only present on three trees: number 7 (see Fig. 9.1) with 49,000 cfu.bud^{-1}, number 33 with 13,000 cfu.bud^{-1}, and number 37 with 10 cfu.bud^{-1}.

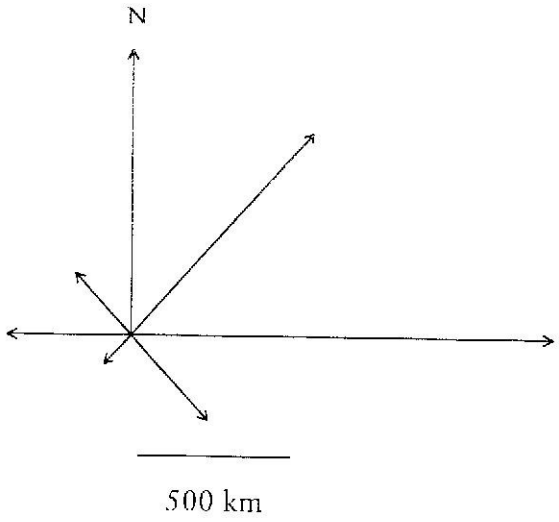

Fig. 9.12
Windrun direction, at Sonning Farm, on days with precipitation higher than 1 mm, for the period between 12 July and 8 November 1995.

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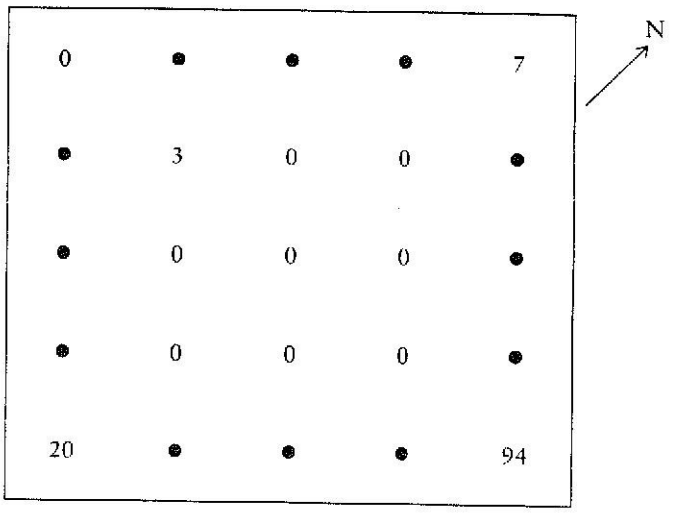

Figure 9.13

Diagram of S-C plot (control plot) illustrating the leaf surface population of *P. syringae* on 18 September 1995. The numbers are expressed in cfu.cm^{-2}.

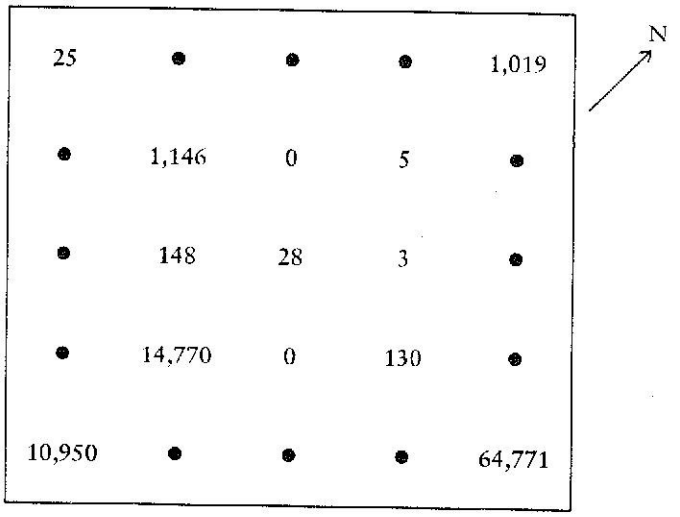

Figure 9.14

Diagram of S-C plot (control plot) illustrating the leaf surface population of *P. syringae* on 14 November 1995. The numbers are expressed in cfu.cm^{-2}.

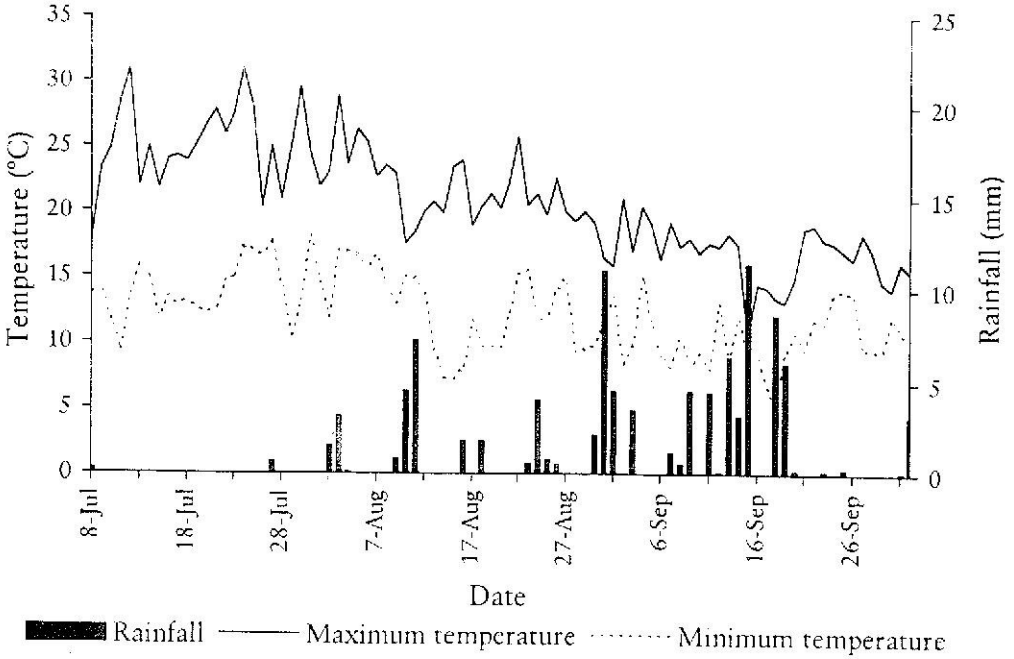

Figure 9.15

Maximum and minimum temperatures, and precipitation at Whiteknights Campus (The University of Reading) during the period between 8 July and 3 October 1994.

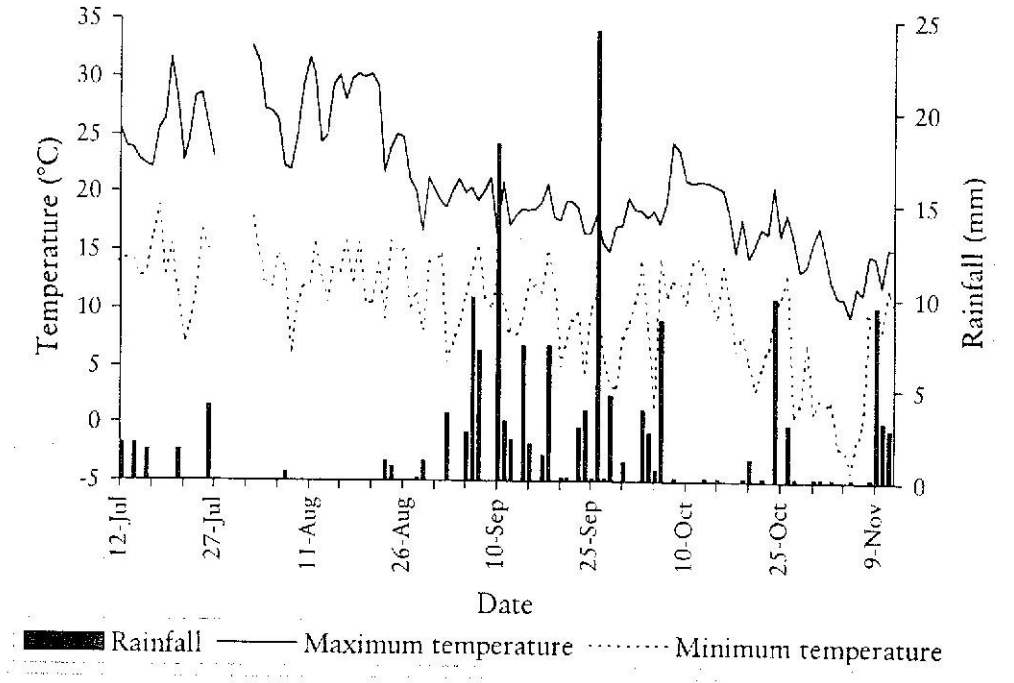

Figure 9.16

Maximum and minimum temperatures, and precipitation at Shinfield Unit during the period between 12 July and 12 November 1995.

In April 1996, *P. syringae* was found in the buds of all the plots, including the control plot which had 2,200 cfu.bud^{-1} on tree number 21 (see Fig. 9.2). In the other plots the numbers of *P. syringae* were much larger and are presented on Figs. 9.17 to 9.19. The S-A plot had bacteria in the buds on 71% of the trees sampled, while S-B had bacteria on 41%, and SON only on 35% of the trees. The number of bacteria was not always larger on the inoculated tree, as can be seen on S-A and S-B. The high population on tree number 43 in S-B possibly indicates an exogenous source of bacteria, although no apparent cankers were detected.

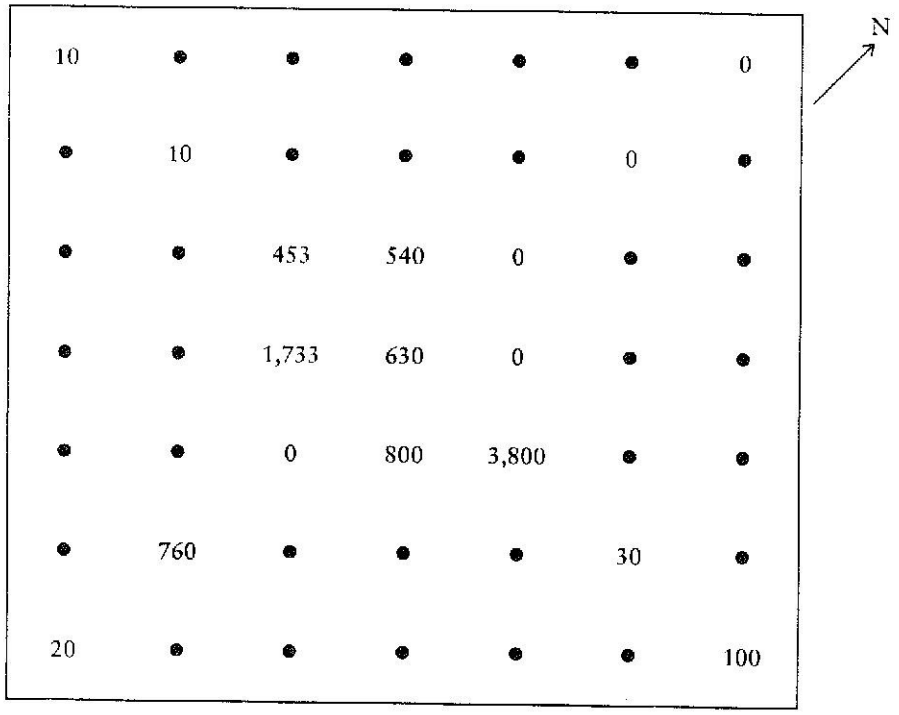

Figure 9.17
Diagram of S-A plot illustrating the population of *P. syringae* present in buds, on 11 April 1996. The numbers are expressed in cfu.bud^{-1}.

The REP-PCR patterns showed that none of the bacteria analysed had a similar pattern to the bacteria inoculated, except the colonies isolated from the leaves, on 8 November 1995, and from the buds, on 10 April 1996, from the inoculated tree at SON (Fig. 9.20). In no other case, including the inoculated trees, were the patterns the same as bacteria sprayed onto the leaves.

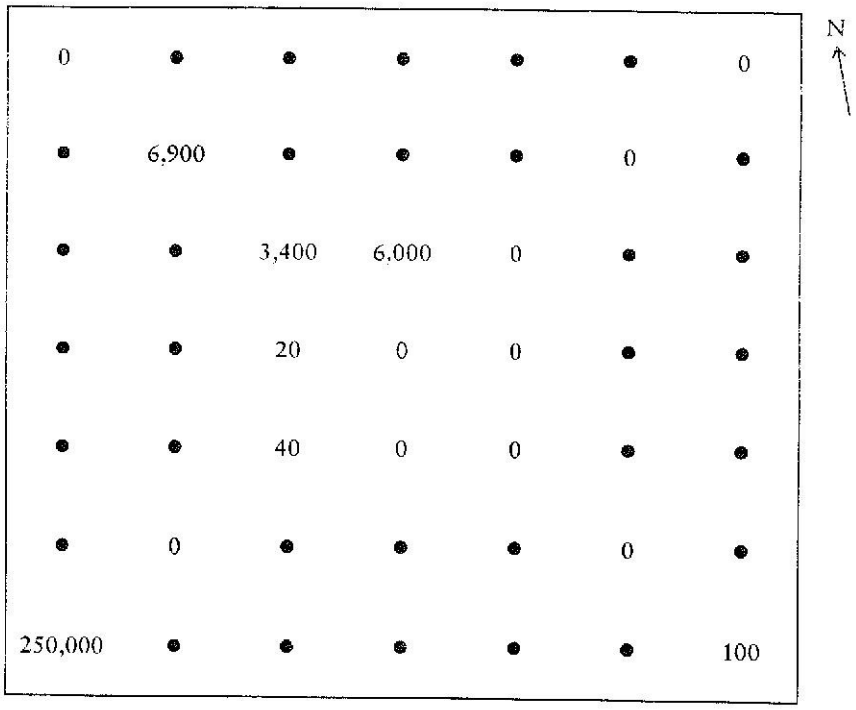

Figure 9.18

Diagram of S-B plot illustrating the population of *P. syringae* present in buds on 13 April 1996. The numbers are expressed in cfu.bud^{-1}.

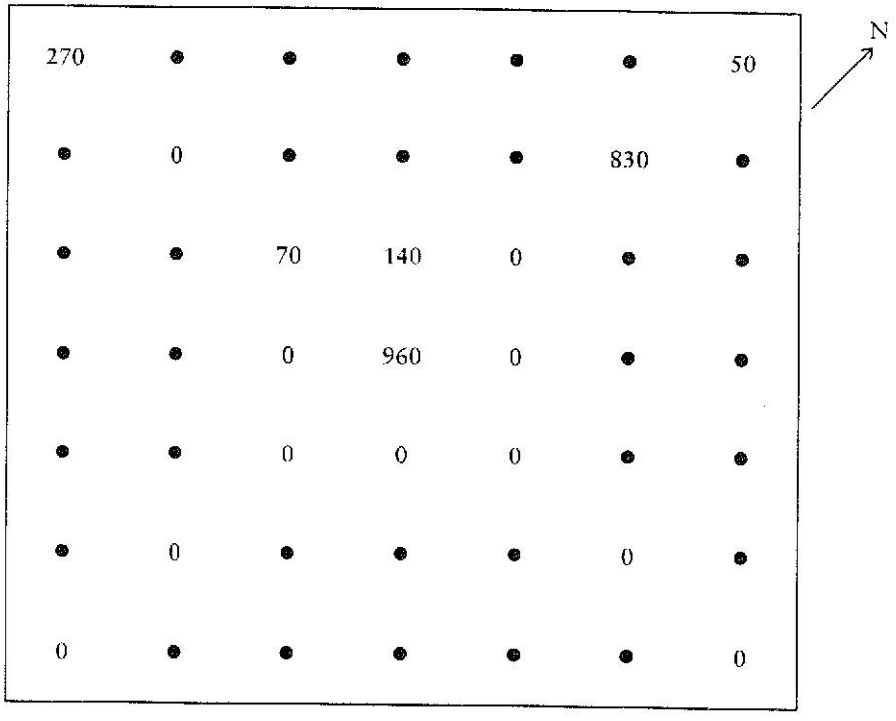

Figure 9.19

Diagram of SON plot illustrating the population of *P. syringae* present in buds on 10 April 1996. The numbers are expressed in cfu.bud^{-1}.

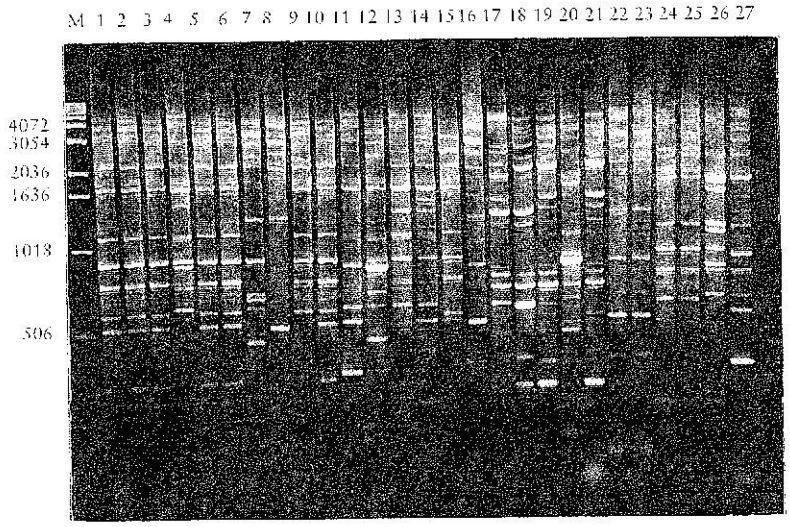

Figure 9.20

Agarose gel electrophoresis of REP-PCR fingerprint patterns obtained from genomic DNA of *P. syringae* isolated from cherry. The resulting electrophoretic patterns of isolates from leaves of SON, on 8 November 1995, from trees number 1 (lane 1);7 (lane 2); 13 (lane 3); 17 (lane 4); 18 (lane 5); 19 (lane 6); 24 (lane 7); 25 (lane 8); 26 (lane 9); 31 (lane 10); 32 (lane 11); 33 (lane 12); 37 (lane 13); 43 (lane 14);and 49 (lane 15); from the inoculated control bacteria *P.s.* pv. *morsprunorum* (NCPPB 1462) in lane 16; and from SON buds, on 10 April 1996, from trees number 1 (lanes 17 and 18); 9 (lanes 19 to 21); 25 (lanes 22 and 23); 31 (lanes 24 and 25); and 43 (lanes 26 and 27) are shown. The left lane (M) contain DNA size markers (1 kb ladder); their sizes are indicated in base pairs.

9.4 Discussion

Almost no relationships among the strains could be found by REP-PCR, therefore it was not possible to be sure of the origin of the bacteria detected. Nevertheless, the pattern of spatial distribution leaves little space for doubt that the major source of bacteria was the central inoculated tree in the inoculated plots and an exogenous source in the control plot. The high genotypic variation observed, although unexpected, is not unusual among pseudomonads (DOWLER & WEAVER, 1975).

Despite constant environmental stress, significant variability arises from differential effects of environmental stress within a population (KNUDSEN, 1991). There is ample time during the lifetime of any leaf for measurable shifts within *P. syringae* populations to accrue from relatively small selections per bacterial generation. Such intraspecific shifts apparently do occur (HIRANO & UPPER, 1990).

Both temperature and solar radiation have direct effects (for example, mortality) and indirect effects (metabolic rates, mutations, etc.) on numbers and types of bacteria on above-ground plant parts. There are also numerous interactions among physical factors (KNUDSEN, 1991).

Populations of cells tend to become asynchronous over time with respect to physiological state, due to changes in microenvironment as well as genetic differences among individuals. Because bacterial cells have a genetic constitution that is not immutable, most "pure" cultures are in reality genetically heterogeneous. When the environment changes, it may select for variants within that population. Due to shading, populations in the different canopy levels became greatly divergent over time (KNUDSEN, 1991). Phyllosphere populations may be regarded as representing a broad range of genotypes, each likely to respond in a slightly different manner to the selection pressure presented by any given set of environmental conditions (HIRANO & UPPER, 1992).

The rather small or undetectable populations after the inoculation were not unexpected because the inoculations were made in summer and, in the field, epiphytic bacteria tend to die (HIRANO & UPPER, 1983) immediately after they are artificially introduced onto plant surfaces (usually by spray application). Populations of *P. s.* pvs. *syringae* and *morsprunorum* rapidly declined when the bacteria were sprayed onto sour cherry leaves maintained at low or high RH, but without visible moisture on the leaves (LATORRE & JONES, 1979b). A model for *Burkholderia cepacia* (Burkholder) Yabuuchi *et al.* assumed that mortality was very high (95%) on the day that cells were applied, but that thereafter cells died at a slower exponential rate during periods of RH less than 95% and that the population increased slowly under conditions of high humidity (KNUDSEN, 1991).

Although dilution plating has been the method most widely used to quantify epiphytic bacteria, its reliability as a means to estimate population sizes of bacteria in nature has frequently been questioned. Nevertheless, the recovery of *P. syringae* from leaves by dilution plating following the application of known densities of the bacterium to the leaves has given no reason to doubt the adequacy of this method for estimating phyllosphere population sizes of *P. syringae* (HIRANO & UPPER, 1992). One of the distinct advantages of the plating techniques is that specific components of the total epiphytic bacterial population can be distinguished, enumerated separately, and isolated for further testing if desired (CROSSE, 1966).

The small numbers of bacteria in early October, in 1994, and in September, in 1995, were to some extent already expected because no leaf spots were present and during summer the numbers of epiphytic bacteria present are very small. Furthermore, the summer of 1994 and especially the summer of 1995 were very dry and hot which are detrimental conditions for the development of epiphytic populations. Also, the cultivar Stella used in the plots, although susceptible to the disease, is not one of the most affected cherry varieties (GARRETT, 1982). The choice of a cultivar with medium susceptibility to bacterial canker, like Stella, was made to prevent possible death of the young trees. Epiphytic pathogenic populations are generally lower on resistant compared with susceptible cultivars of a given host species. This has been found with *P. s.* pv. *morsprunorum* on sweet cherry (CROSSE, 1963) and *P. s.* pvs. *syringae* and *morsprunorum* on sour cherry. Field resistance of certain cultivars to epiphytic pathogenic populations may be a function of the inability of that epiphytic organism to colonise and establish large populations on resistant compared to susceptible cultivars (HIRANO & UPPER, 1983).

The difficulty in isolating *P. syringae* organisms during the summer has been reported by other researchers and likely reflects a lower population of bacteria present then (DOWLER & WEAVER, 1975). The population of *P. s.* pv. *syringae* was low or undetectable during summer under warm (daily minimum 8°C to 15°C) and dry conditions (LATORRE *et al.*, 1985). Low populations during mid and late summer can be attributed to high temperatures, low rainfall and humidity, and high UV radiation (WIMALAJEEWA & FLETT, 1985).

Changes in population sizes in *P. syringae* can occur rapidly in response to changes in the weather, which in turn, may fluctuate tremendously within a given day and from day to day. The general pattern of fluctuation of *P. s.* pv. *syringae* on leaves is similar to that reported in England for *P. s.* pv. *morsprunorum* (CROSSE, 1963), with peaks occurring during late spring to early summer, and during late autumn (HIRANO & UPPER, 1994).

The larger population sizes of *P. syringae* present on leaves and other aerial parts of many stonefruit trees during spring and autumn as compared to summer have been attributed to the cooler temperatures and frequent rains prevailing during the former seasons. WIMALAJEEWA & FLETT (1985) observed that populations of *P. syringae* on apricot and cherry were higher during spring than during autumn in Victoria, Australia, although both seasons had very similar average maximum and minimum temperature ranges and similar average rainfalls. The authors suggested that

temperature and rain are secondary to availability of substrate in determining population levels of *P. s.* pv. *syringae* on stonefruit trees.

CAMERON (1970) suggested a defined seasonal cycle for *P. syringae* on cherry trees. The number of sites in the tree where bacteria are found increases rapidly during the spring (February-April) and then declines through summer (May-August). There is an increase in autumn and early winter followed by a low incidence in the coldest part of the season (January). There is a rapid increase in bacteria from January to March, a gradual decline through summer (April-August), and an increase in autumn (August-November). Because moisture levels are usually replenished more rapidly that they are depleted, the bacterial population tends to increase more rapidly than it decreases. If other factors, such as temperature are favourable, between 3 and 12 days are needed after an increase in available moisture before an increase in bacterial population is observed (CAMERON, 1970).

Rainfall appears to be the environmental factor that has the greatest effect on the temporal variability of *P. syringae*. CROSSE (1966) considered that rain provides simultaneously for the mobilisation, distribution, and penetration of inoculum, and is the most important environmental factor in infections of stone-fruit trees. Rain apparently affects population sizes of *P. syringae* in two ways. First, substantial numbers of bacteria are removed from the phyllosphere by wash-off. Bacterial concentrations of *P. s.* pv. *morsprunorum* exceeding 10^6 cfu.ml^{-1} have been collected from rainwater draining down sweet cherry trees (FREIGOUN, 1974). Second, rain also appears to trigger the onset of growth of *P. syringae* that remain on the leaf surface. The observed large increases in population sizes of *P. syringae* were frequently preceded by rain. The mechanism by which rain triggers growth of *P. syringae* is not entirely understood. The momentum of raindrops appears to be important for triggering growth of *P. syringae* on bean leaflets (HIRANO *et al.*, 1987). Not all rains will trigger growth of *P. syringae*. For example, the momentum of raindrops in a gentle drizzle rain is insufficient to trigger growth of the bacterium (HIRANO & UPPER, 1994). The mechanism by which raindrop momentum triggers growth of *P. syringae* remains to be elucidated. The momentum of rain may trigger the plant to release nutrients necessary for rapid growth of *P. syringae*. Alternatively, the leaf surface may be cleansed of compounds or microbes that are unfavourable for rapid growth of *P. syringae* (HIRANO & UPPER, 1992).

Because no bacteria were present on the control plot during the first year of plantation, it was presumed that no exogenous sources of inoculum were interfering

with the dispersal of the epiphytic populations within the S-A plot during the first year.

As showed in Figures 9.21 to 9.23, there was a rapid decline in number of bacteria from the inoculated tree to the surrounding trees. According to HIRANO & UPPER (1990), immigration is more important in delivering small numbers of cells of *P. syringae* to aerial parts of plants than in causing large increases in *P. syringae* population sizes. It is likely that large population increases are due to growth (i.e. multiplication) rather than immigration since rates of deposition of bacteria from aerosols are orders of magnitude too small to explain shifts of this size (HIRANO & UPPER, 1990). The doubling times for *P. s.* pv. *morsprunorum* growing epiphytically on leaves were 3.6 h at 25°C and 5.2 h at 15°C (FREIGOUN, 1974).

The dispersion of bacteria by rain splash is a mechanism only for local dispersal (a few metres); it cannot be expected to result in a net increase in the number of bacteria on plants in a large field. Indeed, the net movement of leaf-associated bacteria during rain is downward. At least for bacterial brown spot of snap bean, disease follows rain, not because of splash dispersal of large numbers of bacteria but because of pathogen multiplication (HIRANO & UPPER, 1990). These mechanisms are probably more effective for redistributing bacteria within a given canopy. In the presence of water, motile bacteria can move to more favourable sites in or on the leaf (HIRANO & UPPER, 1983).

The trend observed in the control plot (Fig. 9.24) was completely the opposite, with more bacteria on the outer part of the plot. This indicates a possible exogenous source of *P. syringae* from south and east quadrants, migrating because of the prevailing south winds. However, no cherry trees were growing within at least 300 m from these quadrants.

In this work, the erratic colonisation of the tissues by saprophytic bacteria might also have contributed to the marked fluctuations of *P. syringae* populations. According to BILLING & BAKER (1963), with the selective procedures used for enumerating *P. s.* pv. *morsprunorum*, one of the most common saprophytic organisms isolated from the leaves of stone fruit trees is a Gram-negative, with characters very similar to *Erwinia* described from cankers on deciduous fruit trees. These bacteria were also present in my work in great numbers on several plates. Crosse (1966) affirms that although the *Erwinia*-like organisms are now regarded as an integral component of the disease complex in the field, their role in field infection is uncertain. They are less frequent on the leaf surfaces than the pathogen, except during prolonged wet weather, when their numbers have been observed to increase substantially. It is possible that under

these conditions they may interfere with infection, particularly on more resistant varieties.

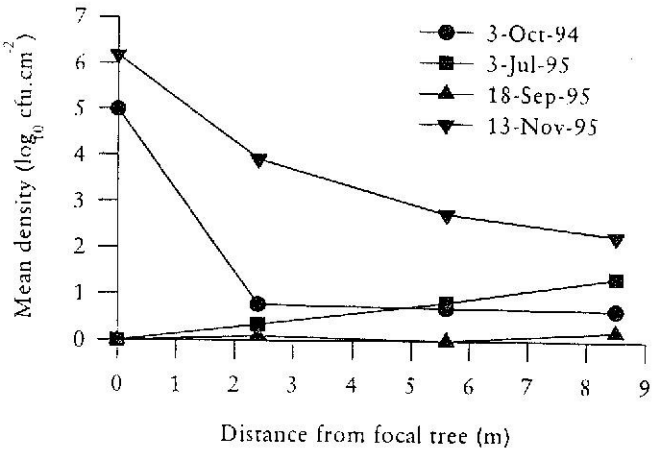

Fig. 9.21
Relation of mean density of epiphytic population of *P. syringae*, at S-A plot on different dates, according to the distance from focal inoculated tree.

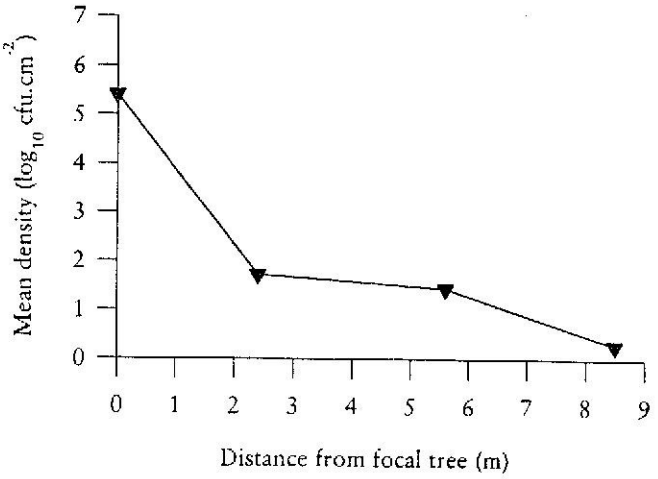

Fig. 9.22
Relation of mean density of epiphytic population of *P. syringae*, at S-B plot on 13 November 1995, according to the distance from focal inoculated tree.

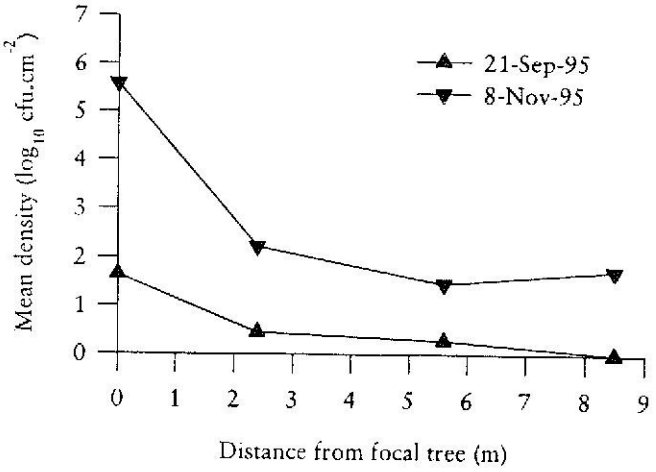

Fig. 9.23

Relation of mean density of epiphytic population of *P. syringae*, at SON plot on different dates, according to the distance from focal inoculated tree.

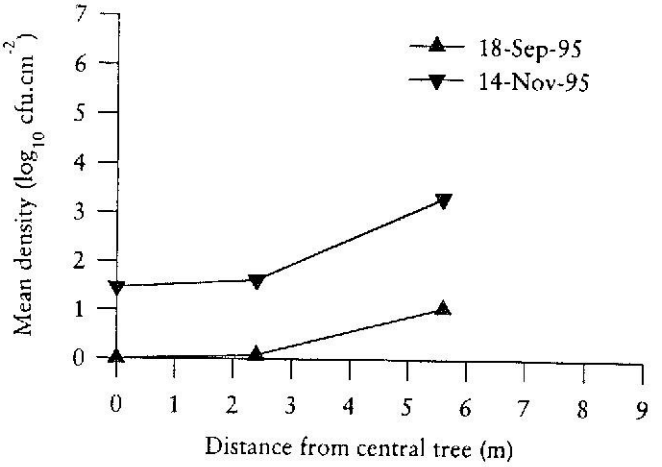

Fig. 9.24

Relation of mean density of epiphytic population of P.syringae, at S-C plot on different dates, according to the distance from central tree.

The absence of leaf spots or cankers suggests that disease expression is not essential for the survival of *P. syringae*. Despite the common presence of the pathogen on leaf surfaces, leaf spots develop rarely, if at all, in South African orchards. Epiphytic populations of *P. syringae* are not restricted to the external surface of leaves. MANSVELT & HATTINGH (1989) postulated that substomatal chambers subsequently serve as protected sites that enable the pathogen to survive adverse atmospheric conditions during warm, dry spells (HATTINGH *et al.*, 1989).

In April 1995, *P.syringae* was already resident in the buds on S-A, although none was detected in the control plot, possibly because of the smaller numbers during autumn. In the next year, and likely because of the inoculation in October 1995, the numbers were quite large in some trees. Nevertheless, the pattern of spatial distribution was not clear and only in SON was the number of bacteria higher on the inoculated tree. In S-B, *P.syringae* could not even be detected on the inoculated tree or on the majority of the surrounding trees. In the control plot, *P.syringae* was only found in the buds of an outer tree, which had already presented a large number of bacteria on the leaves, in November 1995.

Other authors have also found *P.syringae* in the buds of trees. DOWLER & WEAVER (1975) have also detected *P. s.* pvs. *syringae* and *morsprunorum* in apparently healthy bud and stem tissues of their hosts. GUNAWARDENA (1981) found *P. s.* pv. *morsprunorum* resident in dormant buds during winter. This is confirmed by observations for *P. s.* pv. *syringae* and it has been assumed that the populations of the pathogen are resident predominantly in the buds rather than the stems (WIMALAJEEWA & FLETT, 1985).

P. s. pv. *syringae* was isolated most frequently from bud swell until budbreak in Chile, when cool weather (minimum air temperature 0°C-5°C) prevailed (LATORRE *et al.*, 1985). Bacterial populations greater than 10^4 to 10^5 cfu/g of buds were only recorded during wet periods, and invariably populations rapidly declined to very low or undetectable levels during dry periods. *P. s.* pv. *syringae* may colonise cherry buds and develop high populations there during dormancy. Therefore, these epiphytic populations may constitute an important inoculum source for outbreaks of bacterial canker in areas characterised by wet weather. However, BACA & MOORE (1987) found no indication that *P. syringae* was colonising the interior of dormant buds.

Cankers that persist through the winter have also been suggested as sources of inoculum of phytopathogenic bacteria on leaves and other aerial parts of plants in spring (DOWLER & WEAVER, 1975).

Borders of grass around the trees may also contribute to the spread of *P. syringae* throughout the plantations and surrounding fields. The near absence of *P. syringae* from many woody plant species during the extremes of winter, and in some woody hosts during the hot days of summer, followed by a rapid increase in the spring, may be due to transfer of *P. syringae* from the adjacent grasses to the trees. Despite the nonsusceptibility of grasses, they still harbour epiphytic populations of pathogenic *P. syringae* that could serve as an inoculum source for adjacent woody plants. Besides this indiscriminate spread from the weeds to the trees, insects may also transfer bacteria from tree to tree (BACA & MOORE, 1987).

P. s. pv. *syringae* has been commonly isolated from grasses and broad-leaf herbaceous plants and from plant refuse collected under cherry trees. It is possible that *P. s.* pv. *syringae* may overwinter on weeds. *P. s.* pv. *morsprunorum* is much less widespread than *P. s.* pv. *syringae*, and is usually regarded as occurring only on the cherry tree itself. Thus, the disease phase of the life cycle of *P. s.* pv. *syringae* appears to be casual and not essential for its survival in cherry orchards, while that of *P. s.* pv. *morsprunorum* is more important for its survival. Weeds and plant refuse are possibly sources of primary inoculum for bacterial canker incited by *P. s.* pv. *syringae* (LATORRE & JONES, 1979b). It is desirable to know to what extent the bacteria are disseminated by splashing rain from contaminated weeds and plant debris to cherry.

9.5 Conclusions

The population of *P. syringae* had the cyclical temporal pattern found by so many other authors. During summer the numbers were extremely low or undetectable and there was a great increase in autumn. The lack of any kind of clear symptoms supports the idea that asymptomatic trees, but harbouring large numbers of bacteria, can spread epiphytic bacteria to a whole plantation quite rapidly, and possibly cause disease. Even with an intermediately susceptible cultivar of sweet cherry, the number of trees that hosted populations of *P. syringae* was very high. With the technique used to estimate epiphytic bacteria, even in the first year *P. syringae* reached the outer trees of the inoculated plots.

As the control plot hosted bacteria after the second year of plantation, it can be assumed that *P. syringae* is ubiquitous and there are several sources around. It seems likely that these include species other than cherry. It took only slightly more than one year for the noninoculated plot to host epiphytic forms of the bacteria. Therefore, the

spacing of trees in new plantations does not seem to be crucial to avoid dispersion of *P. syringae*.

The genetic fingerprinting technique used, the REP-PCR, to elucidate the relationships among isolates seem to be of a too fine resolution, showing up differences among individuals. The spatial patterns observed make inconceivable the idea that all the bacteria isolated were immigrants. Consequently, it seems that bacteria populations should undergo severe changes and evolve fast as soon as they are sprayed onto the leaves and change environment.

The spatial patterns observed did not correlate clearly with the wind data. There were no distinct patterns of spread that indicated that wind was spreading the bacteria in the direction of the prevailing winds. Knowledge of the distribution directions of individual wind gusts is probably necessary to better understand the spatial patterns. The daily average values of wind data are possibly not enough to show correlations.

The number of bacteria present in early autumn makes it conceivable that the summer bacteria refuges will be on the trees. Although drying kills metabolically active cells of most bacteria, at the same time metabolically inactive cells are much more able to survive desiccation (KNUDSEN, 1991).

The absence of cankers and the common detection of *P. syringae* in the buds indicate that the major overwintering shelter may be the buds.

Forestry weeds, especially trees and shrubs, could play an important role in the spread of *P. syringae* within cherry tree plantations but that aspect still needs clarification.

CHAPTER 10
CONCLUSIONS

The wild cherry trees in some parts of Britain have been quite affected by bacterial canker. The disease has been reported several times since 1984 to the Forestry Authority and preliminary diagnosis has been made. Nevertheless, an accurate diagnosis can be quite difficult and the usual isolation methods not always produce the causal agent. In 24 wild cherry sites investigated for the presence of bacterial canker during 1994 and 1995, only from eight was it possible to isolate the bacteria *Pseudomonas syringae*, determined as the causal agent in 1931. Although a few of the visited sites were apparently free of disease symptoms, the large majority of them were showing dieback and gum exudations, a few of them even with very severe symptoms and with several dead or dying trees. The worst cases were almost invariably connected with the enclosure of the trunks in corrugated plastic guards (tree shelters). Other workers have also encountered the same problem in pear trees (SPOTTS & CERVANTES, 1994). The saprophytic bacteria *Erwinia herbicola*, sometimes a weak pathogen (BRADBURY, 1986), was also apparently causing large necrotic lesions when these trunk guards were used.

From the eight sites where *P. syringae* was isolated, 23 cultures were obtained and used later for testing the diagnostic techniques. In addition, 52 *P. syringae* cultures, acquired from the HRI, the NCPPB, and the CSL, were employed to investigate diagnostic techniques.

The only nursery visited during this work was severely affected by bacterial canker in three year old wild cherry trees and two cultures were isolated from this material. This finding is of considerable concern since it suggests that the planting material could be one of the main sources of the disease. This is probably the reason why the disease was so prevalent in several three-four year old plantations. The level of contamination by *P. syringae* pv. *syringae* of stonefruit material in nurseries in

Victoria, Australia, has also been considered a significant factor in the epidemiology of bacterial canker (WIMALAJEEWA & FLETT, 1985).

Another interesting finding was that in several of the affected stands, with trees around 40 years old, it seemed that the disease had occurred quite recently. This suggests that a particular circumstance had triggered the outbreak, presumably something like a strong wind, similar to the great gale of autumn 1987, which could have opened several avenues for the infection.

The classical cultural patterns of the two pathovars, *P. syringae* pvs. *morsprunorum* and *syringae*, involved in this disease were confirmed in this study and the pathogenicity of the majority of the isolates was proved by tobacco hypersensitivity reaction. Three cultures from sweet cherry trees identified previously as *P. s.* pv. *morsprunorum* did not show the clear typical pattern for this pathovar and should not be considered typical forms. Only one of the *P. s.* pv. *syringae* isolates did not show the typical response of this pathovar to the classical nutritional tests. D,L-lactic acid utilisation gave the most discriminatory results of all the tests.

The cultures from wild cherry trees, both the ones identified by other workers as *P. s.* pv. *morsprunorum* and the ones isolated during this work, only identified as *P. syringae*, had a response between both pathovars with the classical nutritional tests.

The nutritional tests were further developed by the use of a commercial diagnostic kit (Biolog's GN MicroPlate system). In this, 95 carbon sources were tested for all the bacteria isolates and the identification was confirmed by comparison with the Biolog library. Identification at the pathovar level, using the library available at the moment, was not possible, but identification at the species level seemed quite accurate. It was even possible to differentiate a few saprophytes which had been misidentified with *P. syringae* because of their levan production on NSA plates.

Still, the best merit of the Biolog system was to provide characters for numerical taxonomy. The quantitative results of the carbon source utilisation, obtained in an optical density format, were adequate for cluster analysis. This revealed the presence of three clusters in the *P. syringae* isolates analysed. Cluster I mainly formed by the *P. s.* pv. *syringae* isolates, cluster III mainly formed by the typical *P. s.* pv. *morsprunorum*, and the intermediate cluster II mainly formed by wild cherry isolates either isolated during this work or obtained from HRI. This latter cluster is more similar to cluster I than to cluster III, which could mean that most of the wild cherry strains are a group of *P. s.* pv. *syringae*, rather than belonging to *P. s.* pv. *morsprunorum*, where they have usually been included. However, it seems that this

group is formed by a population still too varied to allow a distinct classification and it should remain in *P. syringae*, without assignment to any of the existing pathovars, until further taxonomical work is accomplished. However, there were a few wild cherry isolates which had a characteristic *P. s.* pv. *syringae* behaviour and a also a few which reacted like *P. s.* pv. *morsprunorum*.

One reason for the variation may be that sweet cherry has been grown as clonal material in orchards for many years where a single pathogen type has been able to evolve and the populations are now quite stable. On the other hand, in the wild cherry with its intrinsic variation because of the material origin, there has not been opportunity for a distinct pathovar associated with wild cherry to evolve and become a stable, rather uniform population. It is only very recently that there has been an attempt to develop wild cherry clonal material with the most desirable characteristics for farm woodland planting.

An interesting detail of the cluster analysis is that in the phenograms obtained a sub-cluster of cluster III formed by almost all of the plum isolates could be discriminated from the rest of the cluster. This shows the sensitivity of the cluster analysis when using Biolog absorbance readings.

From the diagnostic techniques tested for *P. syringae*, the best was undoubtedly the conjugated *Staphylococcus aureus* agglutination. The simplicity, celerity (30-45 seconds is enough for a response), and cost of this technique makes it ideal for large surveys in routine use. However, to rely unequivocally on this technique good specific antisera have to be produced. The antiserum raised during my work was quite specific when using the *S. aureus* agglutination test, but not with immunofluorescence slide test. The former test allowed the identification of several colonies obtained in the epidemiological part of the work. This identification would not have been possible without the help of this quick test. Although the bacteria had to be cultured prior to identification, there was no need for subsequent isolations because the colonies which were difficult to identify on the primary plates were easily screened in a well of a multitest slide with the working reagent of this assay.

However, the production of a more specific antiserum is very desirable and it should be a major goal in future diagnostic techniques research. After the production of specific antisera, other immunoidentification techniques which do not need isolated cultures of the micro-organisms, like the fluorescent antibody colony staining (VAN VUURDE, 1987), can be developed.

The other diagnostic techniques used like the DNA hybridisation probe and REP-PCR can also be very useful. The hybridisation probe used, although it only formed part of the original probe, hybridised strongly with almost all the typical *P. s.* pv. *morsprunorum* isolates and discriminated them rather well from *P. s.* pv. *syringae* isolates. However, if this probe just distinguishes the typical forms of *P. s.* pv. *morsprunorum*, epidemiological work in wild cherry situations would be severely restricted or impossible. The same considerations apply to REP-PCR. Almost all typical *P. s.* pv. *morsprunorum* isolates had the same band patterns. The *P. s.* pv. *syringae* isolates, known to be quite different from each other, and the wild cherry cultures were not possible to identify with REP-PCR fingerprints because the band patterns were too diverse.

While almost all the typical forms of *P. s.* pv. *morsprunorum* had the same fingerprinting patterns when they were done from cultures under cultivation, once they had been passed through their natural host, sweet cherry, the patterns changed quite a lot. These results were obtained when analysing the spatial and temporal distribution of epiphytic populations in a simulated plantation. Almost all the *P. syringae* cultures recovered from the trees adjacent to the inoculated one, harboured epiphytic populations but they were different from each other and also different from the inoculated culture. Even on the inoculated tree, bacteria with the same band patterning as the one inoculated were seldom isolated. The patterns of bacterial dispersion and abundance make it unthinkable that almost all the epiphytic bacteria found on trees were immigrating strains, hence the REP-PCR differences should be attributed to rapid change in the bacteria.

In the epidemiology part of this work, besides the cyclical pattern already found by so many other authors, it seems probable that the summer survival of the bacteria is done epiphytically on the leaves, because of the great increase in the population after the first rains. The frequent presence of bacteria in buds and the absence of cankers during winter in the plots, suggest that the major overwintering shelter may be the buds.

As asymptomatic trees were hosting large numbers of epiphytic bacteria which spread in around one year to the whole plots, ultimately causing disease, a useful detection tool is very important if disease monitoring is to be achieved. Apparently, the bacteria spread for more than 8 m from a known source in less than one year. However, the bacteria seem to be ubiquitous as the control (noninoculated) plot also hosted the bacteria after the second year of planting, even without any known sources in the nearest 300 m. Forestry weeds and insects may also play an important role in

the epidemiology of the disease. Hence, spacing of the trees in new plantations does not seem to assist in escaping the epiphytic forms of *P. syringae*. Trees and shrubs, mainly *Prunus* species, like *P. laurocerasus, P. cerasus* L., and *P. spinosa* L. should be carefully looked at as possible sources of epiphytic forms of the bacteria. This could allow the reduction of different sources and therefore reduce the numbers of bacteria.

Further study in taxonomy should be carried out, mainly within the ill-defined *P. s.* pv. *syringae*. As pathovars are based on pathogenicity, cross-infections between the different groups of isolates and their respective hosts should be made. Also, *Pseudomonas syringae* isolated from *Prunus laurocerasus*, the causal agent of cherry-laurel "shot-hole", should be cross-infected onto sweet and wild cherry clones.

As the majority of chemical and cultural control measures are very difficult to implement in woodlands, control work elsewhere has stressed genetic improvement and hopefully wild cherry clones resistant to bacterial canker will become available. However, resistant material must be carefully screened against a large panel of different wild cherry isolates, otherwise resistance might be short-lived. Given the variation in the pathogen a strategy of planting clonal material must seem dubious.

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