A STUDY OF STRAIN VARIATION IN *ERWINIA SALICIS* IN RELATION TO THE EPIDEMIOLOGY OF WATERMARK DISEASE

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Anadolu Üniversitesi
Merkez Kütüphane

Dedicated to my husband Alâattin and my son Alper Tuna

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ABSTRACT

Erwinia salicis (Day) Chester is the causal agent of watermark disease in several species of *Salix* L. (willow) particularly in *Salix alba* var. *caerulea* (cricket-bat willow). Virtually nothing is known about the epidemiology of watermark disease and the infection route, infection process, transmission and survival of *E. salicis* is not known. The work presented here investigates the epidemiology of the disease by distinguishing between isolates of *E. salicis* and then examining their geographical distribution.

The first step in this study was to reliably identify the isolates without a pathogenicity test. This was done by comparing the cultural, biochemical and serological characteristics of authentic isolates of *E. salicis* whose pathogenicity has been confirmed, with those of the suspect cultures. The most important tests were ELISA and API 20E profiling, described in Chapter 2.

Chapter 3 included the carbon source utilisation assay, bacteriophage sensitivity assay and bacteriocin assays. Three bacteriophages specific for *E. salicis* were isolated from natural sources and a total of 5 bacteriophages were used in a bacteriophage typing scheme. Bacteriophage sensitivity assays and galactose utilisation tests demonstrated the differentiation between Dutch and English isolates of the bacterium, but did not reveal sufficient variation for use in the epidemiological study.

Serotyping was employed in Chapter 4 and two antisera were raised against two isolates of *E. salicis*. Only two groups, based on presence or absence of heat-stable 'O' antigen, were identified with one of the antisera, indicating the serological homogenicity of *E. salicis*.

Chapter 5 describes the development and optimization of multilocus enzyme electrophoresis typing for *E. salicis*. A total of seven enzymes were chosen for the typing study.

Chapter 6 describes the application of multilocus enzyme electrophoresis typing for the differentiation of *E. salicis* isolates.This method indicated extensive variation between the isolates tested and 23 ETs could be identified in a population of 78 isolates of *E. salicis*. It was concluded from this study that in Wiltshire, in a severely diseased plantation, the disease did not spread from tree to tree and infection has presumably arisen from infected propagating material.

This hypothesis was supported by the immunoisolation study presented in Chapter 7. Two techniques, immunofluorescence colony staining (IFC) and immunomagnetic separation (IMS), were employed and *E. salicis* could be isolated from symptomless infected

trees by IMS. Although the IMS technique needs to be optimised, initial indications suggest that this technique, in connection with the other techniques, could be applied in further studies of the epidemiology of watermark disease.

Contents

Page
Acknowledgements I
Abstract II
Contents III

Chapter I Introduction and Literature review

1. 1 General Introduction 1
1. 2 The cricket-bat willow *Salix alba var. caerulea* (Sm.) Sm. , and its possible origin 1
1. 3 The propagation and growing of cricket-bat willow 4
1. 4 The discovery of watermark disease 4
1. 5 The symptoms of watermark disease 7
1. 5. 1 Macroscopic symptoms 7
1. 5. 2 Microscopic symptoms 12
1. 6 The causal organism, *Erwinia salicis* (Day) Chester 12
1. 6. 1 The pathogen 12
1. 6. 2 Cultural characteristics 13
1. 6. 3 Strain variation in *E. salicis* 13
1. 6. 4 Synonymy 14
1. 6. 5 Host range 14
1. 7 The transmission of watermark disease 15
1. 7. 1 Infected propagating material 15
1. 7. 2 Soil, root grafts 16
1. 7. 3 Cutting tools 16
1. 7. 4 Insects 17
1. 7. 5 Rain and wind 17
1. 8 The distribution of watermark disease 17
1. 9 Control of the disease 18
1. 10 Bacterial typing for epidemiological studies 21
1. 10. 1 Physiological profiling 21
1. 10. 2 Bacteriophage typing 22
1. 10. 3 Bacteriocin typing 23
1. 10. 4 Serotyping 24
1. 10. 5 Restriction fragment length polymorphism (RFLP) analysis 26
1. 10. 6 Multilocus enzyme electrophoresis typing 26
1. 11 Isolation methods for bacteria 27
1. 12 Aim of research 29

Chapter II Isolation and Identification of *E. salicis*

2. 1 Introduction 30

2. 2 Materials and methods 32
2. 2. 1 Source and maintenance of plant material 32
2. 2. 2 Origin and maintenance of bacterial cultures 32
2. 2. 3 Evaluation of media for growth of *E. salicis* 33
2. 2. 3. 1 Preparation of the inoculum 33
2. 2. 3. 2 Selective media 33
2. 2. 3. 3 Cultivation of *E. salicis* in aerobic and anaerobic conditions 36
2. 2. 4 Isolation of the pathogen 36
2. 2. 5 Biochemical tests and motility 37
2. 2. 5. 1 Gram's staining reaction 37
2. 2. 5. 2 Oxidase test 37
2. 2. 5. 3 Test for Oxidative – Fermentative (O/F) metabolism 37
2. 2. 5. 4 API 20E test 38
2. 2. 5. 4. 1 Preparation of the API 20E test strips.......38
2. 2. 5. 4. 2 Preparation of the inoculum 38
2. 2. 5. 4. 3 Preparation of the reagents 38
2. 2. 5. 4. 4 Inoculation of the test strip 38
2. 2. 5. 4. 5 Reading of the strip 39
2. 2. 5. 4. 6 Identification of the profiles 40
2. 2. 5. 5 Motility test 41
2. 2. 6 Production of a specific antiserum 41
2. 2. 6. 1 Antigen preparation 41
2. 2. 6. 2 Antiserum production 41
2. 2. 6. 3 Separation and preservation of the rabbit sera 42
2. 2. 6. 4 Determination of the titre of the sera 42
2. 2. 7 Agglutination tests 43
2. 2. 8 Competitive ELISA tests 43
2. 3 Results 44
2. 3. 1 Evaluation of media for growth of *E. salicis* 44
2. 3. 2 Biochemical tests and motility 46
2. 3. 3 Agglutination tests 54
2. 3. 4 Competitive ELISA tests 54
2. 4 Discussion 56

Chapter III Differentiation of *E. salicis* isolates according to carbon source utilisation and bacteriophage sensitivity

3. 1 Introduction 61
3. 2 Materials and methods 62
3. 2. 1 Carbon source utilisation assay 62
3. 2. 1. 1 Preparation of the inoculum 62

3. 2. 1. 2 Preparation of the medium....63
3. 2. 1. 3 Inoculation of the tubes and determination of the growth ..63
3. 2. 2 Bacteriophage assays 63
3. 2. 2. 1 Origin and maintenance of bacteriophages....63
3. 2. 2. 2 Isolation of bacteriophages from natural sources 64
3. 2. 2. 3 Isolation of bacteriophages from lysogenic bacteria....65
3. 2. 2. 4 Purification of bacteriophages....65
3. 2. 2. 5 Propagation and preparation of high titre stocks....65
3. 2. 2. 6 Bacteriophage sensitivity assay 67
3. 2. 2. 7 Isolation of bacteriophage-resistant mutants 67
3. 2. 3 Assay for *E. salicis* bacteriocins 68
3. 3 Results 69
3. 3. 1 Utilisation of carbon compounds....69
3. 3. 2 Isolation of bacteriophages....72
3. 3. 3 Reaction of E. *salicis* isolates to bacteriophages 73
3. 3. 3. 1 Choice of panel of tester bacteriophages....73
3. 3. 3. 2 Bacteriophage typing of *E. salicis* isolates....74
3. 3. 4 Relation between galactose utilisation and bacteriophage sensitivity....75
3. 3. 5 *E. salicis* bacteriocins 75
3. 4 Discussion 78

Chapter IV Serotyping of *E. salicis* with Ouchterlony double-gel diffusion (immunodiffusion) tests

4. 1 Introduction 82
4. 2 Materials and methods....83
4. 2. 1 Production of specific antiserum 83
4. 2. 2 Preparation of antigen for immunodiffusion 83
4. 2. 2. 1 Preparation of antigen with phenol-treatment 83
4. 2. 2. 2 Preparation of antigen with heat-treatment....84
4. 2. 3 Preparation of immunodiffusion gel-plates....84
4. 2. 4 Determination of the working dilution of antiserum....85
4. 2. 5 Immunodiffusion tests....85
4. 2. 6 Cross-absorption tests 85
4. 2. 6. 1 Preparation of the antigen....85
4. 6. 2. 2 Preparation of cross- absorbed antiserum 86
4. 2. 7 Staining of gels 86
4. 2. 8 Photography of the immunodiffusion gel-plates 87
4. 3 Results 87
4. 4 Discussion 94

Chapter V Development of multilocus enzyme electrophoresis method for *E. salicis*

5. 1 Introduction 97
5. 2 Materials and methods 98
5. 2. 1 Evaluation of lysis methods 98
5. 2. 2. 1 Preparation of bacterial cultures 98
5. 2. 2. 2 Use of sonicator for the preparation of lysates 99
5. 2. 2. 3 Use of lysozyme in the extraction buffer 99
5. 2. 2. 4 Use of ballotini beads for the preparation of lysates 99
5. 2. 2. 5 Use of X-press for the preparation of lysates 99
5. 2. 2. 6 Use of French pressure cell for the preparation of lysates ..100
5. 2. 2 Determination of the breakage of cells 100
5. 2. 3 Protein estimation of the samples 102
5. 2. 4 Storage of samples 102
5. 2. 5 Enzyme reactions tried 103
5. 2. 6 Separation of enzymes 104
5. 2. 7 Staining 105
5. 3 Results 108
5. 4 Discussion 117

Chapter VI Application of multilocus enzyme electrophoresis typing for differentiation of *E. salicis* isolates

6. 1 Introduction 120
6. 2 Materials and methods 121
6. 2. 1 Bacterial cultures 121
6. 2. 2 Growth of bacteria and electrophoresis of enzymes 121
6. 2. 3 Scoring the gels 122
6. 2. 4 Statistical analysis 122
6. 3 Results 123
6. 3. 1 Enzyme activities and loci 123
6. 3. 2 Geographical distribution of electrotypes in England 129
6. 3. 3 Electrotype analysis of isolates from Wiltshire 130
6. 3. 4 Genetic relationships of electrotypes 134
6. 4 Discussion 136

Chapter VII Immunoisolation of *E. salicis* from *Salix alba*

7. 1 General introduction 139
7. 2 Immunofluorescence colony staining (IFC) 140
7. 2. 1 Introduction 140
7. 2. 2 Materials and methods 141
7. 2. 2. 1 ELISA test 141

7.2.2.2 Preparation of fluorescein isothiocyanate coupled anti *E. salicis* antiserum (FITC-As) 142
7.2.2.2.1 Production of a specific antiserum....142
7.2.2.2.2 Purification of immunoglobulin (Ig G) for conjugation to FITC 142
7.2.2.2.3 Conjugation of Ig G with FITC....143
7.2.2.2.4 Separation of free FITC after conjugation ..143
7.2.2.2.5 Evaluation and storage of the conjugate.....143
7.2.2.3 Immunofluorescence (IF) cell staining of *E. salicis* in pure and mixed cultures 144
7.2.2.4 Source and maintenance of plant material....144
7.2.2.5 Preparation of plant samples 145
7.2.2.6 Isolation of *E. salicis* by conventional methods....145
7.2.2.7 Preparation of dilutions of pure and mixed cultures of *E. salicis* and plant samples for IFC staining....145
7.2.2.8 Preparation of agar plates for IFC staining 146
7.2.2.9 Determination of the working dilution of FITC-As for IFC staining....147
7.2.2.10 IFC staining of the plates 147
7.2.2.11 Microscopy 147
7.2.3 Results 148
7.2.3.1 Specificity of the FITC-As 148
7.2.3.2 IFC staining of pure cultures of *E. salicis* 148
7.2.3.3 IFC staining of mixed cultures of *E. salicis* 148
7.2.3.4 Assay of plant samples....150
7.2.3.4.1 ELISA assay 150
7.2.3.4.2 IFC staining assay 150
7.2.4 Discussion 153
7.3 IMS 155
7.3.1 Introduction 155
7.3.2 Materials and methods 155
7.3.2.1 Coating immunomagnetic particles with anti-*E. salicis* antiserum (IMP-As) 155
7.3.2.1.1 Production of a specific antiserum 155
7.3.2.1.2 Purification of Ig G for attachment to ...... immunomagnetic particles 155
7.3.2.1.3 Attachment of anti-*E. salicis* antiserum to IMP....155
7.3.2.1.4 Agglutination reaction with IMP-As 156
7.3.2.2 Isolation of *E. salicis* from pure cultures with IMP-As 156
7.3.2.3 Isolation of *E. salicis* from artificially inoculated plant samples with IMP-As ('Reconstruction assay') 157
7.3.2.4 Assay of plant samples....158

7. 3. 2. 4. 1 Source and maintenance of plant material ..158
7. 3. 2. 4. 2 Preparation of plant samples 158
7. 3. 2. 4. 3 DAS ELISA test of plant samples 158
7. 3. 2. 4. 4 Isolation of *E. salicis* from plant samples with IMP-As 158
7. 3. 2. 4. 5 Identification of *E. salicis* by bacteriophage sensitivity assay 159
7. 3. 3 Results 159
7. 3. 3. 1 Optimum conditions for the immunomagnetic separation (IMS) of *E. salicis* 159
7. 3. 3. 2 Immunomagnetic separation of *E. salicis* from artificially inoculated wood samples ('Reconstruction assay')..........160
7. 3. 3. 3 Isolation of *E. salicis* from infected plant samples with immunomagnetic separation 162
7. 3. 3. 4 DAS ELISA assay of plant samples at different intervals...166
7. 3. 4 Discussion 168

Chapter VIII General discussion 171

Appendix 176

References 179

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1. 1 General introduction

It is thought that cricket has been played in England for centuries and willow wood has always been used for bat production. In the trade the best quality of bat timber is produced from *Salix alba* var. *caerulea* (Sm.) Sm. (see Plate 1. 1).

The cricket-bat willow is a fast-growing tree, 70-100 feet high, forming a conical crown (Anon, 1974). Its timber combines lightness with a high degree of toughness so extreme as to make it practically unsplinterable while it can easily be cleft for making cricket bats (see Plate 1. 2). It is also resilient and the sapwood is white, but the heartwood varies in shades between red and brown. In the eastern counties of England up to 1,500,000 cricket- bat willows are grown commercially producing wood for more than 90 % of the worlds' cricket bats (Anon, 1974). Watermark disease is the most important disease of willows grown in south eastern counties of England, Holland and Belgium (Anon., 1986). It is a **disease of the xylem tissue which stains the wood making it unsuitable** for bat production and other uses.

Virtually nothing is known about the epidemiology of the disease. In the present investigation, a study was made of strain variation of the causative organism with reference to the epidemiology of the disease.

1. 2 The cricket-bat willow *Salix alba* var. *caerulea* and its possible origin

The cricket-bat willow *S. alba* var. *caerulea* (Sm.) Sm. is a member of the family *Salicaceae*. The real origin of *Salix alba* var. *caerulea* is obscure but it is possibly a hybrid between *Salix alba* L. (white willow) and *S. fragilis* L. (crack willow) (Hutchinson, 1969).

It has been said that all the cricket-bat willows grown in England originated from one single specimen. This specimen was originally found and collected about 1780 in the Lakenheath district of Suffolk by James Crowe, a surgeon and botanist. It has been vegetatively propagated ever since. The botanical name by which the tree is known today was given to it by Sir J. E. Smith, in 1928 (Anon,1974).

Plate 1. 1 Cricket-bat willow, *Salix alba* var. *caerulea*.

Plate 1. 2 Trimmed and stacked clefts for making cricket-bats.

In the trade, the best quality of bat timber is produced from two varieties of willow, referred to as "true bat willows". These are *Salix alba* var. *caerulea* (Sm.) Sm. and another hybrid developed from this which has no botanical name and known as "two coloured bat willow" (Yeates, 1971). Presumably therefore at least two clones of bat willow are in commercial production. Other clones may exist but have not been distinguished by morphological characteristics.

1. 3 The propagation and growing of cricket-bat willow

Willows are dioecious. The majority of female trees do not produce catkins and are therefore sterile and thus can only be propagated by taking cuttings from existing trees.The propagation of cricket-bat willow is done vegetatively in so-called "set beds" (see Plate 1. 3). The set beds contain several hundreds of "mother stools" which are themselves rooted cuttings. Three to 4 years old unrooted cuttings, 3.6 m in length (called "sets") are removed from the mother stools. These are planted 10-12 m apart (see Plate 1. 4). Side shoots are removed from the planted set twice a year for three years after planting to make sure it forms a knot-free clean trunk to give the 'crop' (Preece, 1977).

Cricket-bat willows prefer a moderately dry climate and deep, permeable, rich, heavy loam near to running water. The soil should be well aerated and of adequate depth, so that the tree develops a very strong root system (Anon, 1974). Under favourable conditions *Salix alba* var. *caerulea* reaches a size suitable for cricket bat production in 12 to 15 years.

The cricket-bat willow is mainly grown in Essex, Suffolk and Hertfordshire where the bulk of the timber for bats is produced. Neighbouring counties which also cultivate the cricket- bat willow for economic purposes, but to a lesser degree, are Norfolk, Cambridgeshire, Bedfordshire, Middlesex and Kent (Bryce, 1950).

1. 4 The discovery of watermark disease

Watermark disease was first investigated on the cricket- bat willow trees growing along the river Chelmer in Essex in the early twenties. The first description of the disease was given by Day (1924). He isolated the bacterium from diseased wood and called it *Bacterium salicis* n. sp. recording it as a Gram-positive rod.

Plate 1. 3 A set bed containing mother stools with 2-3 years old shoots which will be cut and planted elsewhere the next growing season.

Plate 1. 4 Young cricket-bat willow trees which have been planted recently.

A similar disease was reported as occurring in the Netherlands on the white willows *Salix alba* and other *Salix* species by Lindejer in 1932 (Gremmen and Kam, 1970). She also isolated a bacterium from diseased wood but described it as Gram-negative, in disagreement with Day, and called it *Pseudomonas saliciperda* n. sp. having one polar flagellum.

The disease was re-investigated by Dowson (1937) to confirm Day's work and provide a more complete description of *Bacterium salicis* than appears in Day's paper. Dowson found the causative organism to be Gram-negative. He also proved the pathogenity of the bacterium and found that it has five to seven peritrichous flagella. Afterwards, the organism was re-named as *Erwinia salicis* (Day) Chester by Chester in 1939 (Buchanan and Gibbons, 1974). Gremmen and Kam (1970) compared Dowson's isolate with that of Lindejer and with other Dutch isolates. They concluded that *Erwinia salicis* is the causative organism of watermark disease on *Salix* species in England and in the Netherlands.

1. 5 The symptoms of watermark disease

1. 5. 1 Macroscopic symptoms

The symptoms of the disease have been described by Day (1924), Webster (1927), Metcalfe (1940), Dowson (1957) and Preece (1977).

The first sign of the disease is the wilting of the leaves on an isolated branch or on a group of branches in spring. The wilted leaves become reddish brown (see Plate 1. 5) and red leaf symptom is the most external symptom of the disease. In successive years affected branches die back producing a stag head appearance (see Plate 1. 6). Occasionally creamy-white bacterial ooze may drip from the crotch regions of the affected branch (see Plate 1. 7). If an affected branch is cut through, the diseased wood stains reddish brown or brownish black contrasting with the white healthy tissue (see Plate 1. 8). When the diseased wood is cut it appears wet, due to congestion with bacterial growth and, upon exposure to air, the surface of the infected wood turns dark brown. The name watermark comes from this appearance of the diseased wood.

Plate 1. 5 A cricket-bat willow showing the typical watermark disease symptom of red leaves.

Plate 1. 6 A cricket-bat willow showing the typical watermark disease symptom of stag head appearance.

Plate 1. 7 A portion of cricket-bat willow branch showing bacterial ooze flowing out from the 'crotch' as indicated by an arrow.

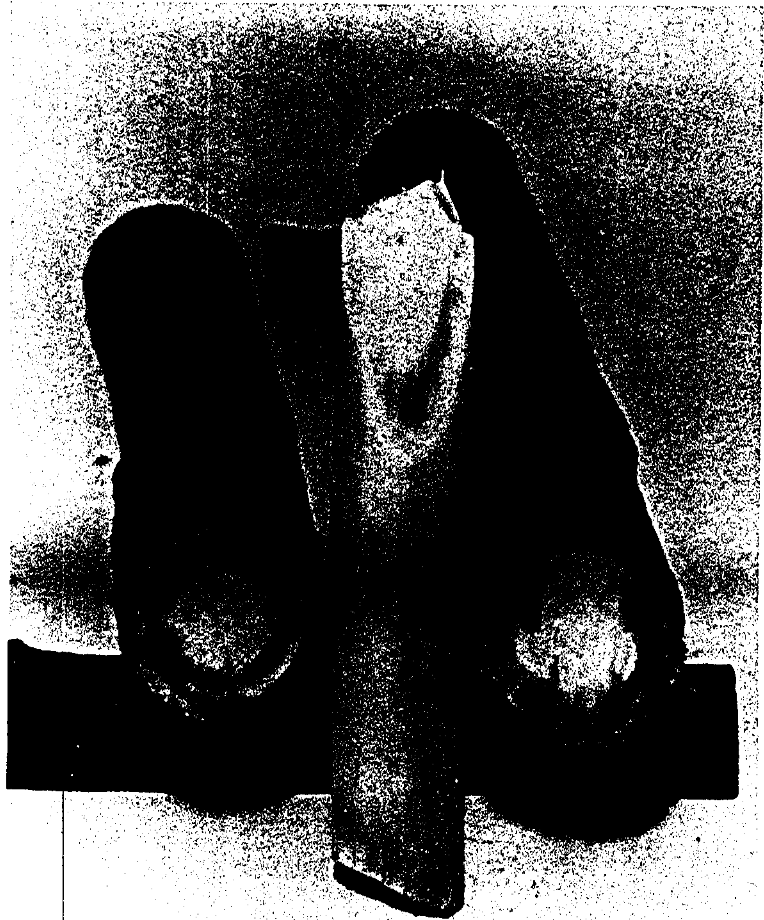

Plate 1. 8 Infected cricket-bat willow wood showing the typical watermark stain. The colour intensifies and becomes black or very dark brown within 24 hour on exposure to the air. The shoot on the left shows the appearance of the freshly cut surface while the shoot on the right shows the darkened colour. The middle specimen shows the pale brown colour of infected wood just after peeling off the bark.

1. 5. 2 Microscopic symptoms

Day (1924) first pointed out that *E. salicis* was confined to the vessels and xylem parenchyma cells. Wong (1974) and Adegeye (1975) confirmed Day's findings but Adegeye also showed by using the indirect immunofluorescence technique that the bacteria are occasionally present in ray cells, fibres and wood parenchyma. In a study by Preece *et al.* (1979), using the toluidine blue staining method, *E. salicis* was found in the watermark stained wood and only a few vessels were filled with the bacteria within the stained wood. Tyloses are produced in some of the vessels as a result of infection (Day, 1924; Metcalfe, 1940, 1941; Wong, 1974). Wong also working on the mechanisms of the dark stain production stated that the responsible substances were mixtures of various polyphenolic compounds, lignin-like compounds, pectate and cellulosic substances. He also noted that infected vessels seemed to have thickened walls. This thickening is caused by the deposition of coloured lignin-like compounds (Wong and Preece, 1978).

1. 6 The causal organism, *Erwinia salicis* (Day) Chester

1. 6. 1 The bacterium

The genus *Erwinia* is in the family *Enterobacteriaceae* and comprises 15 species (see Table 1. 1). These are divided into three groups which reflect the ecological diversity of these bacteria.

Table 1. 1 The genus *Erwinia* (Buchanan and Gibbons, 1974).

Group	Species
Amylovora (vascular wilts)	*E. amylovora, E. tracheiphila, E. nigrifluens, E. quercina, E. rubrifaciens, E. salicis*
Herbicola (epiphytes and pathogens)	*E. herbicola* var. *herbicola, E. herbicola* var. *ananas, E. stewartii, E. uredovora*

Carotovora (soft rots)	*E. carotovora* var. *carotovora, E. carotovora* var. *atroseptica, E. chrysanthemi, E. cyripedii, E. rhapontici.*

E. salicis (Day) Chester is a Gram-negative rod, occurring singly or in pairs but rarely in chains. It is motile by means of 5 to 7 long peritrichous flagella. It is a facultatively anaerobic non-spore forming, capsulated rod (0.5-0.7 x 0.8-1.7 μm) (Dowson, 1937), living in the xylem vessels of *Salix* sp.

1. 6. 2 Cultural characters

On 1 % glycerol nutrient agar moderate growth occurs and after five days incubation at 26 °C the bacteria form small (1- 2 mm) translucent, smooth, golden yellow coloured colonies. *E. salicis* may also be grown on 5 % sucrose nutrient agar, on which it forms characteristic mucoid colonies (Slade and Tiffin, 1984). *E. salicis* gives very poor growth on nutrient agar and its growth in some media is inhibited by agar (Dye, 1968). The yellow pigment is best produced with subcultures on potato plugs or 0.5 % starch potato agar at a pH 6.5, but, most of the isolates lose the ability to produce the yellow pigment after repeated subculture on glycerol nutrient agar (Preece *et al.*, 1979). Descriptions of the biochemical features of the bacterium are given by Dowson (1937), Wong (1974) and Preece *et al.* (1979).

1. 6. 3 Strain variation in *E. salicis*

A few variation have been reported between the isolates of *E. salicis*. In a study of Kam (1976), it was noted that none of the Dutch isolates tested produced yellow pigment on potato plugs and used galactose and raffinose as sole carbon sources. However, English isolates of *E. salicis* grew on galactose and some produced yellow pigment on potato plugs. Only the yellow pigment-producing isolates of *E. salicis* used raffinose as a carbon source. Yellow pigment production seems an unreliable determinative character to distinguish strains of *E. salicis* because Wong (1974) found that some of the pigment-producing isolates lost this ability after repeated subculture. Wong (1974) also showed that English isolates were variable in sensitivity to bacteriophage. He used the NCPPB 1467 phage in his bacterio-

phage sensitivity test and found 4 out of 13 of the English isolates of *E. salicis* reacted negatively.

1. 6. 4 Synonymy

Below is a list of the names used at various times for the causal agent of watermark disease, now known as *Erwinia salicis* (Day) Chester. These are cited by Bradbury (1986).

E. salicis (Day) Chester, 1939
Bacterium salicis Day, 1924
Chromobacterium salicis (Day) Krasil'nikov,1949. He attributes this species to Dowson and considers it different from Day's organism.
Erwinia amylovora var. *salicis* (Day) Martinec and Kocur, 1963
Phytomonas salicis (Day) Magrou 1937
Pseudobacterium salicis (Day) Krasil'nikov, 1949
Pseudomonas saliciperda Lindeijer, 1932
Bacterium saliciperda (Lindeijer) Burgvits, 1935
Phytomonas saliciperda (Lindeijer) Magrou, 1937
Phytobacterium saliciperda (Lindeijer) Magrou and Prévot, 1948 a

1. 6. 5 Host range

Not only has watermark disease been reported in the cricket-bat willow *S. alba* var. *caerulea* but also in other species of *Salix*. Other willow species on which the disease has been found are *S. alba* L. (white willow) and its hybrids, *S. fragilis* L. (crack willow), *S. caprea* L. (goat willow), *S. vitellina* L.(golden ousier), and *S. cinerea* L. (common sallow) (Day, 1924; Metcalfe, 1940; Wong and Preece,1973).

Miller-Jones (1979) inoculated cuttings of *S. alba* var. *caerulea, S. alba* L., *S. cinerea* spp. *atrocinerea, S. caprea* spp. *caprea, S. fragilis* L., *S. viminalis* L., *S. pentendra* L., *S. purpurea* L., *S. alba Sericea, S. repens* L. and *S. triandra* L. with a suspension of *E. salicis*. He concluded that all 11 species tested were susceptible to the disease and *E. salicis* was re-isolated from all 11 species 6 to 12 months after inoculation. *S. viminalis* was indicated to be more resistant to the disease than the other species.

In the Netherlands, Kam (1984) investigated the resistance of willow species to the

watermark disease, both by field observations and artificial inoculations. He observed high mortality in trees of *S. alba* and *S. alba* x *S. fragilis*. Occasional wilting was reported in *S. fragilis, S. viminalis, S. caprea* and *S. cinerea*. The disease has been induced experimentally in *S. amygdalina* and *S. purpurea*.

E. salicis has also been isolated from healthy aspen (Bacon and Mead, 1971).

1. 7 Transmission of watermark disease

Insects, rain splash, the transport of infected planting materials, infected fruit and seeds and the use of contaminated cutting tools are the most important agents of spread of many bacterial plant diseases. However, the infection route, infection process, transmission and survival of *E. salicis* is not known.

It has been shown that the bacterium can be transmitted in more than one way. Whatever the transmission route is, the disease only occurs in the xylem and this tissue must be infected by the bacterium. Artificial inoculations are rarely successful. Adegeye and Preece (1978) reported the lack of movement of *E. salicis* in the young willow shoots artificially inoculated. The only effective inoculation method has been to introduce the bacterium into deep wounds in mature wood and even then less than 10 % of the inoculations resulted in disease (Wong, 1974).

There are several possible ways in which the disease might be therefore transmitted:

1. 7. 1 Infected propagating material

The disease might spread by means of propagation material, since the cricket-bat willow is always propagated vegetatively. *E. salicis* was isolated from “symptomless” trees and from “symptomless” shoots growing on diseased stumps (Wong and Preece, 1973). These symptomless infected trees showed neither wilting nor the typical watermark stain in the wood. Gremmen and Kam (1975) showed that although the stools may function as sources of disease, *E. salicis* does not normally migrate into the one or two year old cuttings. As a result of this work, willow growers were advised to use one and two year old propagation material only. Although one-year old cuttings were used for many road plantations in the Netherlands, these were seriously attacked by the watermark disease. Kam (1983) carried out a new study using a direct immunofluorescence detection technique. He concluded that *E. salicis* was only transmitted by means of one year old cuttings in excep-

tional cases, thus confirming the results of Gremmen and Kam, 1975.

One recent study on the epidemiology of watermark disease has significant results (Turner *et al.*, 1992) which appear to question the conclusion of Kam (1983). With the use of an extremely sensitive double antibody sandwich ELISA for *E. salicis,* based on polyclonal antibody, the bacterium was detected in the symptomless 1-4 year old sets and in the symptomless stools producing these sets.These results indicate that *E. salicis* is present in the symptomless wood. Infected stools having no external symptoms of watermark disease could therefore be a source of the disease which would be transmitted via infected symptomless sets.

1. 7. 2 Soil, root grafts

Wong (1974) showed that when freshly cut shoots were inserted into heavily contaminated soil, the bacterium was able to enter the willow cuttings. This was supported by data from a field survey carried out in Essex in 1972 (Wong *et al.*, 1974). Three hundred and one cricket-bat willows were found to be diseased. Half of the infected trees (154) were either planted amongst the roots of old felled diseased trees or were very near (1.0 to 7.6 m) to a diseased stump. When the root system of a newly infected tree was dug out and examined, it had many root grafts with the living stump of a diseased tree which had been felled two years before. *E. salicis* was isolated from both sides of the graft union in five typical unions examined in the laboratory.

1. 7. 3 Cutting tools

Cricket-bat willows are regularly pruned to keep the lower part of the trunk clean from side shoots and to obtain good quality timber. The possibility of transmitting the disease via contaminated secateurs used for cutting twigs was tested by Wong (1974). Thirty cuts were made into shoots on ten rooted willow cuttings with infected secateurs which had been used to cut diseased wood just before a healthy shoot was cut. The pathogen was isolated from 1 out of 30 shoots 180 days after inoculation. It is apparent that although cutting tools may carry the bacterium, they do not significantly transmit the disease.

1. 7. 4 Insects

The work of Lindejer, 1932 on the disease in the Netherlands showed that the willow weevil, *Crytorrhynchus lapathi,* was a vector. She allowed the beetle to feed on a watermark diseased willow and subsequently transferred it to a healthy tree. She saw typical disease symptoms in the tree but, she could not isolate the bacterium from the wood (Callan, 1939). However, attempts by Callan, 1939 to repeat to Lindejer's experiments with *C. lapathi* gave negative results.

The willow wood wasp, *Xiphydria prolongata,* was thought as a vector by Gray (1940). But he could not succeed in obtaining transmission. Willow gall midge, willow sawfly, goat moth, several species of weevil and small birds are suspected as vectors but it has not yet been proved (Nash, 1963).

1. 7. 5 Rain and wind

Van Der Zweep and Kam (1982) detected *E. salicis* on a *Crataegus* shrub and on an *Alnus* tree standing close to a "wilting" *Salix* by using the direct immunofluorescence technique. Bacteria were also seen on leaves of *Fraxinus excelsior, Populus tremula, Sambucus nigra, Holcus lanatus* and *Dactylus glomerata* around a "wilting" tree. *E. salicis* could not be found in a locality where the disease was absent. It was concluded that *E. salicis* was dispersed from a wilting tree to the whole surrounding vegetation by rain and wind.

1. 8 The distribution of watermark disease

Watermark disease occurs in the Netherlands and in the East Anglian counties of Great Britain and it has the potential to cause considerable financial loss in both countries. In the U.K surveys of the distribution of the disease have been carried out by Dowson and Callan (1937), Wong *et al.* (1974) and Miller-Jones (1979). The disease have been reported in Suffolk, Norfolk, Cambridgeshire, Essex, Bedfordshire and Hertfordshire.

The disease was reported in most parts of the Netherlands and especially on one variety of the white willow *Salix alba* var. *Liempde* (Amels, 1981). It is more severe in Holland than in England. In Amsterdam alone, from 1970 to 1986 the total loss was 30 % (Couenberg, 1989).

The disease also occurs in the northern part of Belgium (Rijckaert *et al.*, 1984).

Bradbury (1986) recorded the disease in Japan and Austria. Austrian trees were found with symptoms but the disease has not been confirmed there.Unconfirmed report also suggests that watermark disease may occur in the United States (Hartley and Crandall, 1935).

1. 9 Control of the disease

The only known method of controlling watermark disease is to destroy diseased trees and stools. This practice is enforced by a Statutory Instrument (Preece and Wortley, 1979). The first watermark Disease Order was introduced in 1933 to authorise the Essex County Council to appoint officers to inspect any willow trees, shrubs, stools and sets growing in that county. If a willow is found which has a typical symptom, an affected branch is cut off and checked for the presence of watermark stain. If watermark is confirmed in the tree, the owner is notified and he is required to burn the tree on the spot. Similar orders were than brought into force in the adjoining counties of Suffolk (1953), Cambridgeshire (1958), Norfolk (1959), Bedfordshire (1962) and Hertfordshire (1967) (Wong, 1974). By arrangement with the local authorities concerned, the fieldwork in all counties was carried out by officers on the staff of Essex County Council. Twenty three thousand,one hundred and seventy two diseased willows and 911 mother trees (stools) were destroyed (see Plate 1. 9) during 1955-1972 (Wong *et al.*, 1974). In the early years the order proved its effectiveness. A significant reduction was observed in the number of watermark diseased stools from 1955 to 1978 (see Fig. 1. 1) and in the number of watermark diseased cricket-bat willows found during inspection from 1955 to 1985 (see Fig. 1. 2) (Wortley, 1989). There have been markedly fewer diseased trees since 1966 and the number of new diseased trees found each year has not altered greatly since 1974 (Wortley, 1989)

The disease occurs in various wild *Salix* species in England (Metcalfe, 1940; Wong and Preece, 1973). Experimental work has shown that other *Salix* species and hybrids are very susceptible to the disease (Miller-Jones, 1979). Therefore, breeding for disease resistance is not likely to be a solution to the problem.

Several bactericides will reduce the disease, but the necessary injection process, as in the control of Dutch Elm disease, is technically difficult and very expensive (Preece and Wortley, 1979).

Heat, gamma radiation and chemicals were tested for the control of watermark disease in a series of laboratory experiments (Amels, 1981). None of the three methods investigated proved to be completely successful.

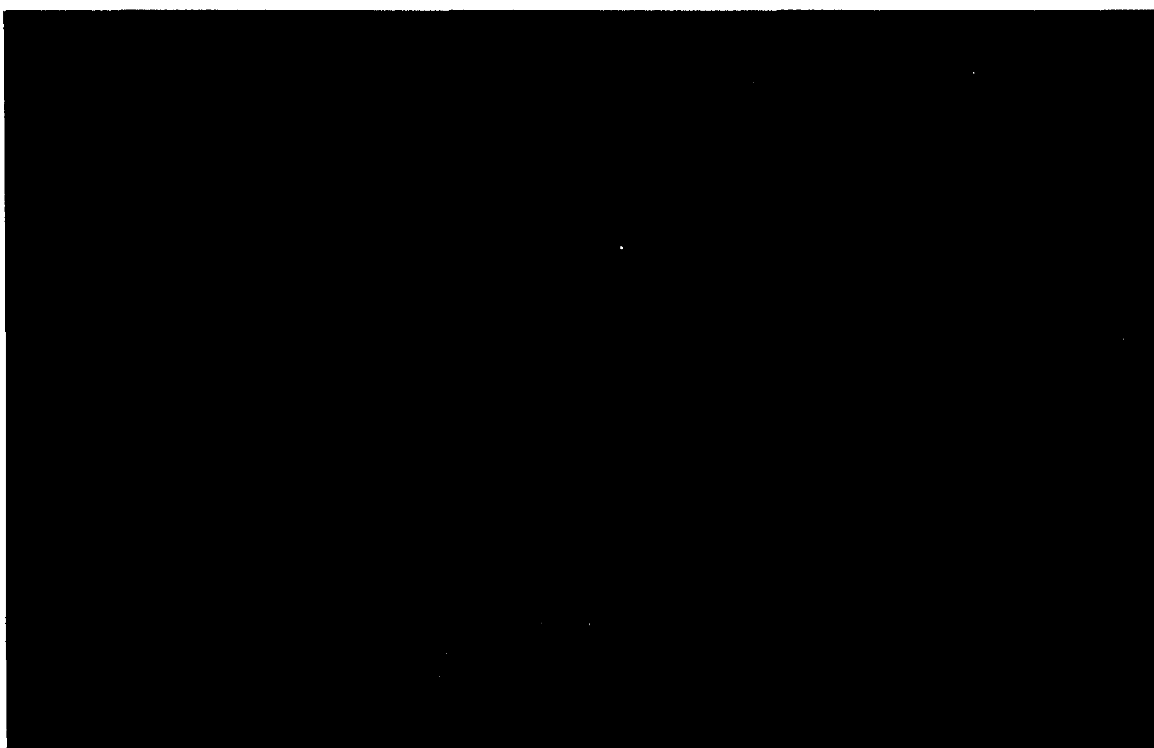

Plate 1. 9 Transverse section of a felled cricket-bat willow which had watermark disease.

No of diseased stools

85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5

1038

1955 '56 '57 '58 '59 '60 '61 '62 '63 '64 '65 '66 '67 '68 '69 '70 '71 '72 '73 '74 '75 '76 '77 '78
year

Figure 1. 1 Number of *Salix alba* stools with watermark disease symptoms found in Essex, Suffolk and Hertfordshire during inspection from 1955 to 1978 (Wortley, 1989).

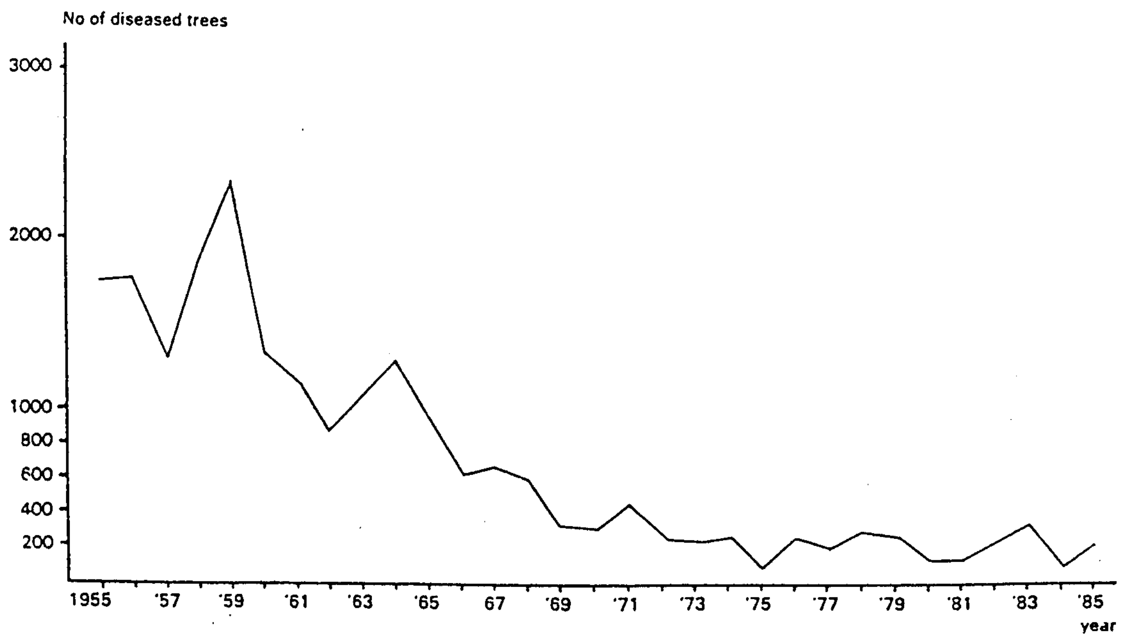

Figure 1. 2 Number of watermarked cricket-bat willows found during inspection from 1955 to 1985 (Wortley, 1989).

1. 10 Bacterial typing for epidemiological studies

Many bacterial diseases are controlled by cultural techniques designed to prevent transmission of the pathogen.

One of the factors hindering progress in control of watermark disease is our ignorance of the mechanism by which the disease is transmitted, and of the epidemiology of the disease. In order to achieve effective control of watermark disease it is therefore important to know the epidemiology of the disease.

A "strain" or a "clone" is a population of genetically identical cells. A strain is a pure culture and individual species of bacteria consist of many clonal populations or strains. Variation between strains of a bacterial pathogen can provide markers for epidemiological studies. For example, by examining a large number of *Listeria monocytogenes* isolates collected from diverse geographic locations in the United States it was possible to identify a single common source (Mexican-style cheese) as a cause of a listeriosis outbreak (Bibb *et al.*, 1990). The epidemiological investigations of this type therefore normally require bacterial characterisation at the subspecies level. Clonal populations or strains can be distinguished by one or a combination of molecular and physiological methods, e.g. physiological profiling, bacteriophage typing, bacteriocin typing, serotyping, RFLP typing and multilocus enzyme electrophoresis typing. Many of these techniques have been developed in the medical field for human pathogens and then adopted by the plant bacteriologist. Some show considerable promise for plant pathogenic bacteria.

1. 10. 1 Physiological profiling

Metabolic properties of a bacterium are detected and determined by studying the utilisation of nutrients, the production of metabolites, or the presence of typical enzymes. The pattern of utilisation of carbon sources plays an important role in the differentiation of species. For example, the ability of certain microorganisms to utilise organic acids-such as acetate, citrate and malonate-as sole source of carbon for metabolism has been useful especially in differentiation of Gram-negative rods. In thiobacilli, the utilisation and formation of sulphur compounds serves in identification (Kuenen and Tuovinen, 1981).

In diagnostic bacteriology such specific biochemical tests are routinely performed for the identification of bacteria. The determination of the biochemical reaction pattern (biotype) is commonly used in clinical laboratories. The usefulness of biotype profiles depends

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on the number of the tests performed and the reproducibility of the individual tests. The commercial development of compact biochemical systems for the identification of bacteria makes it relatively simple to conduct a large number of tests for biotyping. These tests have been designed for medical diagnosis and do not necessarily serve the needs of the plant bacteriologist. One of these is the Analytab Enteric (API 20E) test system which consists of a miniaturised set of 20 biochemical tests. API 20E series is designed for the diagnosis of diseases caused by members of *Enterobacteriaceae,* which contains the plant pathogenic erwinias. However, *Erwinia* spp. are not mentioned in the diagnostic manuals or computer programmes which accompany the kits (Lelliott and Stead, 1987).

The API 20E system of physiological profiling has been successfully tested in many clinical laboratories (Murray, 1978; Freeman *et al.*, 1981) and for the identification of Gram-negative bacteria of brewing origin (Ingledew *et al.*, 1980). As yet little has been published on their use for diagnosis of bacterial plant pathogens. An exception is the genus *Erwinia* which can be identified by the use of the API 20E system (Mergaert *et al.*, 1984 and Verdonck *et al.*, 1987).

1. 10. 2 Bacteriophage typing

Many plant pathogenic bacteria are difficult to distinguish from closely related pathogens and from associated epiphytic bacteria. Conventional cultural and physiological tests are sometimes sufficient to identify the pathogen. However, such tests are often inadequate to distinguish strains of the bacterium.

Bacteriophages often multiply only in a restricted range of host bacteria and they affect their bacterial hosts in one of two ways: a 'virulent' bacteriophage lyses (disrupts and kills) its host cell, while a 'temperate' bacteriophage can establish a stable relationship which is called 'lysogeny' with the (living) host cell. The genetic material of temperate bacteriophages is integrated into the host genome and is thus duplicated along with the host material at the time of cell division, being passed from one generation of bacteria to the next. However, under certain conditions the lysogenic relationship breaks down and the bacteriophage initiates a lytic cycle, destroying its host. Such bacteria, which appear uninfected but have the hereditary ability to produce phage, are called 'lysogenic'. The host-specificity of bacteriophages forms the basis of many useful techniques. Bacterial strains can be typed by differential patterns of sensitivity to bacteriophages and are called "lysotypes". With the bacteriophage typing method it is possible to study the spread of bacterial infections using lyso-

types as stable markers and to relate the occurrence and distribution of the disease to particular inoculum sources. Bacteriophage typing done with care and consistency is useful in epidemiological studies because it is a relatively simple and reliable technique for differentiating numerous strains. A useful introduction to the principles of bacteriophage-typing, whatever approach is used, is given by Adams (1959).

In human and animal pathology, bacteriophage typing has proved of great value in epidemiological studies of some bacterial pathogens, e.g. *Staphylococcus epidermidis, S. aureus, Listeria* and *Pasteurella multocida* (Gershman *et al.* , 1988; Hartstein, 1989; Audurier *et al.*, 1984; Nielsen and Rasdahl, 1990).

Although bacteriophages specific for many plant pathogenic bacteria have been identified, few bacteriophage-typing systems have been developed for phytobacteria (Billing and Garret, 1980). Forecasting outbreaks of leaf blight of rice has been aided by the use of specific bacteriophages (Mizukami and Wakimoto, 1969). An increase of bacteriophage specific for *Xanthomonas oryzae* in nursery, paddy field or irrigation water was observed prior to an outbreak of disease. Thus, it was possible to forecast disease in the field by monitoring bacteriophage populations in nursery water. The differentiation of cherry and plum strains of *Pseudomonas syringae* pv. *morsprunorum* by bacteriophage typing was used in the epidemiological studies on bacterial blight of stone fruit trees in South-East England. The unique sensitivity of cherry strains to bacteriophage A7 and the stability of cherry and plum phage strains during passage through both hosts (Crosse and Garrett, 1970) enabled cross inoculation and mixed infection experiments to be monitored (Persley, 1983). Bacteriophages of *Erwinia amylovora* distinguished capsulated from noncapsulated strains (Billing, 1960) and to some extent virulent from avirulent strains (Bennett and Billing, 1978).The geographical distribution of *Xanthomonas campestris* based on a bacteriophage typing scheme with 11 specific bacteriophages of the bacterium was reported by Liew and Alvarez (1981). Gross *et al.* (1991), developed a bacteriophage-typing system for surveying the diversity and distribution of strains of *Erwinia carotovora* in potato fields.

1. 10. 3 Bacteriocin typing

Bacteriocins are non-replicating, proteinaceous antibiotics with specificity generally restricted to closely related bacterial strains and species. Small amounts are produced spontaneously in cultures of bacteriocinogenic strains, but the substances also can be induced by treating cells with ultraviolet light or mitomycin C. In many of their properties, particularly

in their specificity of action, bacteriocins resemble bacteriophages. They differ principally from bacteriophages in that they are not capable of reproducing in host bacterial cells (Bradley, 1967). Because bacteriocins specifically kill the cells of sensitive bacteria, bacterial isolates from distinct sources can be differentiated or typed by their sensitivity to panels of bacteriocins.

Bacteriocins have found widespread application in epidemiological studies as specific markers for bacteria. Various typing schemes have been based upon either the production of, or sensitivity to, a range of different bacteriocins (Tagg *et al.*, 1976).

Typing by bacteriocins has been very useful in epidemiological studies of human pathogens. The first publication demonstrating the epidemiological relevance of bacteriocin typing was by Shannon (1957) who reported his method of typing *E. coli* 055:B5 strains by means of colicin production. Combined bacteriocin production/ sensitivity typing has been used for the fingerprinting of human oral streptococci (Kelstrup *et al.*, 1970). By use of 10 producers and 10 indicators it was possible to demonstrate individual patterns of reactivity associated with 38 of 40 strains tested.The bacteriocin activity of an epidemic strain of *Staphylococcus aureus* was used as a marker for the rapid screening of staphylococci from cases and carriers and was said to have aided the management of the outbreak (Anthony *et al.*, 1972).

Bacteriocins of phytopathogenic bacteria have been little studied (Okabe and Goto, 1963 and Vidaver *et al.*, 1972) and most of the few studied are listed by Vidaver (1976). Bacteriocin production by *Corynebacterium michiganense* was studied by Echandi (1976). Typing of *C. michiganense* isolates with the four selected strains permitted differentiation of 10 types which included 96 % of the isolates. No correlation was found between bacteriocin type and virulence to tomato plants. Echandi and Moyer (1979) divided 18 strains of *Erwinia chrysanthemi* into five types using three strains as bacteriocin producers.

The possibility of using bacteriocin typing in plant epidemiological work, as has been done with human and animal pathogens, remains to be explored (Starr and Chatterjee, 1972).

1. 10. 4 Serotyping

An "antigen" is a substance that elicits a specific immune response when introduced into the tissues of an animal. A bacterial cell contains many antigens i.e. lipopolysaccharides, proteins, mucopolypeptides. An "antibody" is a type of globulin protein that is

formed during the immune response. Studies of antigen- antibody reactions constitute the methods of serology. A variety of different kinds of serological reactions can be observed, depending on the natures of antigen and antibody and on the conditions chosen for reaction.The antigenic components of a particular organism are characteristic and are likely to comprise both organism-specific and group-specific determinants. When strains are distinguished primarily on the basis of differences in their antigens they are referred to as "serotypes". The major limitation of serodiagnostic tests is the ability of antibodies to react "in vitro" with antigens of slightly different structures. This can result in cross-reactions where the serum raised against one bacterium reacts with antigens of another strain or species. This necessitates the strict use of controls in serological tests. Serological methods provide quick and accurate tests for the identification and differentiation of bacteria.

Serological methods may be divided into three broad categories;

a. The formation of insoluble antigen-antibody aggregates which can be seen either directly (e.g. in tube or microprecipitin tests, ring interface test, gel diffusion assays) or indirectly by using inert particulate markers (e.g. latex and chloroplast agglutination methods).

b. Individual antigen units or small numbers of such units, are reacted with antibody. They are immobilised onto a solid phase and either visualised directly (e.g. immunospecific electron microscopy) or via an associated label (e.g. enzyme-linked immunosorbent assay).

c. Miscellaneous other assays (e.g. growth inhibition, infectivity neutralisation, deformation assays).

Serological methods for detection and identification of pathogens have been used by medical diagnosticians for many years. Serotyping has been utilised and has proved useful in the epidemiological study of antigenically related strains of *Vibrio cholera* and *V. mimicus* (Sakazaki and Donovan, 1984), *Neisseria gonorrhoeae* (Tramont *et al.*, 1976), *N. meningitidis* (Griffiss *et al.*, 1977) and *Legionella pneumophila* (Brown *et al.*, 1985 and Edelstein *et al.*, 1986).

Serological methods for detection and identification of pathogens have been available to plant pathologists for many years (Schaad, 1979). Serological techniques and its applications in plant pathology has been reviewed by Barbara and Clark (1986), and Miller and Martin (1988).The first report on the use of serology to identify a plant pathogenic bacterium was published in 1918 when Jensen showed that a strain of *Agrobacterium tumafaciens* from Denmark could be differentiated from a strain of *A. tumafaciens* from the United States, by the agglutination test (Schaad, 1979).

Ouchterlony double diffusion technique permits identification of antigens in a mix-

ture and can establish relationships between antigens. It can be used to arrange strains of bacteria into serogroups without complete knowledge of all the antigenic groups that may be present. It was used to distinguish strains of *Erwinia carotovora* var. *atroseptica* (Tanii and Akai, 1975), *E. carotovora* (Stanghellini *et al.*, 1977 and De Boer *et al.*, 1979) and *E. chrysanthemi* (Yakrus and Schaad, 1979).

1. 10. 5 Restriction fragment length polymorphism (RFLP) analysis

RFLP analysis makes use of restriction enzymes to fragment DNA, which is then separated by agarose-gel electrophoresis. Each restriction enzyme recognises a specific nucleotide sequence and cuts the DNA specifically every time the sequence occurs. The fragmented DNA is separated by gel electrophoresis, and the banding pattern is made visible by staining (ethidium bromide) or by autoradiography. The differences in the size and number of bands can result from inserts or deletions between existing restriction-enzyme sites. The observed differences in restriction fragments are known as restriction length polymorphisms (RFLPs) and RFLP analysis detects differences between the genotypes.

RFLP analysis have been used for diagnosis of human diseases e.g. sickle cell anaemia (Miller-Martin, 1988) and epidemiological typing of *Yersinia enterolitica* to identify different RFLP types within biogroups/serogroups which were undistinguishable by other means (Andersen and Saunders, 1990).

RFLP analysis have also been used to distinguish strains of plant pathogenic bacteria, e.g. *Xanthomonas campestris* (Gabriel *et al.*, 1988 and Graham *et al.*, 1990), *Pseudomonas solanacearum* (Cook *et al.*, 1989), the symbiotic bacterium, *Rhizobium leguminosorum* (Young *et al.*, 1987) and *E. salicis* (Hall, 1990).

These studies showed that the population structure of the bacteria was clonal and many of the strains distinguished correspond to biologically significant subgroupings of the species.

1. 10. 6 Multilocus enzyme electrophoresis typing

Multilocus enzyme electrophoresis typing (M.E.E.) analyses the variation between clones and provides extensive data for systematics and useful marker systems for epidemiology, by using enzymes as markers of variation in the underlying genes. A change in the underlying genes often leads to an amino acid substitution in the resulting polypeptide. This in

turn may change the net surface charge of a protein and thereby alter its mobility through on electrophoresis gel matrix. Electromorphs (mobility variants) of enzymes can be equated with alleles at the corresponding structural gene loci. Individuals from the population with distinct and characteristic sets of enzyme mobilities are referred as electrophoretic types (ETs).

Multilocus enzyme electrophoresis has long been a standard method in eucaryotic population genetics and systematics. It has been used in large-scale studies to estimate genetic diversity and structure in natural populations of a variety of species of bacteria (Selander *et al.*, 1986), and has recently been used to elucidate the epidemiology of several species of clinical bacteria e.g. *Pseudomonas aeroginosa* (Levin *et al.*, 1984), *Legionella pneumophila* (Selander *et al.*, 1985) and *Listeria monocytogenes* (Bibb *et al.*, 1990). A short review on the use and efficacy of multilocus enzyme electrophoresis in bacterial population genetics, systematics and epidemiology of several human pathogenic bacteria has been given by Selander *et al.*, (1987).

Multilocus enzyme electrophoresis has been used as a typing method for plant pathogenic bacteria, for the differentiation of genera of phytopathogenic bacteria (El-Sharkawy and Huisingh,1971 a), *Xanthomonas* species (El-Sharkawy and Huisingh, 1971 b), *Corynebacterium* spp. (Robinson, 1966), and isolates of *Pseudomonas solanacearum* and *P. cepacia* (Baptist *et al.*, 1971). It has also been used for the identification of *Rhizobium* strains (Mytton *et al.*, 1978). Results demonstrate the applicability of the technique for differentiating between phytobacterial genera and between species of a given genus.

1. 11 Isolation methods for bacteria

Since indirect evidence indicates that *E. salicis* may be present in the symptomless wood, it is important to test this by isolation of the bacterium from symptomless wood. Ideally the bacteria which will be used in a typing scheme should be isolated from not only diseased wood having external symptoms of the disease but also from symptomless infected trees, sets and stools.

The isolation of plant pathogenic bacteria is often done by direct 'streaking' suspension of plant material onto agar media; direct isolation from ooze and exudates, soaking of infected plant tissue in water or buffer, or maceration of plant tissue and preparing a suspension of bacteria from the infected material and streaking loopfuls of the suspension onto agar

plates. Subsequently, selected colonies can be purified by several subcultures. Conventional isolation methods are sufficient to isolate the pathogen from heavily infected samples having typical external symptoms of the disease.

Detection and isolation of bacteria in the symptomless host is often difficult. Low numbers of the target organism, coupled with high populations of contaminating organisms may hinder the recovery process and may prevent determination of the pathogen in the host.

The selectivity of isolation methods has been improved by a number of procedures, including the use of selective media (McGuire *et al.*, 1986), use of host tissue for the selective enrichment of a target organism (Jones *et al.*, 1981) and enrichment in the culture medium (Meneley and Stanghellini, 1976).

Immunoisolation, one new approach, combines the advantages of specificity of serological assay with conventional isolation techniques. New techniques e.g. column immunoaffinity isolation, CIAI, (Hranitzky *et al.*, 1980), immunosorbent dilution plating, ISDP, (Van Vuurde and Van Henten, 1983) and immunosorbent enrichment, ISE, (Van Vuurde *et al.*, 1987) have been described and experiments involving many plant pathogenic bacteria showed the value of ISDP for isolation of *Pseudomonas syringae* pv. *phaseolicola* and *Corynebacterium michiganense* spp. *michiganense* from a mixture of these bacteria (Van Vuurde and Van Henten, 1983).

The reliable isolation of a target bacterium present in low concentrations still may not be possible due to cross-reacting colonies of different species. Recently, new serological colony characterisation techniques, direct immunodiffusion, antiserum mixed agar plating and immunofluorescence colony staining (IFC), have been developed for identification of colonies directly on the plating medium and IFC proved more suitable and reliable than the other two above methods for further serological characterisation of the target colony in the agar (Van Vuurde, 1987).

Immunofluorescent colony staining combines both cultural and serological characteristics of the pathogen. The technique uses fluorescent dye-conjugated antibodies for staining of colonies of the pathogen, and serologically related bacteria, and detects them with an incident ultraviolet light microscope at low magnification. Colonies giving a positive reaction still contain viable cells and can be subcultured to isolate the target bacterium for further research. Experiments with the IFC have demonstrated its value in detecting various plant pathogenic bacteria, representatives of plant pathogenic *Erwinia* spp. (Van Vuurde and Roozen, 1990), *Pseudomonas syringae* pv. *phaseolicola* , *Clavibacter michiganensis* subsp. *michiganensis* in complex natural sources (Franken and Van Vuurde, 1990) and

Xanthomonas campestris pv. *begoniae* in washings of begonia leaves (Van Vuurde, 1990 a).

Immunomagnetic separation, IMS, was also recently developed to trap specific cells from heterogeneous cell suspensions using antibody-coated magnetisable beads (Lea *et al.*, 1985). Subsequently, separation of the target cells from the suspension is carried out with a high strength magnetic field. The technique was applied for cell sorting, subcellular organalle fractionation (Lea *et al.*, 1985, 1988 and Vartdal *et al.* , 1986) and DNA separation (Uhlen, 1989). The technique has been used for detection and isolation of some bacteria e.g. *Escherichia coli* (Lund *et al.*, 1988), *Staphylococcus aureus* (Johne and Jarp, 1988), *Listeria monocytogenes* (Skjerve *et al.*, 1990) and plant pathogenic *Xanthomonas campestris* pv. *pelargonii* (Jones and Van Vuurde, 1990).

1. 12 Aim of research

It is not known whether the watermark disease is spread from one tree to another or is transmitted in infected propagating material. The lack of information on the epidemiology of watermark disease arises partly, because;

– artificial inoculations have very small chance of success ca 10 % (Wong, 1974),

– the symptoms of the disease have not been seen in the trees younger than 4-5 years old (Day, 1924; Dowson, 1937) and only quite exceptionally in trees younger than 7 or 8 years old. Thus, in the experimental work trees more than 4-5 years old are needed,

– even in the case of achieving artificial infection it can take more than 1 year to see the symptoms of the disease.

Because of these reasons, the study of epidemiology of the disease by conventional means is extremely difficult, and therefore a different approach is required. An alternative way to investigate epidemiology of the disease is to examine the geographical distribution of strains of *Erwinia salicis*. This, together with information on the history of sites may provide information on the likely mechanism of transmission.

The first aim of this project was to develop typing methods i.e. physiological profiling, bacteriophage typing, bacteriocin typing, serotyping and multilocus enzyme electrophoresis typing for *Erwinia salicis* and to employ these in an epidemiological study of watermark disease. The second aim was to test for the presence of latent infections of the disease.

CHAPTER II
ISOLATION AND IDENTIFICATION OF *E. SALICIS*

2. 1 Introduction

The isolation of pure cultures of *E. salicis* from infected wood, and its identification, has formed an essential part of this investigation. However, there are some difficulties in isolating pure cultures of *E. salicis* from watermark diseased willow trees.

Old die-back lesions on the watermark diseased trees contain a great variety of bacteria. Metcalfe (1940), for example, gave details of four different bacteria which he isolated from diseased willows, only one of which could be shown to be pathogenic, and was *E. salicis*. *E. salicis* is isolated most easily when the red leaf symptoms first appear in the infected tree. Young branches and shoots, preferably still with green bark, from affected parts of the diseased tree are generally recognised to be the best material from which to attempt isolation of *E. salicis*.

Another difficulty in isolating pure cultures of *E. salicis* arise from the cultural characteristics of the bacterium itself. The growth of *E. salicis* on ordinary nutrient agar is slow and agar inhibits the growth in some media (Dye, 1968). *E. salicis* forms small (1-2 mm) translucent smooth colonies on 1 % glycerol nutrient agar after 5 days incubation at 26 °C (Slade and Tiffin, 1984). Isolation of *E. salicis* from watermark diseased willows can therefore be unsuccessful if the faster growing organisms overgrow on the plates. A selective medium which will prevent the contaminants from growing and encourage the growth of all viable cells of *E. salicis* would have greatly assisted many experiments for the detection and identification of the bacterium. Several attempts were made to find a selective medium for the isolation of *E. salicis* from the plant tissue, but none of them was sufficiently selective (Amels, 1981). In the present investigation, media were evaluated for their capacity to support the growth of *E. salicis*.

When pure cultures of bacteria from infected plant tissues have been isolated, the next step in diagnosis is normally to conduct a pathogenicity test to reproduce the disease. However, testing the pathogenicity of *E. salicis* is difficult, due to very long incubation time of the disease and small chance of success with the artificial inoculations (Wong, 1974). Wong (1974) overcame these problems using large inocula of *E. salicis* to produce watermark staining in willow cuttings. He wiped the *S. alba* var. *caerulea* shoots with

ethanol and cut the end off below the surface of sterile water. The cut end was covered with a small plastic tube containing water and transferred to a boiling tube containing 25 ml of a 48 hour nutrient broth culture (approximately 10^9 cells/ml) of the isolate to be tested. The small tube was removed with sterile forceps and the boiling tube was sealed with plastic film and wrapped in light proof plastics. The cuttings were incubated in a controlled environment cabinet and the nutrient broth in the tubes replenished as required. After 10-14 days the discolouration of the wood due to action of *E. salicis* was observed by cutting the base of the shoot longitudinally and exposing it to the air for 5-10 hours. Unfortunately, this pathogenicity test was found to be non-reproducible (Kam, M. and Davis, J., pers. comm.). No pathogenicity test has been included in this study and cultural, biochemical and serological tests have therefore been used to confirm the identification of *E. salicis* .

Some of biochemical tests, e.g. nitrate reduction to nitrite, pectinolytic activity and cellulolytic activity have given contradicted results with *E. salicis* (Dye, 1968, 1969; Lakso and Starr, 1970 and Wong, 1974). To increase the number of characters examined, I have used the API 20E compact biochemical tests, which have been previously used for the identification of *E. salicis* by Mergaert *et al.* (1984) , Rijckaert *et al.* (1984) and Verdonck *et al.* (1987).

Serological methods have been successfully used for the identification and detection of *E. salicis* in crude samples obtained directly from the field and in the pure cultures. Wong and Preece (1973) first used a specific antiserum to detect *E. salicis* in the field by using a slide agglutination test. In addition to agglutination and precipitation methods to identify *E. salicis* in pure culture, new serological techniques, notably an Enzyme Linked Immunosorbent Assay (ELISA) and direct immunofluorescence have been developed to detect *E. salicis* within the host plant and as part of the leaf surface microflora (Kam, 1982 a, b; Van Der Zweep and Kam, 1982 and Kam, 1989).

In the present study I have compared 64 pure cultures isolated from 25 watermarked trees with 17 isolates of *E. salicis*, whose pathogenicity has been confirmed, obtained from the NCPPB. I have used cultural tests e.g. motility, biochemical tests e.g. Gram's staining, oxidation/fermentation and oxidase in conjunction with agglutination tests. Meanwhile, these cultures were subjected to a competitive ELISA test, based on polyclonal antibody, developed for *E. salicis* (Davis, J. , pers. comm.).

Seventeen out of 19 presumptive *E. salicis* isolates determined by the tests mentioned above together with 6 authentic *E. salicis* isolates from the NCPPB were further ex-

amined by the API 20E tests to confirm the identification. As an additional criteria, bacteriophage sensitivity tests developed for the differentiation of strains of *E. salicis* have been included to assist identification.

Fifteen isolates of *E. salicis* obtained from the Netherlands and 27 cultures isolated from the diseased trees were only tested by their cultural characteristics and ELISA for confirmation and identification.

2. 2 Materials and methods

2. 2 1 Source and maintenance of plant material

The majority of samples of *Salix* species were sent by Essex County Council willow inspector Mr. Ken Cutts and the rest of samples were collected from various sites in Suffolk, Essex, and Wiltshire between 1989 and 1990 by J. Davis, Dr. J. G. Turner and myself. The site of collection and host for the isolates are listed in Table 2. 2. The diseased wood was placed in polythene bags and brought back to Norwich. Cuttings, about 2–3 cm diameter by 10–15 cm long, still with green bark were preferred for isolation of bacteria. Diagnosis in the field was based on the " red leaf " symptom, confirmed by the presence of characteristic watermark stain in the wood. Cuttings were stored at – 20 °C for varying lengths of time up to 1 month before the isolation was attempted.

2. 2. 2 Origin and maintenance of bacterial cultures

Seventeen cultures of *E. salicis* were obtained from the National Collection Plant Pathogenic Bacteria (NCPPB), Plant Pathology Laboratory, Harpenden, Herts. , UK. Fifteen cultures of *E. salicis* were kindly obtained from M. Kam, Dorschkamp Research Institute for Forestry and Landscape planning, Wageningen, the Netherlands. Source details of the bacterial isolates used in these studies are given in Table 2. 2. Stock cultures were grown on nutrient dextrose agar (NDA) (see Chapter 2. 2. 3. 2, medium 5), and stored in the refrigerator and sub-cultured monthly. For long-term storage, fresh broth cultures of the isolates were stored in 15 % glycerol at – 80 °C. For use in experiments, bacterial cultures were prepared on NDA freshly by incubation at 28 °C for three days.

2. 2. 3 Evaluation of media for growth of *E. salicis*

2. 2. 3. 1 Preparation of the inoculum

E. salicis isolate NCPPB 2535 was grown in nutrient dextrose broth (NDB) at 28 °C for 2 days on a rotary shaker. A loopful of inoculum having 10^8–10^9 cells/ml was streaked onto the agar plates of different media. Four plates were inoculated for each of the media and a pair of plates were incubated in anaerobic conditions while the other two plates were incubated in aerobic conditions.

2. 2. 3. 2 Selective media

Ten different media were tested. The first six were media commonly used in plant pathology. No modifications were made because it was known that some of the ingredients would inhibit growth of *E. salicis*. A detailed description of the composition of the media tested is given below ;

Medium 1 : Sucrose peptone agar (SPA)

sucrose	20.0 g
peptone	5.0 g
K_2HPO_4	0.5 g
$MgSO_4 .7H_2O$	0.25 g
agar	12.0 g
distilled water	1 l
pH	7.2 – 7.4

Medium 2 : Nutrient yeast glycerol agar (NYGA)

glycerol	20 ml
yeast extract	3.0 g
peptone	5.0 g
agar	12.0 g
distilled water	1 l
pH	7.0

Medium 3 : Yeast peptone glucose agar (YPGA)

yeast extract	5.0 g
protease peptone	5.0 g
glucose	10.0 g
agar	12.0 g
distilled water	1 l
pH	6.5 – 7

Medium 4 : King's medium B (KB)

protease peptone	20.0 g
glycerol	10.0 g
K_2HPO_4	1.5 g
$MgSO_4 . 7H_2O$	1.5 g
agar	15.0 g
distilled water	1 l
pH	7.2

Medium 5 : Nutrient dextrose agar (NDA)

nutrient agar	23 g
D-glucose	10 g
distilled water	1 l

Medium 6 : Glycerol nutrient agar (reference medium, GNA)

glycerol	10 ml
nutrient agar	23 g
distilled water	1 l

Medium 7 : LB agar

tryptone	15.0 g

NaCl	5.0 g
yeast extract	5.0 g
agar	12.0 g
distilled water	1 l

Medium 8 : M9 medium

A.	KH_2PO_4	3.0 g
	Na_2HPO_4	7.0 g
	NaCl	0.5 g
	NH_4Cl	1 g
	distilled water	0.5 l
B.	Glucose	2 g
	$MgSO_4 . 7 H_2O$	0.2 g
	agar	12 g
	distilled water	0.5 l

A and B were mixed after autoclaving.

Medium 9 : P. lachryman

sucrose	12 g
yeast extract	1.0 g
casein hydrolysate	2.5 g
agar	15 g
distilled water	1 l

Medium 10 : Coon's agar

Maltose (dextrose)	4.0 g
KNO_3	2.0 g
$MgSO_4 . 7 H_2O$	1.2 g
KH_2PO_4	2.68 g

agar	20 g
distilled water	1 l

Agar was dissolved in 250 ml of distilled water by autoclaving. Maltose, KNO_3 and Mg $SO_4.7\,H_2O$ were dissolved in 500 ml of distilled water. KH_2PO_4 was dissolved separately in 250 ml of distilled water and these two solutions were added to molten agar.

In the preparation of other media except Coon's agar all ingredients were added prior to autoclaving. The pH of the media was adjusted with 1N sodium hydroxide solution prior to autoclaving.

2. 2. 3. 3 Cultivation of *E. salicis* in aerobic and anaerobic conditions

Duplicate sets of *E. salicis* NCPPB 2535 on each of the media was prepared by streaking out onto agar plates. One set of plate cultures were incubated at 28 °C for 3 days for aerobic growth. Another set of plate cultures was placed in an inverted position inside a GasPak chamber. The anaerobic indicator strip was exposed and placed inside the anaerobic jar so that the wick is visible from the outside. Ten ml of water was added to the gas generator and the lid was quickly sealed. The sealed jar was placed in the incubator at 28 °C for 3 days. A colour change to colourless in the indicator strip indicated the anaerobic conditions.

2. 2. 4 Isolation of the pathogen

For isolation, the bark was peeled from infected material using sterilised forceps. The end of the branch was then cut across with a pair of sterilised secateurs. Sap was squeezed from the freshly cut surface using a vice.The sap was then streaked directly onto glycerol nutrient agar (see Chapter 2. 2. 3. 2, medium 6), and incubated at 28 °C and examined daily. Colonies of the bacterium became visible after 2 to 3 days, and with some samples colonies did not appear until 4-5 days after inoculation. Single colonies were subcultured and their biochemical and serological characteristics were compared with known isolates of *E. salicis* from the NCPPB and the Netherlands.

2. 2. 5 Biochemical tests and motility

2. 2. 5. 1 Gram's staining reaction

Hucker's modification of this staining procedure was used as described by Lelliott and Stead (1987) using 95 % ethanol as a decolourising agent and 0.25 % aqueous safranin as a counterstain. Smears were prepared on the clean microscope slides using discrete colonies from 48 hour-old NDA cultures.

2. 2. 5. 2 Oxidase test

Kovacs' oxidase test depends on the presence in bacteria of enzymes which catalyse the transport of electrons between electron donors in the bacteria and a redox dye, tetramethyl *p*- phenylenediamine . On donating electrons to an electron acceptor the dye is oxidised to give a deep purple colour. The filter paper method (Lelliott and Stead 1987) was used. A freshly prepared 1 % aqueous solution of tetramethyl *p*- phenylenediamine dihydrochloride was used to soak a Whatman's No. 1 filter paper disc and this wet filter paper was placed inside a petri dish. The colony to be tested was smeared on the paper using a microbiological loop. A positive reaction was indicated by an intense deep purple blue, appearing within 30 seconds. The production of a purple colour in 30–60 seconds (called a ' delayed' positive reaction by some workers), after 60 seconds and complete absence of colouration were recorded as negative reactions.

2. 2. 5. 3 Test for Oxidative – Fermentative metabolism (O/F reaction)

Hugh and Leifson's O/F test depends on the use of semi-solid medium containing carbohydrate (glucose in this case) together with a pH indicator. The test bacteria were inoculated in duplicate into Hugh and Leifson agar (Lelliott and Stead, 1987) by stabbing with a needle down to the bottom of the medium. One of each pair was then sealed with sterile paraffin oil to a depth of 1-2 cm to give anaerobic conditions. If glucose was oxidised aerobically, producing acid, the medium in the open tube turned yellow from the top downwards. If glucose was fermented (anaerobic) the indicator in both tubes turned yellow.

2. 2. 5. 4 API 20E test

2. 2. 5. 4. 1 Preparation of the API 20E test strips

The API 20E test kit including API 20E test strips, report sheets and one of API 20E instruction manual was obtained commercially (API System, La Balme Les Grottes, 38390 Montalieu, Vercieu, France) and they were handled as described by the manufacturer with some minor modifications. Test strips were stored at 4 °C until use. An incubation box was prepared and some water was distributed in it to create a humid chamber. Isolate reference was recorded on the elongated tab of the tray. The strip was removed from its packaging and placed in the tray just before using.

2. 2. 5. 4. 2 Preparation of the inoculum

Twenty three isolates of *E. salicis* (see Table 2. 3) were grown on NDA plates at 28 °C for 3 days. Five ml of sterile distilled water without additives was put into a test tube. Bacterial growth on the plates was harvested and cells were suspended in the distilled water at a density of approximately 10^8 cells/ml as recommended by Mergaert *et al.* (1984).

2. 2. 5. 4. 3 Preparation of the reagents

TDA reagent, IND reagent and Voges Proskauer reagents of VP1 and VP2 were prepared freshly in the distilled water. Composition of these reagents is given in Table 2.1.

2. 2. 5. 4. 4 Inoculation of the test strip

Both the tube and cupule of tests CIT, VP and GEL were filled with the bacterial suspension. Only the tubes (and not the cupules) of the other tests were filled with the bacterial suspension. Anaerobiosis was created in the tests ADH, LDC, ODC, URE and H_2S by overlaying sterile mineral oil into the cupules of these tests. The test strips were placed in the humid chamber and incubated at 28 °C, as *E. salicis* isolates were not able to grow at 37 °C.

Table 2. 1 Composition of the reagents used in API 20E test of *E. salicis*.

TDA reagent

ferric chloride	3.4 g
distilled water	100 ml

IND reagent

paradimethylaminobenzaldehyde	5 g
iso–amyl alcohol	75 ml
HCl 37 %	25 ml

Voges Proskauer reagents

VP1	
potassium hydroxide	40 g
distilled water	100 ml
VP2	
alpha naphtol	6 g
ethanol	100 ml

2. 2. 5. 4. 5 Reading of the strip

After 24 hours incubation the API 20E test strips were checked but since no colour development was observed, the test strips were further incubated one more day. After a total of 48 hours incubation at 28 °C, the strips were read by referring to the interpretation table. All the spontaneous reactions were recorded on the report sheet (see Figure 2. 1). If the glucose was positive and /or 3 tests or more are positive, the tests which required the addition of reagents were revealed.

VP test

One drop of VP1 and VP2 reagents were added to VP test cupule for the detection of acetoin. After 10 minutes, development of a bright pink or red colour indicated a positive reaction and was recorded on the report sheet (see Figure 2. 1).

TDA test

One drop of TDA reagent was added to the test cupule for the detection of acetoine. A dark brown colour indicated a positive reaction to be recorded on the report sheet (see Figure 2. 1).

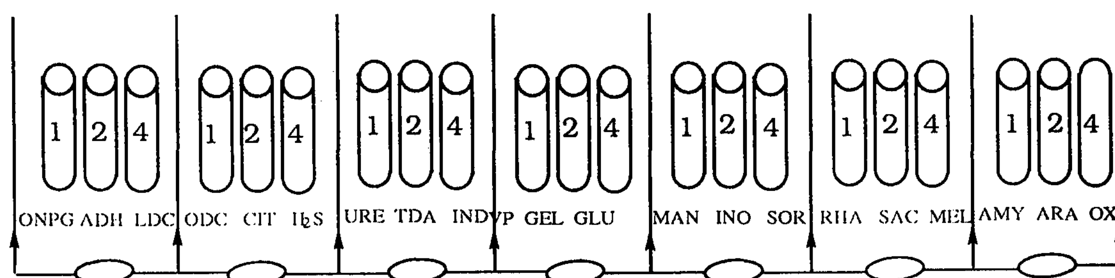
IND test

One drop of IND reagent was added to the test cupule. After 2 minutes development of a red ring indicated a positive reaction of indole to be recorded on the report sheet (see Figure 2. 1).

2. 2. 5. 4. 6 Identification of the profiles

On the report sheet (see Figure 2. 1), the tests were separated into groups of 3 and a number 1, 2, or 4 was indicated for each. By adding the numbers corresponding to positive reactions within each group, a 7-digit profile number was obtained for the 20 tests of the API 20E strip. The oxidase reaction constituted the 21st test, which was carried out in Chapter 2. 2. 5 and would have had a value of 4 if it was positive.

Figure 2. 1 The report sheet of API 20E test.



2. 2. 5. 5 Motility test

The hanging–drop method (Singleton and Sainsbury, 1985) was used to determine motility of test bacteria. The test organism was grown in NDB at 28 °C for 2 days on a rotary shaker and a drop of fresh broth culture containing live, unstained bacteria was placed on a clean cover-slip. A ring plasticine was pressed onto a microscope slide and the slide was inverted and pressed onto the cover–slip. The microscope slide was inverted and examined under the 40x objective of a Zeiss light microscope.

2. 2. 6 Production of a specific antiserum

2. 2. 6. 1 Antigen preparation

Erwinia salicis isolate NCPPB 2535 was grown in NDB at 28 °C for 2 days. The bacterial cells were washed two times with sterile 0.01 M phosphate buffer saline (PBS) pH 7.2 (see appendix for the recipe) by centrifugation at 10 000 rpm for 15 min. The optical density of the suspension was adjusted to A_{550}=0.63 to give 10^9 cells/ml by addition of PBS pH 7.2. One ml aliquots of the shaken bacterial suspension were placed in sterile eppendorf tubes. The antigen preparations were stored at – 20 °C until required for the inoculation of the test rabbits.

2. 2. 6. 2 Antiserum production

Two New Zealand white rabbits of approximately 3 kg weight were injected with an emulsion prepared from equal volumes of the antigen and Freund's adjuvant. The immunisation procedure was as follows :

Day	Route	Volume	Antigen
0	Subcutaneous	1 ml in nape of neck	10^9 cells/ml culture in PBS pH 7.2 emulsified with an equal volume of Freund's Complete Adjuvant
10	Subcutaneous	1 ml in nape of neck	
17	Test bleeding		
20	Intramuscular and	0.5 ml in leg	10^9 cells/ml culture in PBS pH 7.2 emulsified with an equal volume of Freund's Incomplete Adjuvant
	Subcutaneous	0.5 ml in nape of neck	
40	Intramuscular and	0.5 ml in leg	
	Subcutaneous	0.5 ml in nape of neck	

The titre of the antiserum was determined on day 49 and the animals were bled on day 55.

2. 2. 6. 3 Separation and preservation of the rabbit sera

The blood was collected in a sterile glass container and allowed to stand first at room temperature for 2-3 hours and then in a refrigerator at 4 °C overnight for the clot formation. The clot was freed gently using a sterile wooden toothpick. The serum was decanted and red cells were removed by centrifugation at 14 000 rpm for 30 seconds. The sera obtained in this way were stored in 1 ml aliquots at –20 °C. No preservative was used in the storage of the antisera.

2. 2. 6. 4 Determination of the titre of the antiserum

A series of two–fold dilutions of the antiserum (1/2 to 1/1024) were prepared in PBS pH 7.2. Fifty µl of the standard bacterial antigen suspension used for injection was put into each of the 12 wells of a polystyrene microtiter plate. Fifty µl of the dilution series of the antiserum (from tubes 1 to 12) was added to the wells and the two solutions were mixed with the tip. One well contained only PBS pH 7.2. The microtiter plate was allowed to stand at room temperature for 3 hours and was then held at + 4 °C overnight. The titre was the highest dilution at which a definite agglutination could be seen after incubation for 16–18 hours.

2. 2. 7 Agglutination tests

Agglutinations tests were carried out in microtiter plates with round bottom wells. The working dilution of the antiserum was 1/256 which was two steps lower than the maximum titration suggested by Lelliott and Stead, 1987. The test organisms were grown in NDB at 28 °C for two days to give 10^9 cells/ml. Two steps ten–fold dilution series of each test culture were prepared in sterile PBS pH 7.2. Fifty µl of each dilution of the antigen suspension and 50 µl of the antiserum were put into the wells of a microtiter plate and mixed with the tip. One well contained only undiluted antigen suspension and PBS as control. The microtiter plates with lids on were left at room temperature for 3 hours and then in the fridge at + 4 °C overnight. The positive results were recorded as clumping of the cells in the wells.

2. 2. 8 Competitive ELISA test

All the isolates tested were grown on NDA plates and discrete colonies were suspended in 1 ml of 0.01 M phosphate-buffered saline containing 0.5 % Tween-20 pH 7.4 (PBST) (see appendix for the recipe). The concentration of bacterial suspensions was approximately 10^8- 10^9 cells/ml.

To coat the plates, *E. salicis* isolate NCPPB 2535 was grown in NDB and harvested by centrifugation at 10 000 rpm for 15 min. The pellet was resuspended in coating buffer [0.01 M phosphate buffer pH 8.0 (see appendix for the recipe), containing 0.3 % methyl glyoxal] and the protein content of the suspension was determined by the method of Smith *et al.* (1985). Two hundred and fifty µl of bacterial suspension containing 10 µg/ml protein in coating buffer were added to each well of the flat bottom 96 well microtitration plate (Nunc, Nunc-immunoplate maxisorp) and incubated for 3 hours at 37 °C. After washing three times with PBST using a semiautomatic plate washer, unreacted sites were blocked by adding 250 µl of 1 % bovine serum albumin (BSA) in coating buffer without methyl glyoxal. After incubation for two hours at 37 °C the plates were again washed three times with distilled water. The coated plates were air-dried overnight and stored over silica gel desiccant.

Bacterial cultures were tested in a competitive ELISA assay. After coating the plates as above, 100 µl of bacterial suspensions in PBST were added to the wells and 100 µl of a 1:20 000 (v/v) dilution of the antiserum (see Chapter 2. 2. 6) was added to each well. After incubation for 3 hours at 37 °C the plates were washed five times with PBST and 200 µl of 1 :1000 dilution of goat anti-rabbit serum conjugated with peroxidase (Sigma No. A-8275) was applied to each well. After further incubation for 2 hours at 37 °C, the plates were again washed five times with PBST and 200 µl of peroxidase substrate (ABTS) solution [8 mg of 2-2'–Azino-bis (3–ethylbenz–thiazoline–6–sulfonic acid) (Sigma No. A-1888) in 20 ml of 100 mM phosphate-citrate buffer pH 7.4 (see appendix for the recipe) containing 2 µl of 30 % H_2O_2] was added. Optical density of each well was read at 414 nm on a plate-reader (Titertek, Multiscan) after incubation for 2 hours at 37 °C.

2. 3 Results

2. 3. 1 Evaluation of media for growth of *E. salicis*

Bacterial growth was determined qualitatively at the end of the incubation. Apparently, bacterial growth in aerobic conditions was much better than growth in anaerobic conditions. In aerobic conditions *E. salicis* grew moderately on GNA and NDA and growth of *E. salicis* on medium NDA (see Plate 2. 1), which had a nutrient agar base, was equal to that of reference GNA (see Plate 2. 2). No differences were observed in the colony size and speed of growth between GNA and NDA. Colonies of *E. salicis* were small (1–2 mm), translucent and yellow coloured on these media, but *E. salicis* produced fluidal entire, white colonies on SPA. Colonies remained small and yellow on NYGA and 'P. lachryman' media . A poor growth occurred on the plates of YPGA and KB. Growth of *E. salicis* on M9 and LB agar was slower and colonies were very small. No growth of *E. salicis* was observed on Coon's agar. The anaerobic cultivation of *E. salicis* on SPA medium did not affect the colony morphology of the bacterium and colonies were still fluidal, entire and white. But the growth of *E. salicis* was slower than in aerobic cultivation. In anaerobic conditions no bacterial growth was observed on the plates of NYGA, YPGA, LB agar, M9 and Coon's agar; moderate growth of *E. salicis* occurred on the plates of NDA, GNA media and KB medium supported slight growth.

Plate 2. 1 *E. salicis* NCPPB 2535 grown on NDA at 28 °C for 3 days.

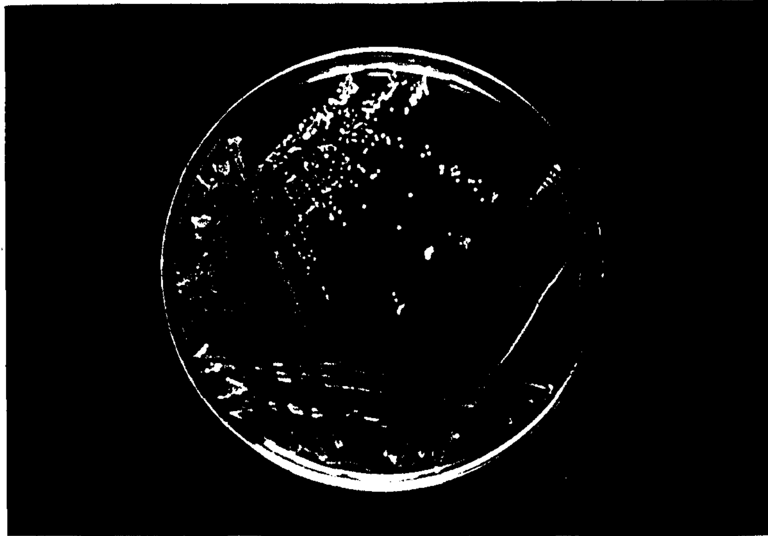


Plate 2. 2 *E. salicis* NCPPB 2535 grown on GNA at 28 °C for 3 days.

2. 3. 2 Biochemical tests and motility

Gram's staining reaction

A total of 81 cultures 64 of which isolated from the diseased willow wood during the course of this study and 17 of NCPPB cultures were tested with Gram's staining reaction. Of these 17 authentic isolates of *E. salicis* received from the NCPPB and 19 cultures **isolated during the course of this study and presumed to be *E. salicis*** were Gram-negative (see Table 2. 2).

Oxidase test

A total of 81 cultures 64 of which isolated from the diseased willow wood during the course of this study and 17 of NCPPB cultures were tested with oxidase test. Of these 17 authentic isolates of *E. salicis* received from the NCPPB together with 19 cultures isolated during the course of this study and presumed to be *E. salicis* were negative (see Table 2. 2), i.e. they failed to reduce the dye, tetramethyl *p* -phenylenediamine, to give a purple colour within 30 seconds.

Oxidation/ Fermentation test

A total of 81 cultures 64 of which isolated from the diseased willow wood during the course of this study and 17 of NCPPB cultures were tested with oxidation/fermentation test. Of these 17 authentic isolates of *E. salicis* received from the NCPPB together with 19 cultures isolated during the course of this study and presumed to be *E. salicis* used glucose as a carbon source. Following incubation, a yellow colour developed in the both sealed (anaerobic) and unsealed (aerobic) tubes with all the isolates tested, indicating a fermentative metabolism (see Table 2. 2). No gas bubbles were trapped in the semi-solid medium of any tube.

Table 2. 2 *E. salicis* isolates and their characteristics.

Number	Isolates	Host	Geographical location	Year	Identification tests					
					Gr	Ox	O/F	Mot.	Agg.	ELISA
1	447***	*S. alba* var. *caerulea*	UK (unknown)	1957	-	-	F	+	+	+
2	1466***	*S. alba* var. *caerulea*	UK (unknown)	1957	-	-	F	+	+	+
3	2310***	*Salix* sp.	UK (unknown)	1970	-	-	F	+	+	+
4	2317***	*Salix* sp.	UK (unknown)	1971	-	-	F	+	+	+
5	2522***	*S. alba* var. *caerulea*	Writtle, Essex	1971	-	-	F	+	+	+
6	2523***	*S. alba* var. *caerulea*	Writtle, Essex	1971	-	-	F	+	+	+
7	2525***	*S. alba* var. *caerulea*	Coggeshall, Essex	1972	-	-	F	+	+	+
8	2526***	*S. alba* var. *caerulea*	Coggeshall, Essex	1972	-	-	F	+	+	+
9	2529***	*S. alba* var. *caerulea*	Bulmer, Essex	1972	-	-	F	+	+	+
10	2530***	*S. alba* var. *caerulea*	Bulmer, Essex	1972	-	-	F	+	+	-
11	2531***	*S. caprea*	Langham, Essex	1972	-	-	F	+	+	-
12	2532***	*S. caprea*	Langham, Essex	1972	-	-	F	+	+	+
13	2533***	*S. caprea*	UK (unknown)	1972	-	-	F	+	+	+
14	2534***	*S. alba* var. *vitellina*	Suffolk (unknown)	1972	-	-	F	+	+	+
15	2535***	*S. alba* var. *caerulea*	Little Baddow, Essex	1972	-	-	F	+	+	+
16	2907***	*S. alba* cv. Liempde	Netherlands (unknown)	?	-	-	F	+	+	+
17	2908***	*S. alba* cv. Liempde	Netherlands (unknown)	?	-	-	F	+	+	+
18	73** (= CCM 2418)	*S. alba* cv. Liempde	Netherlands (unknown)	?	n.t	n.t	n.t	n.t	n.t	+

19	94**	*S. alba*	Netherlands, Kapelse Veer	1977	n.t	n.t	n.t	n.t	n.t	+
20	103**	Unknown	Netherlands, Bemmel	1977	n.t	n.t	n.t	n.t	n.t	+
21	104**	*S. alba*	Netherlands, Ede	1979	n.t	n.t	n.t	n.t	n.t	+
22	118**	*S. alba*	Netherlands, Abcoude	1978	n.t	n.t	n.t	n.t	n.t	+
23	132**	*S. alba*	Netherlands, Amsterdam	?	n.t	n.t	n.t	n.t	n.t	+
24	142**	*S. alba* cv. Lichtenvoorde	Netherlands, Liempde	?	n.t	n.t	n.t	n.t	n.t	+
25	143**	*Crataegus* spec. (near a diseased *S. alba*)	Netherlands, Renkum	1980	n.t	n.t	n.t	n.t	n.t	+
26	144**	*Alnus* spec. (near a diseased *S. alba)*	Netherlands, Renkum	1980	n.t	n.t	n.t	n.t	n.t	+
27	147**	*S. alba*	Netherlands, Biddinghuizen	1980	n.t	n.t	n.t	n.t	n.t	+
28	149**	*S. alba*	Netherlands, Ede	1980	n.t	n.t	n.t	n.t	n.t	+
29	150**	symptomless pollard willow	Netherlands, Abcoude	1980	n.t	n.t	n.t	n.t	n.t	+
30	156**	*S. alba*	Netherlands, Ede	1981	n.t	n.t	n.t	n.t	n.t	+
31	173**	*S. alba* cv. Lichtenvoorde	Netherlands, Liempde	?	n.t	n.t	n.t	n.t	n.t	+
32	174** (=PDDCC 9136)	*Salix* sp.	Witham, Essex	1983	n.t	n.t	n.t	n.t	n.t	+
33	S1A*	*S. alba* var. *caerulea*	Witham, Essex	1989	-	-	F	+	+	+
34	S4A*	*S. alba* var. *caerulea*	Witham, Essex	1989	-	-	F	+	+	+
35	S5A*	*S. alba* var. *caerulea*	Witham, Essex	1989	-	-	F	+	+	+
36	S7B*	*S. alba* var. *caerulea*	Witham, Essex	1989	-	-	F	+	+	⊥
37	S8A*	*S. alba* var. *caerulea*	Wickham Bishops, Essex	1989	-	-	F	+	+	+
38	S9B*	*S. alba* var. *caerulea*	Wickham Bishops, Essex	1989	-	-	F	+	+	+

39	S10B*	*S. alba* var. *caerulea*	Dedham, Essex	1989	-	-	F	+	+	+
40	S11A*	*S. alba* var. *caerulea*	Dedham, Essex	1989	-	-	F	+	+	+
41	S12A*	*S. alba* var. *caerulea*	Dedham, Essex	1989	-	-	F	+	+	+
42	S13B*	*S. caprea*	Long Melford, Suffolk	1989	-	-	F	+	+	+
43	S14A*	*S. alba* (type unknown)	Long Melford, Suffolk	1989	-	-	F	+	+	+
44	S14B*	*S. alba* (type unknown)	Long Melford, Suffolk	1989	-	-	F	+	+	+
45	S15A*	*S. alba* var. *caerulea*	Stebbing, Essex	1989	-	-	F	+	+	+
46	W1B*	*S. alba* var. *caerulea*	Dadford Ln, Wiltshire	1989	-	-	F	+	+	+
47	W2A*	*S. alba* var. *caerulea*	Dadford Ln, Wiltshire	1989	-	-	F	+	+	+
48	W3A*	*S. alba* var. *caerulea*	Dadford Ln, Wiltshire	1989	-	-	F	+	+	+
49	W4A*	*S. alba* var. *caerulea*	Dadford Ln, Wiltshire	1989	-	-	F	+	+	+
50	W6A*	*S. alba* var. *caerulea*	Dadford Ln, Wiltshire	1989	-	-	F	+	+	+
51	27B*	*S. caprea*	Long Melford, Suffolk	1989	-	-	F	+	+	+
52	28BD*	*S. alba* var. *caerulea*	Bungay, Suffolk	1989	n.t	n.t	n.t	n.t	n.t	+
53	28C*	*S. alba* var. *caerulea*	Bungay, Suffolk	1989	n.t	n.t	n.t	n.t	n.t	+
54	29AD*	*S. alba* var. *caerulea*	Bungay, Suffolk	1989	n.t	n.t	n.t	n.t	n.t	+
55	30AD*	*S. alba* var. *caerulea*	Bungay, Suffolk	1989	n.t	n.t	n.t	n.t	n.t	+
56	30C*	*S. alba* var. *caerulea*	Bungay, Suffolk	1989	n.t	n.t	n.t	n.t	n.t	+
57	35B*	*S. alba* var. *caerulea*	Good Easter, Essex	1989	n.t	n.t	n.t	n.t	n.t	+
58	41A1*	*S. alba* var. *caerulea*	Epping, Essex	1989	n.t	n.t	n.t	n.t	n.t	+
59	41B2*	*S. alba* var. *caerulea*	Epping, Essex	1989	n.t	n.t	n.t	n.t	n.t	+
60	90–1*	*S. alba* var. *caerulea*	Witham, Essex	1990	n.t	n.t	n.t	n.t	n.t	+
61	90–8*	*S. alba* var. *caerulea*	Epping, Essex	1990	n.t	n.t	n.t	n.t	n.t	+
62	90–18*	*S. alba* var. *caerulea*	Gt. Yeldham, Essex	1990	n.t	n.t	n.t	n.t	n.t	+
63	90–21*	*S. alba* var. *caerulea*	Witham, Essex	1990	n.t	n.t	n.t	n.t	n.t	+

64	90–23*	*S. alba* var. *caerulea*	Fairstead, Essex	1990	n.t	n.t	n.t	n.t	n.t	+
65	90–30*	*S. alba* var. *caerulea*	Fordham, Essex	1990	n.t	n.t	n.t	n.t	n.t	+
66	90–31*	*S. alba* var. *caerulea*	Beccles, Suffolk	1990	n.t	n.t	n.t	n.t	n.t	+
67	90–36*	*S. alba*	Beccles, Suffolk	1990	n.t	n.t	n.t	n.t	n.t	+
68	90–38*	*S. alba*	Beccles, Suffolk	1990	n.t	n.t	n.t	n.t	n.t	+
69	90–39*	*S. alba*	Beccles, Suffolk	1990	n.t	n.t	n.t	n.t	n.t	+
70	90–40*	*S. alba*	Beccles, Suffolk	1990	n.t	n.t	n.t	n.t	n.t	+
71	90–43*	*S. alba*	Beccles, Suffolk	1990	n.t	n.t	n.t	n.t	n.t	+
72	90–44*	*S. alba*	Beccles, Suffolk	1990	n.t	n.t	n.t	n.t	n.t	+
73	90–45*	*S. alba*	Beccles, Suffolk	1990	n.t	n.t	n.t	n.t	n.t	+
74	90–52*	*S. alba* var. *caerulea*	Hadleigh, Suffolk	1990	n.t	n.t	n.t	n.t	n.t	+
75	90–65*	*S. alba* var. *caerulea*	Writtle, Essex	1990	n.t	n.t	n.t	n.t	n.t	+
76	90–97*	*S. alba* var. *caerulea*	Witham, Essex	1990	n.t	n.t	n.t	n.t	n.t	⊥
77	90–98*	*S. alba* var. *caerulea*	Witham, Essex	1990	n.t	n.t	n.t	n.t	n.t	⊥
78	90–113*	*S. alba* var. *caerulea*	Long Melford, Suffolk	1990	n.t	n.t	n.t	n.t	n.t	+

*** from the NCPPP
** from M. de Kam
* isolated at UEA (the University of East Anglia)
CCM : Czeschlovakian Collection of Microorganisms
PDDCC :New Zealand Collection

Gr : Gram's reaction
Ox : oxidase test
O/F : oxidation/fermentation test
Mot : motility test
Agg. : agglutination test
n.t.: not tested

F : fermentative
- : negative
+ : positive
⊥ : intermediate
[: isolated from the same tree

API 20E Tests

Twenty three isolates of *E. salicis*, 6 from the NCPPB and 17 from our collection, were characterised by the API 20E test and 7-digit code profile of each isolate was given in Table 2. 3. All the isolates of *E. salicis* tested were negative with the following: *ortho*–nitrophenyl-β-D-galactosidase (ONPG), arginine dihydrolase (ADH), lysine decarboxylase (LDC), ornithine decarboxylase (ODC), H_2S production (H_2S), urease (URE), trytophane desaminase (TDA), indole production (IND), gelatinase (GEL) and citrate utilisation (CIT) except one isolate, S7B, was positive with the ONPG test and 2 isolates, NCPPB 2530 and NCPPB 2531, were positive with CIT test.

All the isolates tested produced acetoin from sodium pyruvate in the VP test. All the isolates investigated with the API 20E test system produced acid within 48 hours from glucose (GLU), mannitol (MAN), sucrose (SAC), and amygdalin (AMY) (Table 2. 4) and they were oxidase negative as shown in Table 2. 2. Over 82 % of the *E. salicis* isolates produced acid from inositol (INO), but 87 % did not produce acid from sorbitol (SOR) and rhamnose (RHA). More than 56 % isolates of *E. salicis* tested produced acid from melibiose (MEL) and only 4 % produced acid from arabinose (ARA).

Six phena namely, A, B, C, D, E, and F could be differentiated by only one, two or four positive and negative features (see Table 2. 4). Phenon A consisted of 10 isolates and except for two isolates, NCPPB 2310 and NCPPB 2534, they were our collections. Phenons B, C and D differed from phenon A either only by inositole or melibiose or both inositole and melibiose negative reactions. Phenon B composed of 6 isolates and only one isolate, 2907, was from the NCPPB. Phenon B differed from phenon A by melibiose negative reaction. Phenon C was different from phenon A by inositole negative reaction, and consisted of two cultures isolated from the diseased wood samples. Phenon D had two isolates of *E. salicis*, one was from the NCPPB and another was from our collection, and they differed from phenon A by two negative reactions with inositol and melibiose. Phenons E and F were quite different from phenons A, B, C, and D mainly by sorbitol and rhamnose positive reactions. Phenon E consisted of two NCPPB isolates of *E. salicis* which were NCPPB 2530 and NCPPB 2531 and only phenon E members used citrate as a sole carbon source. Melibiose was not used by phenon E. Phenon F differed from phenons A, B, C, D, and E mainly by ortho-nitro-phenyl-β-D-galactosidase and arabinose positive reactions. Phenon F was similar to phenon E by sorbitol and rhamnose positive reactions,

to phenons A, B and E by inositol positive reactions, to phenons A and C by melibiose positive reaction. Only one isolate of *E. salicis*, S7B, was in phenon F (see Table 2. 4).

Table 2. 3 The API 20E profiles of 23 isolates of *E. salicis*.

isolates	7-digit code profile
2310	0005361
2530	0205731
2531	0205731
2532	0005121
2534	0005361
2907	0005321
S1A	0005361
S7B	1005773
S8A	0005361
S9B	0005361
S10B	0005361
S13B	0005321
S14A	0005161
S15A	0005161
27B	0005321
W1B	0005321
W3A	0005321
W6A	0005361
28BD	0005321
29AD	0005361
30C	0005361
35B	0005361
41A1	0005121

Table 2. 4 Differential tests for the separation of the phenons of isolates of *E. salicis*.

Phenon :	A	B	C	D	E	F
Number of isolates :	10	6	2	2	2	1
Profile :	0005361	0005321	0005161	0005121	020573	1005773
tests						
ONPG	—	—	—	—	—	+
ADH	—	—	—	—	—	—
LDC	—	—	—	—	—	—
ODC	—	—	—	—	—	—
CIT	—	—	—	—	+	—
H_2S	—	—	—	—	—	—
URE	—	—	—	—	—	—
TDA	—	—	—	—	—	—
IND	—	—	—	—	—	—
VP	+	+	+	+	+	+
GEL	—	—	—	—	—	—
GLU	+	+	+	+	+	+
MAN	+	+	+	+	+	+
INO	+	+	—	—	+	+
SOR	—	—	—	—	+	+
RHA	—	—	—	—	+	+
SAC	+	+	+	+	+	+
MEL	+	—	+	—	—	+
AMY	+	+	+	+	+	+
ARA	—	—	—	—	—	+
OX	—	—	—	—	—	—

+ : positive reaction

— : negative reaction

Motility test

A total of 81 cultures 64 of which isolated from the diseased willow wood during the course of this study and 17 of NCPPB cultures were tested with motility test. Of these 17 authentic isolates of *E. salicis* obtained from the NCPPB and 19 cultures isolated during the course of this study and presumed to be *E. salicis* were actively motile (see Table 2. 2).

2. 3. 3 Agglutination tests

The titre of the antiserum was determined at 1/1024. Thus, the working dilution of the antiserum was chosen as 1/256, two steps lower than the least dilution at which optimum agglutination occurs. Agglutination occurred with 17 authentic isolates of *E. salicis* and 19 cultures isolated during the course of this study and presumed to be *E. salicis* (see Table 2. 2).

2. 3. 4 Competitive ELISA tests

In the competitive assay colour development in the well indicated a negative result. This is because absence of the specific antigen from the sample allowed the antibody to react only with solid-phase antigen, which bound the anti-rabbit antibody coupled to peroxidase, and a colour was produced following the addition of the peroxidase enzyme substrate. Competitive ELISA assay used in this study seemed very specific for the identification of *E. salicis*. Seventeen authentic isolates of *E. salicis* received from the NCPPB and 15 isolates from the Netherlands shown in Table 2. 2 gave a positive reaction having an OD_{414} value in the range of 0.0 and 0.2, except for two isolates, *E. salicis* NCPPB 2530 and 2531, which gave an unexpected negative reaction similar to those indicated in Table 2. 5. Forty six cultures isolated from the diseased trees also gave positive reaction, except three isolates which were S7B, 90-97 and 90-98. These isolates had an OD_{414} value between 0.2 and 1.0 indicating an intermediate reaction (see Table 2. 2). Other species of *Erwinia*, other bacteria and unidentified bacteria from the watermark diseased wood samples shown in Table 2. 5 gave a negative reaction indicating no cross-reactivity of the antiserum. Their OD_{414} values were 1.0 or more than 1.0.

Table 2. 5 Specificity of polyclonal antisera raised against *E. salicis* NCPPB 2535 (adapted from Turner *et al.*, 1991).

Bacterium	Number of isolates	Competitive ELISA reaction
E. salicis	78	*
E. rhapontici	1	–
E. herbicola	1	–
E. chrysanthemi pv. *chrysanthemi*	1	–
E. chrysanthemi pv. *diffenbachia*	1	–
E. amylovora	1	–
E. carotovora var. *carotovora*	1	–
Enterobacter agglomerans	1	–
Escherichia coli B	1	–
Pseudomonas syringae pv. *phaseolicola*	1	–
P. syringae pv. *atropurpurea*	1	–
P. syringae pv. *syringae*	1	–
Unidentified contaminating bacteria (not *E. salicis*) isolated from watermarked willow	5	–

* Seventeen isolates were from the National Collection of Plant Pathogenic Bacteria, fifteen isolates were from the Netherlands and forty six isolates were isolated from the diseased trees during the course of this study are given in detail in Table 2. 2. Two isolates received as *E. salicis*, NCPPB 2530 and 2531, were negative in this test are now believed not to be authentic cultures of this species.

2. 4 Discussion

From the results of media evaluation it was observed that *E. salicis* grew slowly on all of the solid media tested. The best growth was observed on NDA and GNA and the former was used as a working medium to grow *E. salicis* in the work represented in this thesis.

For reasons outlined earlier, a pathogenicity test has not been used in this investigation. Therefore, the identification of isolates has relied on cultural, biochemical and serological tests. To develop a simple and reliable test for the identification of *E. salicis*, I have compared isolates from culture collection (NCPPB), whose pathogenicity has been confirmed, with field isolates obtained in the present study.

The results of the determinative tests, including Gram's staining , oxidase test, oxidation/ fermentation of glucose and motility are in complete agreement with those carried out by Dowson (1937), Gremmen and Kam (1970), Wong (1974) and Preece *et al.* (1979). A number of biochemical tests with the API 20E tests system did not give the same results as those given in Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974), e.g. the acidification of inositol, melibiose, rhamnose and sorbitol for the identification of *E. salicis*. However, the results of some of the API 20E tests e.g. inositol, melibiose and citrate described here are in complete agreement with those carried out by Mergaert *et al.* (1984), Rijckaert *et al.* (1984) and Verdonck *et al.* (1987). As pointed out by Wong (1974), biochemical tests are not sufficiently selective and alone can not be used as a diagnostic tool.

In a study of 18 *Erwinia* species (Mergaert *et al.*, 1984) testing by API 20E tests, four different 7-digit code profiles representing 7 *E. salicis* isolates were obtained. Three out of 4 of the profiles belonging to isolates of *E. salicis* were homologous to those described in the present study (see Table 2. 6). NCPPB 2310 and NCPPB 2534 tested in both of the studies and gave the same profile, 0005361, confirming the result of Mergaert *et al.* (1984). NCPPB 2531 did not fall into the same phenon as those other six isolates of *E. salicis*. This isolate gave a profile of 0204731 in Mergaert *et al.* 's study and the profile described in the present investigation was 0205731 differing only by the VP positive test. The test reproducibility within the API 20E system varied from 88 to 100 % in the study carried out by Mergaert *et al.* (1984) and they concluded that API 20E system was a useful and rapid alternative to conventional phenotypic testing for the classification and identification of *Erwinia* species.

Rijckaert *et al.* (1984) characterised 43 isolates of *E. salicis* from England, from the Netherlands and from Belgium with the API 20E test. Five of the Belgium isolates gave a profile similar to the NCPPB, HIM (HIM : Hygiene Institut und Medizinal Untersuchungstamt der Universitåt Marburg, Marburg, B.R.D.) and PDDCC collection strains. Although the profiles and number of isolates were shown in a table of their study, the profile of the each isolate tested was not given in the paper. It is presumed that two distinct profiles, 0205773 and 0205731, belonged to NCPPB 2530 and NCPPB 2531 isolates.

Verdonck *et al.* (1987) expanded the study of Mergaert *et al.* (1984) by testing 421 strains belonging to 21 different *Erwinia* species. Twenty isolates of *E. salicis* from the Netherlands and UK were tested by the API 20E system and 18 isolates were in the subphenon 32 B. *E. salicis* NCPPB 2532 and NCPPB 2530 fell outside the phenon 32 B and displayed API 20E profile numbers different from those of the majority of *E. salicis* isolates, in agreement with Mergaert *et al.*, 1984 and Rijckaert *et al.*, 1984 (Verdonck *et al.*, 1987).

The comparison of some of the 7-digit code profiles of *E. salicis* isolates is summarised in Table 2. 6. In conclusion, the API 20E tests carried out in this study not only supported the identification of isolates of *E. salicis* but also confirmed the previous workers' findings.

Table 2. 6 Comparison of profiles of *E. salicis* isolates obtained by different workers.

Profile	Isolate	Reference
0005361	2534, 447, 2522 and 2310	Mergaert *et al.* (1984)
	2534 (+ 9 others)	Güven (see Table 2. 3)
0205731	2531	Rijckaert *et al.* (1984)
	2531 (+ 2530)	Güven (see Table 2. 3)
0204731	2531	Mergaert *et al.* (1984)
	1466	Mergaert *et al.* (1984)
0005161	23 isolates	Rijckaert *et al.* (1984)
	2 isolates	Güven (see Table 2. 3)
	HIM 588-6	Mergaert *et al.* (1984)
0005121	9 isolates	Rijckaert *et al.* (1984)
	2 isolates	Güven (see Table 2. 3)
0005321	6 isolates	Güven (see Table 2. 3)
1005773	1 isolate	Güven (see Table 2. 3)
1005353	1 isolate	Rijckaert *et al.* (1984)
0205773	1 isolate	Rijckaert *et al.* (1984)
0004121	5 isolates	Rijckaert *et al.* (1984)
0005573	1 isolate	Rijckaert *et al.* (1984)
0005163	1 isolate	Rijckaert *et al.* (1984)

HIM : Hygiene Institut und Medizinal Untersuchungstamt der Universität Marburg, Marburg, B.R.D.

The slide agglutination test has previously been used to detect *E. salicis* either from isolation plates or in sap expressed from infected shoots (Wong and Preece, 1973; Kam, 1976; Preece *et al.*, 1979). Diagnosis by slide agglutination using sap from healthy willow shoots occasionally gave false positive agglutination reactions (Kam, 1976; Wong

and Preece, 1973 and Kam, 1989). Kam (1989) showed that non-specific reactions occur especially in low serum dilutions and in plant liquids of *Salix alba*. *E. salicis* appears to exhibit little serological variation. A few isolates gave noticeably weak agglutination in antiserum to NCPPB 1466 in a study of Preece *et al.* (1979). Kam(1976) using cross-adsorption techniques was unable to detect any serological differences between isolates from England and from the Netherlands.

ELISA has demonstrated its excellent qualifications such as, high specificity and speed for routine application in the medical (Voller *et al.*, 1976 a) and veterinary field (Voller *et al.*, 1976 b) and, more recently in plant pathology (Clarck and Adams, 1977 and Torrance and Jones, 1981). ELISA has been used for the detection and identification of *Corynebacterium sepedonicum* (Claflin and Uyemoto, 1978), *Xanthomonas campestris* pv. *phaseoli* (Malin *et al.*, 1985) and *Pseudomonas syringae* pv. *phaseolicola* (Wyatt *et al.*, 1989). Kam (1982 a, b) first used a double antibody sandwich (DAS) ELISA to detect *E. salicis* within the host plant and on the leaf surface. DAS ELISA detected not only bacterial cells but also soluble antigens of the bacterium.

Competitive ELISA is easier to perform but there are some reports that competitive ELISA is not as useful as DAS ELISA for antigen detection in crude extracts (Lommel *et al.*, 1982). The method used in this study proved its specificity for the reliable identification of *E. salicis* in pure culture.

Kam (1989) reported that ELISA was more specific than agglutination for the identification of *E. salicis*. In the present investigation although all the isolates shown in Table 2. 2 agglutinated with the anti-2535 antiserum two authentic isolates of *E. salicis*, NCPPB 2530 and NCPPB 2531, and 3 cultures isolated during the course of this study, S7B, 90-97 and 90-98, gave a negative or intermediate ELISA reaction respectively (see Table 2. 2).

In addition to cultural, biochemical and serological tests, bacteriophage sensitivity tests carried out in Chapter 3 confirmed the identification of authentic *E. salicis* isolates received from the NCPPB and from the Netherlands and cultures isolated during the course of this study. All the isolates tested except 5 isolates, NCPPB 2530, NCPPB 2531, S7B, 90-97 and 90-98, were typable with any of 4 of the *E. salicis* bacteriophages indicating their host specificity.

Two isolates of *E. salicis*, NCPPB 2530 and NCPPB 2531, have been reported being distinct from other isolates of the bacterium tested (Wong, 1974). They differed by VP positive, MR negative, pectate negative reactions. They also gave a slight agglutina-

tion with the NCPPB 1466 antiserum and were not sensitive to bacteriophage NCPPB 1467. Although they did not fit into the phenotypic pattern of authentic *E. salicis*, it was suggested that they were either mutants or different strains of the bacterium since they were positive with the pathogenity tests. The pathogenicity tests employed however, have been questioned (see section 2. 1).

In the present study these two *E. salicis* isolates, NCPPB 2530 and NCPPB 2531, differed from the others by giving a negative ELISA reaction and falling into a distinct phenon by the API 20E tests. Three isolates obtained from the diseased trees during the course of this study, S7B, 90-97 and 90-98, were also different by an intermediate ELISA reaction. As suggested above, these three isolates together with NCPPB 2530 and NCPPB 2531 could be a different strain or subspecies of *E. salicis*. Unfortunately, we could not carry out a pathogenicity test to confirm their identity as *E. salicis*. Hence, the identity of these five isolates, NCPPB 2530, NCPPB 2531, S7B, 90-97 and 90-98, remains obscure and is discussed in Chapter 8.

In conclusion, ELISA confirmed the identity of isolates of *E. salicis* received from the NCPPB (except NCPPB 2530 and NCPPB 2531) and from the Netherlands and, identified the isolates of *E. salicis* obtained from diseased wood samples during the course of this study in conjunction with the biochemical, cultural and agglutination tests performed. ELISA has therefore proven to be a reliable and simple test for the identification of *E. salicis*. Therefore, I only used ELISA and cultural characteristics to identify further isolates of the *E. salicis*.

The purpose of this study is to examine epidemiology of watermark disease. The first requirement, to develop a reliable method for identification of the bacterium, has been achieved. In the following chapters I describe attempts to distinguish isolates of *E. salicis*, and to interpret the geographical distribution of these strains.

CHAPTER III

DIFFERENTIATION OF *E. SALICIS* ISOLATES ACCORDING TO CARBON SOURCE UTILISATION AND BACTERIOPHAGE SENSITIVITY

3. 1 Introduction

Differences between English and Dutch isolates of *E. salicis* in the utilisation of galactose and raffinose have been reported previously (Kam, 1976). Kam (1976) compared 7 isolates of *E. salicis* from England and 4 isolates from the Netherlands for growth on 1 % carbon source in Ayers, Rupp and Johnson synthetic medium at pH 6.8. After incubation for 4 days at 27-29 °C, he determined the growth turbidimetrically and concluded that only English isolates used galactose as carbon source whereas the Dutch isolates did not. He also reported that only the yellow pigment-producing isolates of *E. salicis* used 0.1 % raffinose as a carbon source, indicating variation among the English isolates of bacterium. The significance of these results was difficult to judge because they were based on a small number of isolates of *E. salicis*.

In the present study 61 isolates of *E. salicis* were tested applying the method of Kam (1976) with minor modifications. The pH of the medium was adjusted to 7.2 with a phosphate buffer and instead of 1, 2–epoxypropane, membrane filters were used to sterilise the carbon source solution.

Not only galactose and raffinose, but also melibiose and inositol were included for these tests because 3 phena were differentiated according to utilisation of these carbohydrates in previous work with the API 20E tests (see Chapter 2), indicating variation in this character in *E. salicis*.

Strain variation of *E. salicis* was also reported by Wong (1974) using bacteriophage sensitivity tests. He tested 13 English isolates of *E. salicis* against one bacteriophage, NCPPB 1467, and found that 4 of the isolates reacted negatively. He noted that two isolates of *E. salicis* isolated from the same tree reacted differently and concluded that different strains of *E. salicis* exist in a single diseased tree.

An aim of the present investigation was therefore to isolate new bacteriophages of *E. salicis* from natural sources and from lysogenic bacteria and develop a bacteriophage typing scheme to distinguish isolates of *E. salicis*.

Bacteriocins, or antagonisms between strains that might be due to bacteriocins, have been reported for *E. salicis* in a review by Vidaver (1976) referring to the study of Hamon and Peron (1961). The original article (Hamon and Peron, 1961) indicates that a total of 9 strains of *Erwinia* sp., 3 strains of *E. carotovora* var. *graminarum,* 2 strains of *E. carotovora* f. sp. *zeae* (syn. *E. chrysanthemi*), 1 strain of *E. carotovora,* 1 strain of *E. aroideae* (syn. *E. carotovora*), 1 strain of *E. lathyri* (syn. *E. herbicola*) and 1 strain of *E. salicis,* were studied to demonstrate the bacteriocin activity by two methods including uv induction. These authors concluded that only 6 strains, 2 strains of *E. carotovora* var. *graminarum,* 2 strains of *E. carotovora* f. sp. *zeae,* 1 strain of *E. aroideae* and 1 strain of *E. herbicola,* showed bacteriocinogeny. No bacteriocinogeny has been reported for *E. salicis* in this article and it seems that the results were misinterpreted by Vidaver (1976). Hence, in the present study 7 isolates of *E. salicis* were examined for bacteriocin activity by employing 3 different methods.

3. 2 Materials and methods

3. 2. 1 Carbon source utilisation assay

3. 2. 1. 1 Preparation of the inoculum

Four isolates of *E. salicis,* 2533 g (grey), 2533 y (yellow), 2534 g (grey) and 2534 y (yellow), differentiated according to yellow pigment production on potato plugs were obtained from M. de Kam, Holland. A total of 61 isolates of *E. salicis* (numbered 1 to 12 and 15 to 59 in Table 2. 2, see Chapter 2) including the four isolates mentioned above were tested for the utilisation of galactose, raffinose melibiose and inositol as sole carbon source. The bacteria tested were grown on NDA medium for 3 days at 28 °C and were examined visually for purity. Each isolate was then grown in NDB for 2 days at 28 °C on a rotary shaker to give approximately 10^8– 10^9 cells/ml.

3. 2. 1. 2 Preparation of the medium

Ayers, Rupp and Johnson's synthetic liquid base was prepared by the method of Lelliott and Stead (1987). The medium was dispensed in the test tubes in 4 ml portions and sterilised by autoclaving at 121 °C for 15 min.

D (+) galactose, raffinose, melibiose and inositol were used as sole carbon source. Five percent solution of the carbon compound was prepared and filter-sterilised. One ml solution of the carbon compound was added to each tube to give one percent final concentration. In the case of raffinose two concentrations, 1 % and 0.1 %, were prepared in the test tubes. Before inoculating the media with a bacterial suspension, the tubes containing base and carbohydrate solution were left for 4 days at 28 °C to confirm the sterility.

3. 2. 1. 3 Inoculation of the tubes and determination of growth

The tube containing synthetic base and carbon source were inoculated with 100 µl of bacterial suspension. Control tubes contained only the liquid base and carbon source solution. After incubation for 6 days at 28 °C the test tube was shaken to disperse bacterial cells and 3 ml was withdrawn. Its absorbance at 550 nm was measured against the control.

3. 2. 2 Bacteriophage assays

3. 2. 2. 1 Origin and maintenance of bacteriophages

Bacteriophage NCPPB 1467, virulent on *E.salicis* isolate NCPPB 1466, was obtained from the National Collection of Plant Pathogenic Bacteria, Plant Pathology Laboratory, Harpenden, Herts., UK. Bacteriophage T3, a bacteriophage of *Escherichia coli* B, was kindly obtained from Dr. C. Clarke, School of Biological Sciences, University of East Anglia, Norwich. Thirty bacteriophages of *E. coli* were kindly obtained from Dr. H. Milch, National Institute of Hygiene, Budapest, Hungary. The origins of the bacteriophages obtained and the propagating bacteria are shown in Table 3. 1. Three bacteriophages of *E. salicis*, bacteriophage L, bacteriophage C and bacteriophage M, were isolated from natural sources using *E. salicis* NCPPB 2535 as host bacterium (see Table 3. 2). All the *E. salicis* bacteriophages were stored in nutrient broth (NB), and *E. coli* bacteriophages were stored in LB broth.

3. 2. 2. 2 Isolation of bacteriophages from natural sources

Soil and diseased willow wood to be used as sources of bacteriophages were collected from several willow plantations in England. Soil was collected from beneath of wil-

low trees bearing symptoms of watermark. Raw sewage, and animal feces e.g. rat, rabbit, goat and duck were also used as natural sources of bacteriophages. Four isolates of *E. salicis*, NCPPB 2535, NCPPB 2530 and Dutch isolates NCPPB 2907 and NCPPB 2908 were chosen as indicator bacteria for the isolation of bacteriophages. The classical methods of Adams (1959) were used for the isolation and purification of bacteriophages. Indicator strains were grown in 100 ml of NDB for 2 days at 28 °C on a rotary shaker. Sixty ml of broth culture was centrifuged at 10 000 rpm for 15 minutes to harvest the cells. The pellet was resuspended in 5 ml of NDB on a rotary mixer and added to 100 ml of sterile NDB in a flask. Before using diseased wood samples were chopped to pieces by a scalpel and 10 gram of solid or 10 ml of fluid test material was added to the flask for the enrichment of bacteriophage in the test sample. After incubation for 2 days at 28 °C on a rotary shaker, most of the bacteria were removed by centrifugation at 10 000 rpm for 15 minutes. To inactivate or remove the bacteria left in the suspension two methods were employed:

a. Half of the suspension (50 ml) was filter-sterilised by a 0.45 μm pore size membrane filter (Millipore).

b. The other part was sterilised by the addition of 5 ml of chloroform. After giving a short and vigorous shake the suspension was allowed to stand for a few minutes and decanted into a sterile flask to prevent prolonged contact.

Two methods were employed to plate the test suspension:

a. One hundred μl of broth culture of host bacterium and 100 μl of test suspension were added to 3 ml of 0.5 % molten agar at 45 °C. The mixture was shaken immediately without causing bubbles and then poured over the surface of NDA base. The plate was rocked gently so that the surface of the agar was completely covered, and incubated for two days at 28 °C.

b. Two ml of bacterial suspension was dispensed onto the surface of a NDA plate and surplus was decanted rapidly. After drying the plate in a laminar flow hood for 30 minutes, 20 μl of the test suspension was spotted on the lawn and the plate was allowed to dry for 30 minutes. After incubation for two days at 28 °C the presence of a clear spot or only a few distinct plaques were observed.

3. 2. 2. 3 Isolation of bacteriophages from lysogenic bacteria

Six isolates of *E. salicis* from the NCPPB, NCPPB 447, NCPPB 2317, NCPPB

2529, NCPPB 2535, NCPPB 2907 and NCPPB 2908, were grown in 100 ml of NDB for 2 days at 28 °C on a rotary shaker. A bacterial lawn of each isolate was prepared on NDA in duplicate by pipetting 2 ml of bacterial suspension on NDA and then drying the plates as mentioned above. On the other hand, 10 ml of bacterial suspension was centrifuged at 10 000 rpm for 15 minutes and the supernatant was collected. One ml of chloroform was added to supernatant to remove the viable cells and 20 µl supernatant of each isolate was spotted onto the lawn. After incubation for two days at 28 °C, the plates were examined visually.

3. 2. 2. 4 Purification of bacteriophages

Single plaque purification was carried out. A distinct plaque or a piece of cleared zone was cut out with a sterile pasteur pipette and put into 1 ml of NB. After leaving at room temperature for 3 hours, the suspension was sterilised by addition of 100 µl of chloroform. Ten-fold dilutions were prepared in NB and spotted onto the lawn of indicator bacterium. After incubation for two days at 28 °C single plaques which differed in plaque size or by the presence of a 'halo' were picked as above and re-diluted and re-spotted onto the bacterial lawn. Single plaque purification was repeated at least 5 times to obtain the purified bacteriophage.

3. 2. 2. 5 Propagation and preparation of high titre stocks

The plate method of Eisenstark (1967) was used to prepare the stocks of bacteriophages. Ten-fold dilution series of each bacteriophage suspension were prepared and 100 µl were mixed with 100 µl of indicator bacterial suspension in 3 ml of molten agar, as described before. After incubation for two days at 28 °C, the dilution which gave a semiconfluent lysis (overlapping plaques) on NDA plates was determined visually for each of the bacteriophage suspensions and this dilution was used to prepare the stock of the bacteriophage.

Ten plates showing semiconfluent lysis of each bacteriophage were prepared. After incubation, 3 ml of NB was added to each plate and these were left for 3 hours at room temperature. The bacteriophage were harvested by gently removing the fluid. Remaining viable bacteria were killed by chloroform and the sterile bacteriophage suspension was placed in 1 ml of Eppendorf tubes. Before storing the bacteriophages at 4 °C, the number of plaque-

forming units (pfu/ml) was estimated. Ten-fold dilution series were prepared in NB and 20 μl of dilution was spotted onto the dried surfaces of plates sown with the indicator bacterium. Plaques were counted after incubation overnight at 28 °C. All the bacteriophages used in this study were propagated every 6 months.

3. 2. 2. 6 Bacteriophage sensitivity assay

Bacteriophage sensitivity tests were done with either an undiluted bacteriophage suspension (10^9–10^{10} pfu/ml) or with 'routine test' diluted bacteriophage suspension (10^4 to 10^6 pfu/ml, depending on the bacteriophage). Each of the bacteriophage suspension was diluted to the routine test dilution (RTD) which is that dilution which just gives confluent lysis with the propagating bacterium (Adams, 1959). Seventy eight isolates of *E. salicis* shown in Table 2. 2 (see Chapter 2), and other bacteria (see Table 3. 4) were tested for sensitivity to 5 bacteriophages. A bacterial lawn of each isolate was prepared in duplicate and 20 μl of each bacteriophage suspension was spotted on specified gridded areas on petri plates with a pipette. After incubation for two days at 28 °C, the plates were examined for the presence of confluent lysis in the gridded drop zones. All isolates of *E. salicis* were typed on at least four occasions to verify the lysotype.

3. 2. 2. 7 Isolation of bacteriophage-resistant mutants

To isolate the mutants of two English isolates of *E. salicis*, NCPPB 1466 and NCPPB 2535, resistant to bacteriophages C and M, the method of Eisenstark (1967) was used. Host bacteria were grown freshly in NDB for 2 days at 28 °C on a rotary shaker and 1 ml of growth containing 10^8 cell/ml was mixed with 1 ml of bacteriophage suspension having 10^9 - 10^{10} pfu/ml. After 10 minutes incubation at 28 °C a small drop of mixture was spread on the surface of the NDA plate. The plate was then incubated for 10 days at 28 °C. Colonies grown after incubation were streaked onto NDA plates, and re-streaking was repeated 3 more times. After confirming the resistance to the homologous bacteriophages the mutant isolate was also tested by the bacteriophage typing scheme, for sensitivity to all 5 bacteriophages (see Table 3. 3). These mutant isolates were next tested for galactose utilisation (see Chapter 3. 2. 1).

Table 3. 1 The origin and propagating strains of bacteriophages obtained for typing of *E. salicis* isolates.

bacteriophage	Propagating strain	Origin
1467	*E. salicis* NCPPB 1466	NCPPB
T3	*E. coli* B	Dr. C. Clarke
F1	*E. coli* C1	Hungary
F2	*E. coli* C29	Hungary
F3	*E. coli* C31	Hungary
F4	*E. coli* 08	Hungary
F4a	*E. coli* R816	Hungary
F4b	*E. coli* R984	Hungary
F5	*E. coli* C5	Hungary
F6	*E. coli* C23	Hungary
F7	*E. coli* C32	Hungary
F8	*E. coli* F42	Hungary
F9	*E. coli* C26	Hungary
F10	*E. coli* 111/1	Hungary
F11	*E. coli* 111/5	Hungary
F12	*E. coli* C35	Hungary
F13	*E. coli* C8	Hungary
F14	*E. coli* C15	Hungary
F15	*E. coli* C20	Hungary
F16	*E. coli* C19	Hungary
F17	*E. coli* 058	Hungary
F18	*E. coli* 058	Hungary
F19	*E. coli* 55/1	Hungary
F20	*E. coli* R604	Hungary
F21	*E. coli* 111/2	Hungary
F22	*E. coli* C3	Hungary
F23	*E. coli* C7	Hungary
F24	*E. coli* 018	Hungary
F25	*E. coli* C9	Hungary
F26	*E. coli* C17	Hungary
F27	*E. coli* F42	Hungary
F28	*E. coli* C24	Hungary

3. 2. 3 Assay for *E. salicis* bacteriocins

Seven isolates of *E. salicis* namely NCPPB 447, NCPPB 1466, NCPPB 2317, NCPPB 2529, NCPPB 2535, NCPPB 2907 and NCPPB 2908 were tested for bacteriocin activity. Two isolates of *E. coli, E. coli* E9 and *E. coli* W 3110, were kindly given by Dr. R. James, School of Biological Sciences, University of East Anglia, Norwich, and they were included to the bacteriocin tests as controls. Three methods were employed to detect bacteriocinogeny.

a. Test isolate was streaked across the surface of NDA medium in a glass petri plate and then incubated for 3 days at 28 °C(Mayr-Harting *et al.*, 1972). The plate containing bacterial growth was exposed to chloroform vapour by placing a blotting-paper disc in the lid, applying 3 ml of chloroform to the disc, and inverting the base of the plate into the lid, for about 30 minutes. The growth was then scraped off with a glass slide and the agar surface was again briefly exposed to chloroform vapour which was afterwards allowed to evaporate by opening the plate for 60 minutes. The indicator bacteria were next streaked at right angles across the original streak of the test organism and the plate was re-incubated for 3 days at 28 °C.

b. A method of Vidaver et al (1972) was used with minor modifications. NDA medium in the glass petri plates was stab-inoculated with test bacteria and after incubation for 3 days at 28 °C these master plates were transferred with a replicator to fresh plates. The plates were incubated for 3 days at 28 °C and exposed to chloroform vapour by dispensing 3 ml of chloroform in the lid and inverting the base of the plate into the lid for about 60 minutes. The plates were next allowed to stand with lids removed for another hour. Fifty µl of test indicator culture grown in NDB for 2 days at 28 °C was mixed with 4 ml of 0.7 % molten agar at 45 °C and poured over the basal layer. Plates were then incubated again for 3 days at 28 °C.

c. Ultraviolet induction of bacteriocins from the seven isolates was tested by the method of Vidaver *et al.* (1972). Cultures were grown in 10 ml NDB for 2 days at 28 °C on a rotary shaker. Nine ml of the culture was centrifuged at 8 000 rpm for 5 minutes and the pellet was resuspended in 9 ml of 0.8 % sodium chloride and irradiated for 80 seconds with u.v. light (254 nm). Five ml of irradiated culture was added to 5 ml of double strength NDB and incubated on a shaker for 4 hours in the dark. The cultures were then re-centrifuged as before and the supernatant fluids were filtered through a membrane (Millipore 0.45 µm) to

remove bacteria. The indicator test culture in 3 ml of 0.7 % molten agar was prepared as before and 20 µl of supernatant fluid of each isolate was spotted on the indicator layer with a pipette. The plates were then incubated for 3 days at 28 °C.

3. 3 Results

3. 3. 1 Utilisation of carbon compounds

Growth of *E. salicis* on galactose, raffinose, melibiose and inositol was measured as A_{550} (see Figure 3.1- 5). These values for the utilisation of galactose ranged between 0.05–0.33 and two clusters occurred (see Figure 3. 1). All of the Dutch isolates of *E. salicis* together with two English isolates, NCPPB 2530 and NCPPB 2531, gave an $A_{550} <$ 0.10 indicating no bacterial growth. English isolates of *E. salicis* gave an $A_{550} > 0.10$ indicating the utilisation of galactose for the growth of bacteria.

Utilisation of raffinose by the isolates of *E. salicis* was tested at two concentrations of this sugar, 0.1% w/v and 1% w/v. The majority of isolates gave an A_{550} value between 0.11 and 0.31 indicating utilisation of raffinose (see Figure 3. 2). Yellow pigment-producing isolates of *E. salicis*, 2533 y and 2534 y, did not give an A_{550} value significantly different from those non-pigment producing isolates, 2533 g and 2534 g. All isolates of *E. salicis* tested used 0.1 % raffinose as a carbon source, except NCPPB 2530 and NCPPB 2531 which gave A_{550} of 0.08 and 0.07 respectively.

At 1 % raffinose A_{550} was above 0.11 for all the isolates indicating the utilisation of 1 % raffinose by all isolates of *E. salicis* tested (see Figure 3. 3). Strikingly, one isolate of *E. salicis* from our collections, S7B, gave the highest A_{550} value, $A_{550}=0.44$ in 0.1 % raffinose and $A_{550}=0.51$ in 1 % raffinose.

The majority of isolates of *E. salicis* tested used 1 % melibiose as a carbon source except two isolates (see Figure 3. 4). NCPPB 2530 and NCPPB 2531 which did not use melibiose (A_{550} 0.05 and 0.08 respectively).

1 % inositol was used as a carbon source by all isolates of *E. salicis* tested (see Figure 3. 5). The A_{550} values ranged between 0.10 and 0.38.

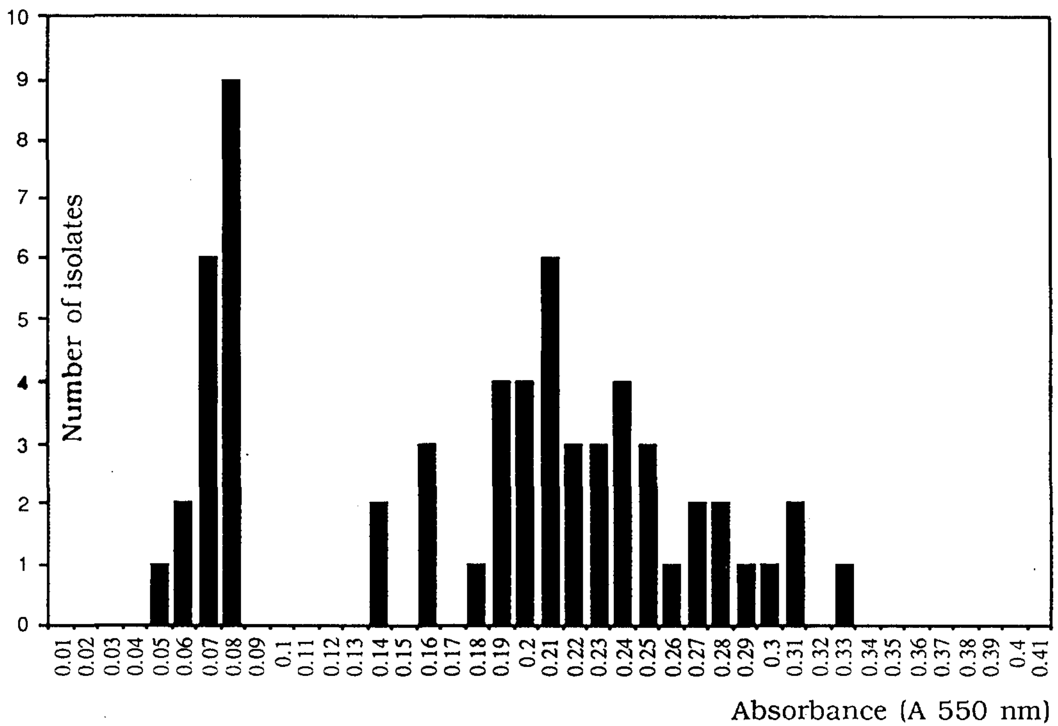


Figure 3. 1 Utilisation of galactose by *E.salicis* isolates.

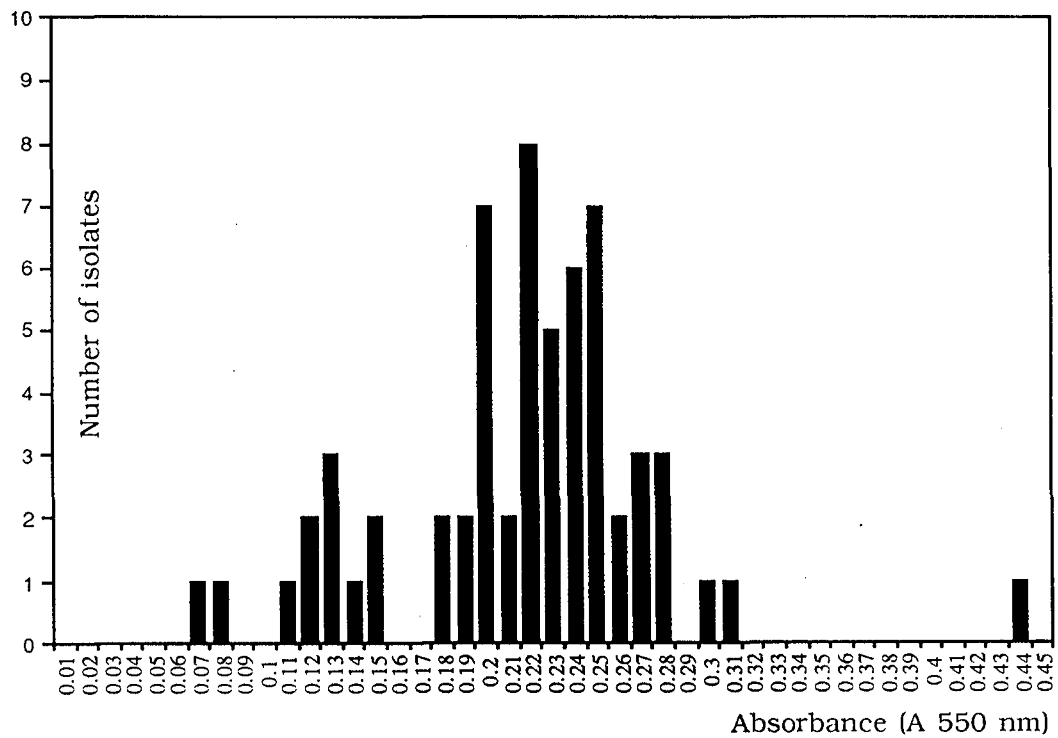


Figure 3. 2 Utilisation of 0.1 % raffinose by *E. salicis* isolates.

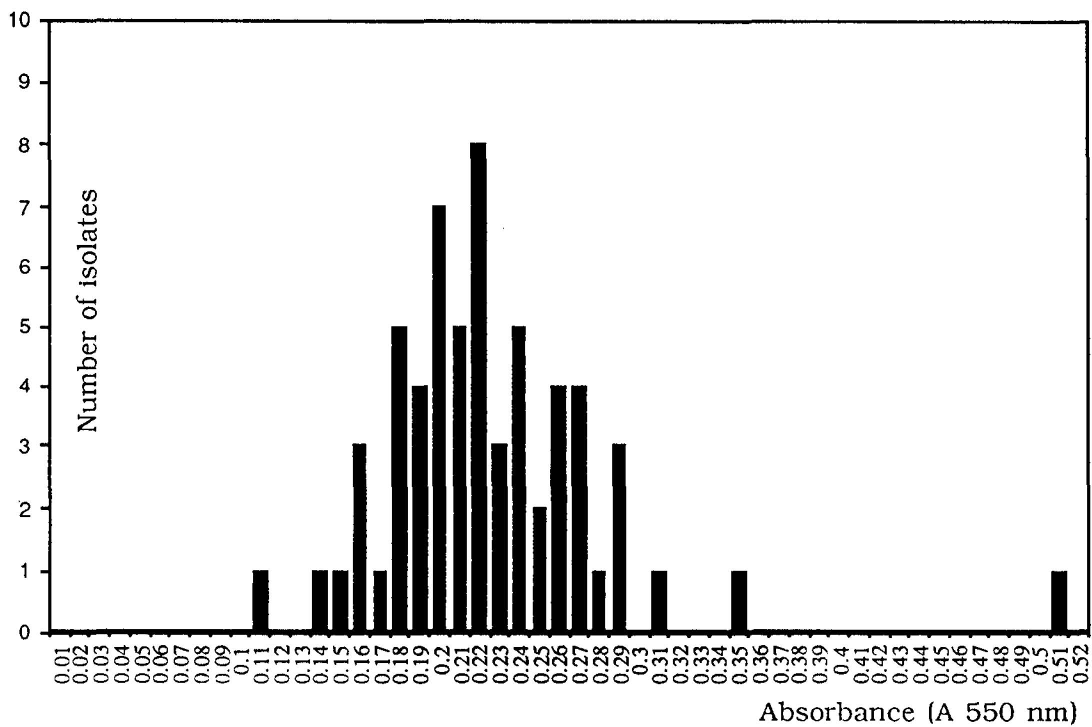


Figure 3. 3 Utilisation of 1 % raffinose by *E. salicis* isolates.

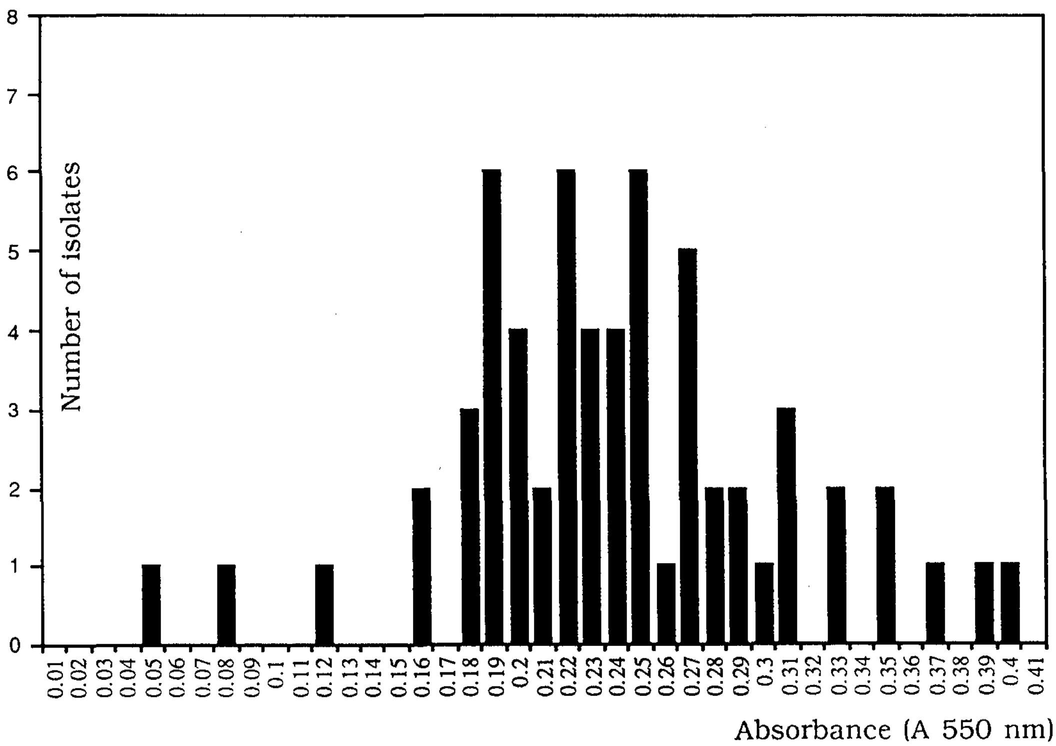


Figure 3. 4 Utilisation of melibiose by *E. salicis* isolates.

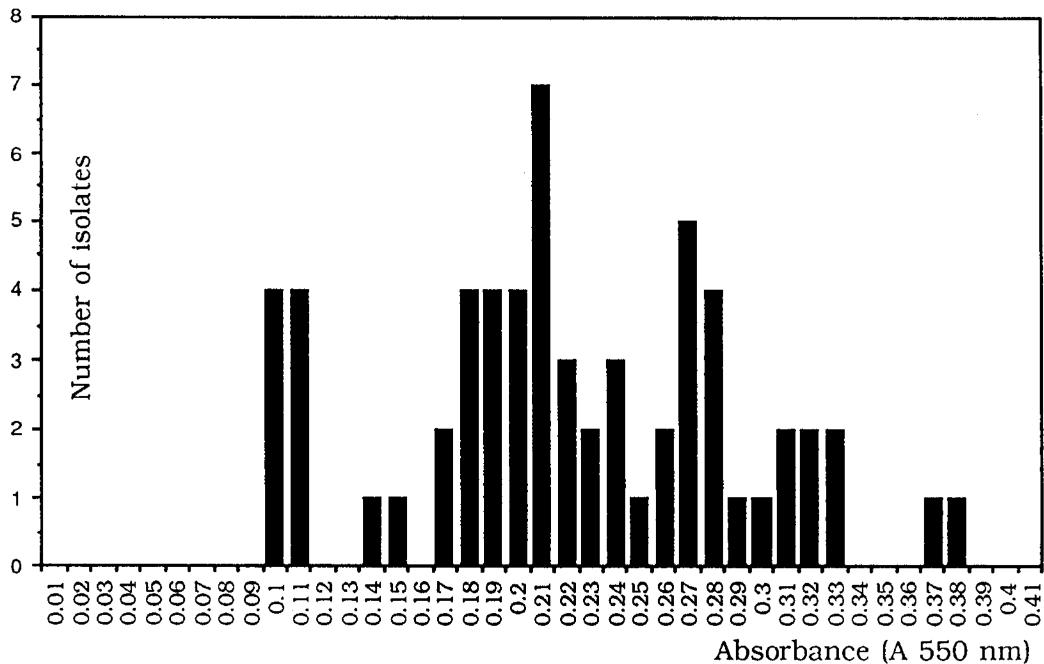


Figure 3. 5 Utilisation of inositol by *E. salicis* isolates.

3. 3. 2 Isolation of bacteriophages

Although several attempts were made to isolate the bacteriophages of *E. salicis* from natural sources and from lysogenic bacteria only 3 virulent bacteriophages could be recovered (see Table 3. 2). The indicator bacterium for all three bacteriophages isolated was English isolate NCPPB 2535. Bacteriophage L was isolated from the raw sewage sample obtained from local treatment plant. The other 2 bacteriophages, C and M, were isolated from soil sample collected from beneath *S. caprea* (goat willow) with symptoms of watermark disease, located in a park area in Long Melford in Sudbury. No bacteriophages could be isolated using three other isolates of *E. salicis,* NCPPB 2530, NCPPB 2907 and NCPPB 2908. No temperate bacteriophage could be isolated from lysogenic bacteria and because I was only interested in naturally occurring lysogeny no effort was made to induce this by irradiation or chemicals.

E. coli bacteriophage T3 gave the largest plaques.The plaques of the other 4 bacteriophages used showed minor differences. Bacteriophage L produced plaques that were larger than bacteriophages C and M, and had sharp edges. The plaques of bacteriophage C were surrounded by an halo. Plaques of bacteriophage NCPPB 1467 was the smallest. All bacte-

riophages tested developed clear plaques with no background growth on the plates after 2 days of incubation. No morphological study was made to characterise the bacteriophages of *E. salicis*.

Chloroform was chosen to kill the remaining bacteria in the routine preparation of stocks of bacteriophages since they were not sensitive to chloroform and the titer was not affected. Good yields upto 10^{10} pfu/ml were obtained by the method described. Routine test dilution was 10^4 for bacteriophage T3 and 10^6 for bacteriophages 1467, L, C and M. No loss in bacteriophage activity was observed within storage upto 6 months and the bacteriophages were propagated every 6 months. After each new propagation, the lytic spectrum of the propagated bacteriophage was tested to ensure the stability of the lytic pattern of the bacteriophage.

Table 3. 2 Bacteriophages isolated for *E. salicis*.

Source	Indicator strain	Bacteriophage
Raw sewage	NCPPB 2535	L
Soil	NCPPB 2535	C
Soil	NCPPB 2535	M

3. 3. 3 Reaction of *E. salicis* isolates to bacteriophages

3. 3. 3. 1 Choice of panel of tester bacteriophage

Seventeen isolates of *E. salicis* from the NCPPB, numbered from 1 to 17 in Table 2. 2 (see Chapter 2), were tested in order to choose a panel of tester bacteriophage. Of the 35 bacteriophage suspensions prepared, a set of five was selected for typing. None of the 30 bacteriophages of *E. coli* (see Table 3. 1) lysed any of *E. salicis* isolates tested. Only one bacteriophage of *E. coli* B, T3, lysed the cells of two English isolates of *E. salicis*, NCPPB

2533 and NCPPB 2534. The panel of tester bacteriophages therefore contained NCPPB 1467, *E. coli* B bacteriophage T3 (see Table 3. 1) and 3 bacteriophages of *E. salicis* isolated from natural sources namely L, C and M (see Table 3. 2).

3. 3. 3. 2 Bacteriophage typing of *E. salicis* isolates

Both the undiluted bacteriophage suspension (10^9- 10^{10} pfu/ml) and routine test diluted suspension (10^4 or 10^6) gave only two types of reaction on the spot-tested plates after incubation for two days at 28 °C. If the isolate was sensitive to bacteriophage a complete lysis was observed on the spotting. If the isolate was resistant to bacteriophage no lysis was observed at all. The percentage of typable isolates of *E. salicis* was 96 %. Only five isolates tested were not sensitive to any of the bacteriophages in the typing scheme.

Based on sensitivity to 5 bacteriophages, it was possible to differentiate lysotypes or bacterial strains with similar lytic patterns. All typable isolates of *E. salicis* were sensitive to bacteriophage L. Two main lysotypes designated α and β could be recognised. While lysotype α included only English isolates of *E. salicis*, lysotype β included only Dutch isolates of the bacterium. Table 3. 3 shows the lysotypes differentiated with reactions to the bacteriophages. Within lysotype α, 3 subtypes could be distinguished by differences in reactions to the bacteriophages T3, C and M. Lysotype α_1 included the majority of English isolates of *E. salicis* and they were sensitive to four bacteriophages, except bacteriophage T3 (see Plate 3. 1). Lysotype α_2 contained 5 English isolates of *E. salicis* namely, NCPPB 2533, NCPPB 2534, 90-30, 90-31 and 90-40 and, they were sensitive to bacteriophages L and T3. Lysotype α_3 included 2 English isolates of *E. salicis,* 35B and 41A1, from our collections and they were typed by the bacteriophages L, 1467 and C. Lysotype β containing only Dutch isolates of *E. salicis* was sensitive to only bacteriophage L (see Plate 3. 2). Five English isolates of the bacterium resistant to all bacteriophages were determined as nontypable in the present bacteriophage scheme. These isolates were namely, NCPPB 2530, NCPPB 2531, S7B, 90-97 and 90-98.

Strain variation in *E. salicis* was observed even in a single diseased tree. Nine isolates of *E. salicis* namely, NCPPB 2529 and NCPPB 2530 (Wong, 1974), NCPPB 2531 and NCPPB 2532 (Wong, 1974), 41A1 and 41B2 together with 90-38, 90-39 and 90-40

had been isolated from single trees (see Table 2. 2 in Chapter 2)) but they fell into different lysotypes with different lytic patterns, indicating that different strains of the bacterium may exist in a single tree.

Other bacteria were not lysed by any of 4 bacteriophages of *E. salicis* indicating the host specificity of the bacteriophages (see Table 3. 4).

3. 3. 4 Relation between galactose utilisation and bacteriophage sensitivity

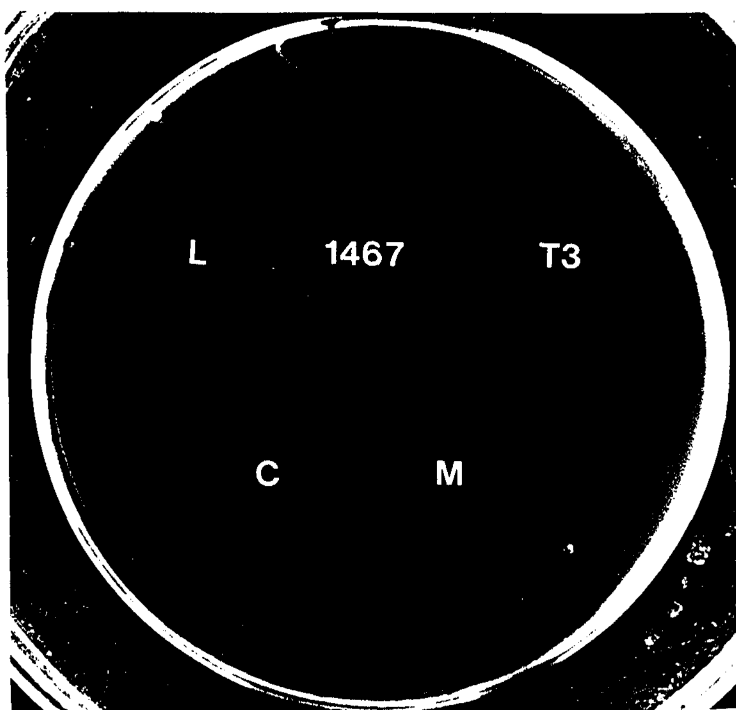
The galactose utilisation test distinguished English and Dutch isolates of *E. salicis* (see Figure 3.1).

The bacteriophage typing scheme confirmed the differentiation of English and Dutch isolates of the bacterium, giving two main lysotypes, α and β (see Table 3. 3). These results raised the question of any relation between the galactose utilisation and bacteriophage sensitivity of isolates of *E. salicis*. To test this, two mutant colonies of *E. salicis* NCPPB 2535 resistant to bacteriophages C and M respectively, were isolated. After confirming the resistance to the homologous bacteriophages they were found to be sensitive to only bacteriophage L, giving the bacteriophage typing pattern of Dutch isolates of *E. salicis*, although they were of course originally English isolates. In the galactose utilisation test they used galactose as a carbon source exactly as did those English isolates of the bacterium. Therefore, galactose utilisation was not associated with sensitivity to bacteriophages C and M.

3. 3. 5 *E. salicis* bacteriocins

No bacteriocin production could be detected with 7 of *E. salicis* isolates although control tests showed that the two methods employed worked very well. Bacteriocinogeny of *E. coli* isolates was confirmed but, attempts to induce the bacteriocins of *E. salicis* failed and therefore no bacteriocin typing scheme could be developed for *E. salicis*.

(a)



(b)

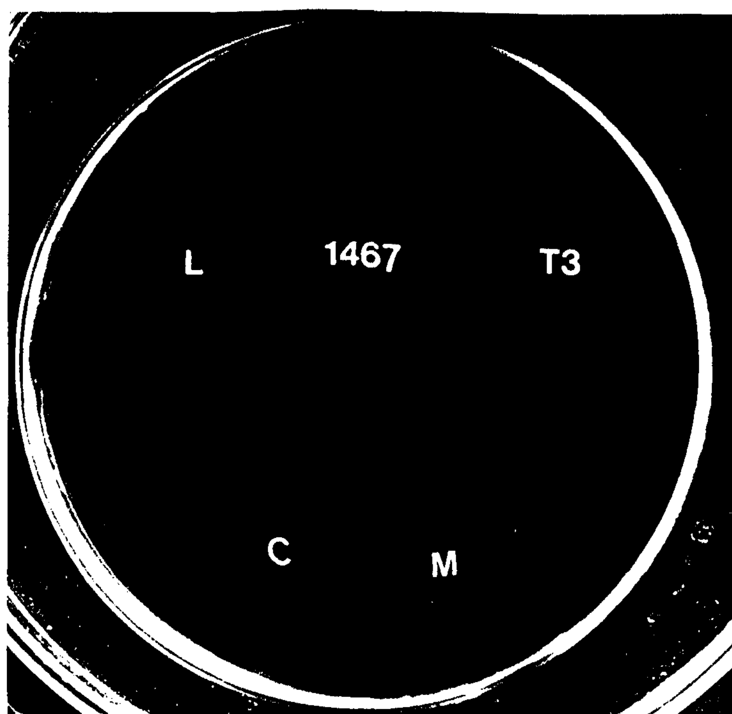


Plate 3. 1a. b Bacteriophage typing pattern of (a) *E. salicis* NCPPB 2535, a typical English isolate, a representative of lysotype α_1 and (b) *E. salicis* NCPPB 2907, a typical Dutch isolate, a representative of lysotype β. Five bacteriophages were spotted onto the bacterial lawn and incubated at 28 °C for 2 days.

Table 3. 3 Lysotypes of *E. salicis* differentiated by bacteriophage reaction.

Lysotype	Number of isolates	Reactions of bacteriophage L	1467	T3	C	M	Country of origin
α_1	50	+	+	–	+	+	England
α_2	5	+	–	+	–	–	England
α_3	2	+	+	–	+	–	England
β	16	+	–	–	–	–	Holland
NT	5	–	–	–	–	–	England

α_1 : *E. salicis* isolates 447, 1466, 2310, 2317, 2522, 2523, 2525, 2526, 2529, 2532, 2535, S1A, S4A, S5A, S8A, S9B, S10B, S11A, S12A, S13B, S14A, S14B, S15A, W1B, W2A, W3A, W4A, W6A, 27B, 28BD, 28C, 29AD, 30AD, 30C, 41B2, 174, 90-1, 90-8, 90-18, 90-21, 90-23, 90-36, 90-38, 90-39, 90-43, 90-44, 90-45, 90-52, 90-65 and 90-113.

α_2 : *E. salicis* isolates 2533, 2534, 90-30, 90-31 and 90-40.

α_3 : *E.salicis* isolates 35B and 41A1.

β : *E. salicis* isolates 2907,2908,73, 94,103,104, 118, 132, 142, 143, 144, 147, 149, 150, 156 and 173.

NT (not typable): *E. salicis* isolates 2530, 2531, S7B, 90-97 and 90-98.

+ : sensitive (confluent lysis)

– : resistant

Table 3. 4 Specificity of *E. salicis* bacteriophages

Isolate	Reaction to the bacteriophages of *E. salicis*			
	L	8	C	M
*Erwinia salicis**	+	+	+	+
Erwinia chrysanthemi	–	–	–	–
E. coli B	–	–	–	–
Pseudomonas phaseolicola	–	–	–	–
Ps. syringae	–	–	–	–
Ps. maculicola	–	–	–	–
Ps. lachrymans	–	–	–	–
Ps. fluorescens	–	–	–	–
Ps. syringe var. *atropurpurea*	–	–	–	–

* : Seventy three isolates of *E. salicis* differentiated in Table 3. 3.

— : no lysis

3. 4 Discussion

The results obtained with the 45 English isolates and 16 Dutch isolates of *E. salicis* suggest that utilisation of galactose test is a simple discriminatory test which demonstrates the strain variation of *E. salicis* and indicates the geographical origin of the bacterium.

Bacteriophages are generally isolated from natural habitats of the host bacterium. Sources of bacteriophages for phytopathogenic bacteria include lesions on diseased plants, infected seeds, soil beneath diseased plants, irrigation water and lysogenic bacterial strains. Generally, bacteriophages isolated from diseased tissues are more specific than those isolated from soil (Okabe and Goto, 1963). However, highly host-specific bacteriophages for phytopathogenic bacteria have been isolated from soil beneath diseased plants (Billing and Garrett, 1980 and Persley, 1983). There are many instances of successful isolation of bacteriophages from soil or sewage that might be free from plant pathogens (Okabe and Goto,

lytic spectrum of each bacteriophage was never solely restricted to the propagating strain and bacteriophage L isolated from sewage was capable of lysing 94 % of *E. salicis* isolates. Although bacteriophages C and M were isolated from the same soil collected beneath a diseased willow tree, they showed their distinct host ranges in lysotype α_3. Only 5 isolates were found to be untypable with the present typing scheme. Bacteriophages isolated for *E. salicis* were quite specific and they did not lyse any of the other species of 8 bacteria tested (see Table 3. 4).

The bacteriophage typing of *E. salicis* also confirmed Wong's findings (1974) that different strains of the bacterium exist in a single tree.

The most significant result obtained with the bacteriophage typing system developed has been the correlation of lysotypes of *E. salicis* with the results of galactose utilisation tests (see Table 3. 5). The six strains thus distinguished proved the geographical distinction of *E. salicis* isolates. Strains E and F were extremely different from other strains. It is proposed that these two strains be regarded as a subspecies of *E. salicis*.

Table 3. 5 *E. salicis* strains distinguished by galactose utilisation and bacteriophage typing.

Strain	No. of isolates	Reaction to bacteriophage					Galactose utilisation	Country of origin
		L	1467	T3	C	M		
A	50	+	+	—	+	+	+	England
B	5	+	—	+	—	—	+	England
C	2	+	+	—	+	—	+	England
D	16	+	—	—	—	—	—	Holland
E	2	—	—	—	—	—	—	England
F	3	—	—	—	—	—	+	England

+ : sensitive to bacteriophage, positive with galactose utilisation

— : resistant to bacteriophage, negative with galactose utilisation

Among other microorganisms it is well known that the predominant bacteriophage types change over the years (Nielsen and Rosdahl, 1990), therefore the necessity for isolating bacteriophages which will subdivide the Dutch isolates of the bacterium is obvious.

The present typing scheme indicated more variation in the English isolates of *E. salicis* than those in the Dutch isolates. Since we tested large number of English isolates, it is possible that more strains of the bacterium might be identified if a large number of Dutch isolates were included to bacteriophage sensitivity test.

In conclusion, our present typing scheme indicates that the use of bacteriophage typing is a quite promising means of discriminating and differentiating *E. salicis* isolates despite the difficulty of isolating bacteriophages of *E. salicis* from natural sources. To differentiate a larger proportion of isolates of *E. salicis* serological methods of strain identification need to be employed.

CHAPTER IV

SEROTYPING OF *E. SALICIS* WITH OUCHTERLONY DOUBLE DIFFUSION TESTS

4. 1 Introduction

The agar double diffusion precipitin (immunodiffusion) test was introduced by Ouchterlony (1948) and have been widely used to identify or differentiate strains of plant pathogenic bacteria since 1960 (Schaad, 1979). Species-specific antigens of *Pseudomonas tabaci* were detected by the Ouchterlony double diffusion tests (Lovrekovich and Klement, 1961). Serological variation and identification of *P. lachrymans* and other phytopathogenic *Pseudomonas* nomenspecies were studied by the double diffusion tests and three distinct serotypes were differentiated among nearly 300 isolates of *P. lachrymans* (Lucas and Grogan, 1969). Approximately 450 isolates of *P. syringae* were tested by Ouchterlony double diffusion tests and 10 distinct serotypes based on the reaction of their heat-stable antigens were detected (Otta and English, 1971). Pathogenic variability of *Xanthomonas cyamopsidis* was demonstrated by the immunodiffusion tests (Orellena and Weber, 1971). Immunodiffusion tests also revealed the antigenic differences between infective and non-infective strains of *Rhizobium trifolii* (Dazzo and Hubbell, 1975). Lipopolysaccharide composition of *P. syringae* pv. *syringae* and *P. syringae* pv. *morsprunorum* was compared by the immunodiffusion tests to distinguish between these two closely related bacteria (Zamze *et al.*, 1986).

Ouchterlony double diffusion tests were also used for *Erwinia* species. The relationships of the variants of *E. herbicola* were studied by the test (Chatterjee and Gibbins, 1971). Serological relationships among 27 isolates of *E. chrysanthemi* were determined by Ouchterlony double diffusion tests and 4 serotypes occurred. The test was concluded to be an ideal method for determining the relatedness of strains of this species (Yakrus and Schaad, 1979).

Enterobacteria have several surface antigens, including O, H, K and others (Orskov and Orskov, 1978). 'O' antigen is heat-stable lipolpolysaccharide components in the outer membrane and they have been found the most useful for primary serological grouping. 'O' antigen has been used to group some strains of *E. carotovora* (De Boer *et al.*, 1979) and

1001 isolates of *E. carotovora* were categorised into 18 serogroups based on the similarities in the lipopolysaccharide 'O' antigen.

Kam (1976) first used Ouchterlony double diffusion tests to reveal serological variation in *E. salicis* using 4 Dutch isolates and 7 English isolates of the bacterium. He reported that 2 Dutch isolates reacted less distinctly than the other isolates at the same cell concentration; however serotypes could not be demonstrated by means of cross-absorption experiments. Therefore he concluded that immunodiffusion test has detected only quantitative strain differences. However, the test has been found to have excellent specificity for identification of *E. salicis* (Kam, 1989).

In the present study, a serotyping scheme for grouping of *E. salicis* into serogroups on the basis of diffusible heat-stable somatic antigens was developed using 59 isolates of *E. salicis*. Two antisera were raised against two English isolates, representing strain A and strain C differentiated by the bacteriophage typing and galactose utilisation tests (see Chapter 3). Different methods e.g. phenol-treatment and heat-treatment were employed to prepare antigens of the isolates.

Cross-absorbtion tests were carried out to reveal the relatedness of the between two isolates.

4. 2 Materials and methods

4. 2. 1 Production of specific antiserum

Two antisera were raised against two English isolates of *E. salicis,* NCPPB 2535 and 35 B. Two New Zealand white rabbits were injected for each antiserum production. Antigen preparation, antiserum production, separation and preservation of the sera and determination of titre of the sera have been described (see Chapter 2. 2. 6).

4. 2. 2 Preparation of antigen for immunodiffusion tests

4. 2. 2. 1 Preparation of antigen with phenol-treatment

Preliminary immunodiffusion tests were made with phenol-treated bacterial suspensions prepared by the method of Kam (1989). The isolates tested, 36 isolates of *E. salicis* numbered 1 to 36 in Table 4. 1, were grown on NDA plates for 3 days at 28 °C. The bacteri-

al growth was suspended in 1 ml of 0.01 M phosphate buffered saline (PBS) pH 7.4 (see appendix for the recipe) to give approximately 10^9 cells/ml. A drop of 10 % phenol aqueous solution was added to the cell suspension just before using and thoroughly mixed.

4. 2. 2. 2 Preparation of the antigen with heat-treatment

Two methods were employed to prepare heat-stable antigens of *E. salicis* :

a. Preliminary preparations of heat-treated antigen suspensions were made by the method of Lucas and Grogan (1969). Five isolates of *E. salicis*, NCPPB 447, NCPPB 1466, NCPPB 2310, NCPPB 2317 and NCPPB 2535 were grown on NDA plates for 3 days at 28 °C. Bacterial cells were suspended in distilled water to give 10^9 cells/ml and heated at 100 °C for 1 hour.

b. The method of Lelliott and Stead (1987) was used to prepare heat-treated antigen suspensions of 63 isolates (Table 4. 1) containing 59 isolates of *E. salicis* from the NCPPB, from the Netherlands and from our collection together with *Erwinia herbicola* NCPPB 2971, *E. rhapontici* NCPPB 1578 and 2 unknown epiphytes isolated from watermark diseased trees were grown on NDA plates for 3 days at 28 °C. Bacterial colonies were suspended in 0.1N sodium hydroxide solution. The suspension containing approximately 10^9 cells/ml was centrifuged at 10 000 rpm for 15 minutes. The supernatant containing only the soluble antigens of the bacterium was autoclaved at 121 °C for 15 minutes. No preservative was added to the antigen suspension and it was stored at 4 °C until use.

4. 2. 3 Preparation of immunodiffusion gel-plates

Difco purified agar was used to prepare immunodiffusion gels. Agar suspension (0.8 %) containing 0.85 % sodium chloride and 0.05 % sodium azide as a preservative was prepared and 15 ml of medium was poured into a petri dish 9 cm in diameter. The plates were allowed to dry for 24 hours at room temperature and then stored at 4 °C until needed. Prior to use, circular patterns with 6 wells 6 mm in diameter and 7 mm apart, surrounding a central well, were cut with a sterile pasteur pipette. Agar discs were removed by a sterile toothpick.

4. 2. 4 Determination of working dilution of antiserum

Two-fold dilution series, 1/2, 1/4 and 1/8, of anti-2535 antiserum and anti-35B antiserum were prepared in PBS pH 7.4. Fifty µl of the diluted antisera was added to the central well. Fifty µl of homologous antigen suspension was added to the two opposite peripheral wells. The remaining four wells were filled with antigen suspensions of isolates NCPPB 447, NCPPB 1466, NCPPB 2310 and NCPPB 2317. The plates were covered with the lids and placed in the polythene bag containing damp cotton to create high humidity. The plates were incubated overnight at room temperature and then for 5 to 7 days at 4 °C.

4. 2. 5 Immunodiffusion tests

Anti-2535 antiserum was diluted to 1/8 in PBS pH 7.4. Fifty µl of the antiserum was put into the central well. Fifty µl of homologous antigen suspension of *E. salicis* isolate NCPPB 2535 was put into two opposite peripheral wells. The remaining four wells were used to test isolates. The plates were covered and placed in polythene bags containing damp cotton to create high humidity and incubated as above. Further experiments were done with the second antiserum, anti-35B antiserum, and absorbed antiserum.

4. 2. 6 Cross-absorption tests

4. 2. 6. 1 Preparation of the antigen

Heat-treated soluble antigens of the *E. salicis* isolates NCPPB 2535 and 35B were prepared in 3 ml of 0.1 N sodium hydroxide as described previously. After autoclaving at 121 °C for 15 minutes the cell suspension was dialysed overnight at 4 °C against several changes of distilled water. The dialysed antigen preparation was freeze-dried in Eppendorf tubes as 250 µl volumes overnight and stored at –20 °C until use. Before use in the cross-absorption tests the activity of the freeze-dried antigen preparations was tested. Freeze-dried antigen preparations, 2535 and 35B, were taken up in their original volume (250 µl) of 0.1 N sodium hydroxide. Two wells 6 mm in diameter were cut equidistant from a central well in an agar plate.

Central well was filled with either 50µl of the neat anti-2535 antiserum or anti-35B antiserum . One of the peripheral wells contained 50 µl of heat-treated homologous antigen suspension. Another outer well was filled with 50 µl of freeze-dried antigen suspension. After incubation overnight at room temperature and then at 4 °C in a polythene bag the plates were examined to confirm that an identical precipitin band had formed with the two antigen preparations, indicating the activity of freeze-dried homologous antigen samples.

4. 2. 6. 2 Preparation of cross-absorbed antiserum

After confirming the antigenic activity of freeze-dried samples, the two antisera were cross-absorbed with heterologous antigens. Undiluted anti-2535 antiserum (250 µl) was added to 500 µl of freeze-dried antigenic suspension of *E. salicis* isolate 35B. Undiluted anti-35B antiserum (250 µl) was added to 500 µl of freeze-dried antigenic suspension of *E. salicis* 2535. These two samples in the Eppendorf tubes were incubated for 30 minutes at room temperature and then overnight at 4 °C. After incubation, antigen and antiserum mixtures in the Eppendorf tubes were centrifuged at 14 000 rpm for 7 minutes and the supernatant collected was used as cross-absorbed antiserum.

4. 2. 7 Staining of the immunodiffusion gel

An attempt was made to use the method of M. de Kam (pers. comm.) to stain the immunodiffusion gels. After development of precipitin bands following incubation, the gel was taken out from the petri dish and placed inside of the lid. Ten filter papers cut to the size of the lid were placed on top of the immunodiffusion gel. The gel was pressed with 250 ml flask containing water. After 10 minutes the filter papers were renewed with the dry filter papers and the flask was put on top. The procedure was repeated until the agar was 1-2 mm in thickness but still flexible. Staining solution containing 1 % amido black and 7 % acetic acid was poured over the agar. After the agar was swollen with the staining solution, the above procedure was repeated to remove the staining solution until the gel was 1-2 mm thick. Destaining was made with several changes of 7% acetic acid. When the background was clear and only the precipitin bands were stained in the gel the destaining solution was removed by pressing with filter papers. The gel was dried when it was 1-2 mm thick and

stuck onto white card.

4. 2. 8 Photography of the immunodiffusion plates

The lighting technique of Reed (1960) was used with some minor modifications for the photography of immunodiffusion plates. The stage or platform for the mounting the petri plate was a heating tripod 9 in. high, with a diameter of 7.5 in., to the top of which a disk of black cardboard was stuck. A central hole in the cardboard was made, 7 cm diameter in size, and the plate was put on it. A lighting box was used as illuminant. The face of the box was covered with a cardboard having a hole 10 in. in diameter in the centre of which was placed a black cardboard disk approximately 7.5 in. in diameter taped to the glass. The resultant ring of fluorescent illumination was approximately 1.25 in. wide. The tripod was then placed directly over the black cardboard disk, the light was turned on, and the camera was focused on the plate. Ilford, PanF film was used in the photography of the plates.

4. 3 Results

Agglutination titers of both antisera raised against *E. salicis* NCPPB 2535 and 35B were 1/1024. Immunodiffusion gels could not be successfully removed from the petri plates for staining and this method was not found to be useful. However, satisfactory results were obtained with the photographic method which was used to record all gels.

Immunodiffusion tests with phenol-treated antigen preparations showed that all had at least two antigens in common (see Plate 4. 1). The precipitin lines were weak and poorly defined therefore it was difficult to detect any antigenic difference between isolates by phenol-treated antigenic preparations.

Preliminary tests with heat-treated antigenic preparations (100 °C for 1 hour) gave similar results to those with phenol-treated antigenic preparations. Hence these two preparations could not be used to develop an immunodiffusion typing scheme for *E. salicis*.

Heat-stable soluble antigenic preparations obtained by the method of Lelliott and Stead (1987) gave at least three, strong, well defined precipitin lines when they interacted with anti-2535 antiserum (see Plate 4. 2). Common precipitin bands disappeared when 1/8 diluted antiserum was used and as a result, a single precipitin band was obtained for each isolate (see Plate 4. 3).

Fifty nine isolates of *E. salicis*, 17 isolates were from the NCPPB, 15 isolates were

received from the Netherlands and 27 cultures isolated from the diseased trees during the course of this study and presumably *E. salicis,* and 4 isolates of other bacteria (see Table 4. 1) were tested with anti-2535 antiserum. Two types of immunodiffusion reactions were observed with the cultures tested: Either a precipitin band developed and fused with that of the homologous antigen NCPPB 2535 without spur formation and the isolates were considered to contain the heat-stable antigen (HSA) or there was no reaction and the isolate was considered not to contain heat-stable antigen (HSA) (see Plate 4. 4). On this basis two groups of 59 *E. salicis* isolates were distinguished. A total of seven isolates of *E. salicis* three from the NCPPB, 2530, 2531 and 2907, two isolates received from the Netherlands, 73 and 104, and two cultures isolated during the course of this study, 35B and 41A1, lacked heat-stable antigen (HSA). Fifty two isolates of *E. salicis* contained heat-stable antigen (HSA) (see Table 4. 1). Two epiphytes (not *E. salicis*) isolated from watermark diseased willow trees and *E. herbicola* NCPPB 2971 and *E. rhapontici* NCPPB 1578 did not react with anti-2535 antiserum indicating the specificity of the antiserum in the immunodiffusion tests.

Further immunodiffusion tests were carried out with anti-35B antiserum. Heterologous heat-stable antigen preparations gave two precipitin lines with this antiserum while this antiserum gave only a very weak single precipitin band with the homologous heat-stable antigen preparation. Anti-35B antiserum therefore only distinguished antigen 35B from other antigens, and was of limited value for a typing scheme. Hence, it was not used further.

Serological relatedness between isolates *E. salicis* NCPPB 2535 and 35B was revealed by the cross-absorbed antisera. Six outer wells were cut equidistant from two centre wells (see Plates 4. 5-6). Center wells contained antisera and outer wells were filled with the heat-treated antigenic suspensions.

Plate 4. 5 shows the reaction of absorbed and unabsorbed anti-2535 antiserum. Central well no. 1 contained 50 μl of unabsorbed anti-2535 antiserum and well no. 2 contained 50 μl of cross-absorbed anti-2535 antiserum which was absorbed with excess of 35B antigen. Outer wells denoted 'a' were filled with an equal volume (50 μl) of antigenic preparation of 2535. Outer wells denoted 'b' contained 50 μl of antigenic suspension of 35B. Unabsorbed anti-2535 antiserum gave three precipitin bands two of which were strong and sharp while the third one was very weak with the homologous antigen suspension (2535). Heterologous antigen suspension (35B) had only the weak band with unabsorbed anti-2535 antiserum.This weaker band was common with both of the antigenic preparations.

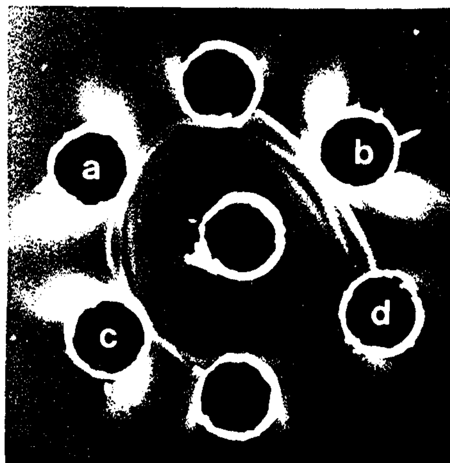


Plate 4. 1 Ouchterlony double diffusion patterns showing the reactions between anti-2535 antiserum (centre well) and phenol-treated cells (the homologous antigen: top and bottom wells of set of six peripheral wells and tests isolates a: NCPPB 2529, b: NCPPB 2526, c: NCPPB 2523 and d: NCPPB 2522).

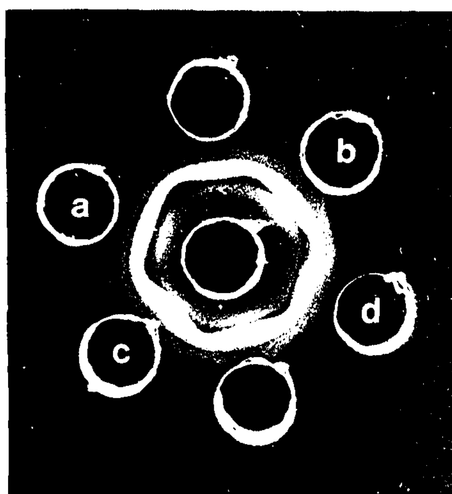


Plate 4. 2 Ouchterlony double diffusion patterns showing the reactions between undiluted anti-2535 antiserum (centre well) and heat-treated cells (the homologous anti gen: top and bottom wells of set of six peripheral wells and tests isolates a: NCPPB 2522, b: NCPPB 1466, c: NCPPB 2310 and d: NCPPB 2317).

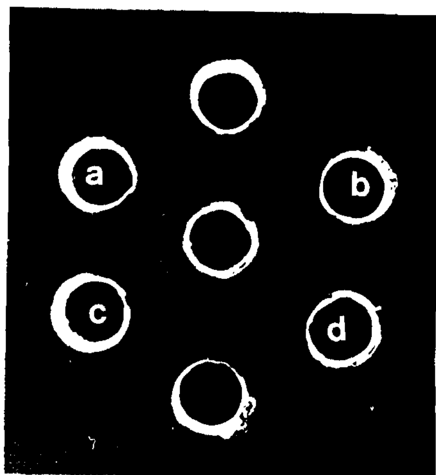


Plate 4. 3 Ouchterlony double diffusion patterns showing the reactions between 1/8 dilut ed anti-2535 antiserum (centre well) and heat-treated cells (the homologous antigen: top and bottom wells of set of six peripheral wells and tests isolates a: 30AD, b: 29AD, c: 28C and d: 28BD).

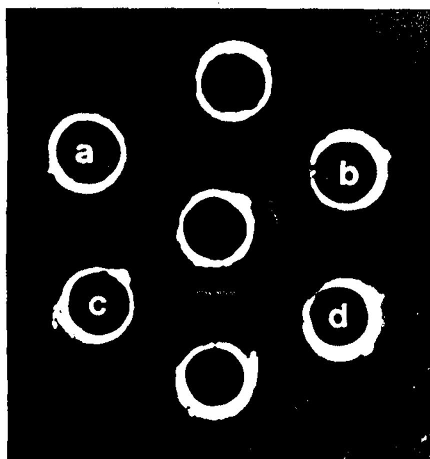


Plate 4. 4 Ouchterlony double diffusion patterns showing the reactions between 1/8 dilut ed anti-2535 antiserum (centre well) and heat-treated cells [the homologous antigen: top and bottom wells of set of six peripheral wells and tests isolates a: 41B2 (HSA positive), b: 30C (HSA positive), c: 35B (HSA negative) and d: 41A1 (HSA negative)].

Two strong bands disappeared when the cross-absorbed anti-2535 antiserum reacted with the homologous antigen preparations. But, the weaker and common band still remained with the both of the homologous and heterologous antigenic preparations.

Plate 4. 6 shows the pattern of immunodiffusion plate for absorbed and unabsorbed anti-35B antiserum. Well no. 1 was filled with 50 μl of unabsorbed anti-35B antiserum and well no. 2 was filled with an equal volume of cross-absorbed anti-35B antiserum which was absorbed with excess of 2535 antigen. Outer wells denoted 'a' contained 50 μl of antigenic preparation of 2535 and outer wells denoted 'b' were filled with 50 μl of antigenic preparation of 35B.

Unabsorbed anti-35B antiserum gave a band with the homologous antigen preparation (35B), while it gave two precipitin bands one of which was strong and sharp with the heterologous antigen preparation (2535). The sharp band disappeared when the cross-absorbed anti-35B antiserum reacted with the heterologous antigen preparation, but a weak immunodiffusion reaction was observed with both of the antigenic preparations. The band obtained with the cross-absorbed anti-35B antiserum was fainter than the band obtained with the unabsorbed anti-35B antiserum.

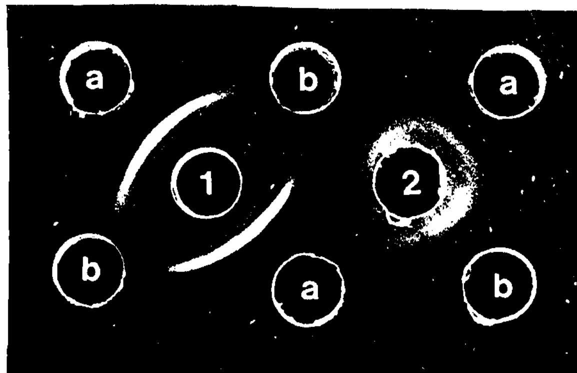


Plate 4. 5 Pattern of an immunodiffusion plate containing unabsorbed and absorbed anti-2535 antiserum. Well no. 1 contains unabsorbed anti-2535 antiserum, well no. 2 contains cross-absorbed anti-2535 antiserum with 35B antigen, wells de noted 'a' contain heat-treated antigen preparation of *E. salicis* 2535, wells de noted 'b' contain heat-treated antigen preparation of *E. salicis* 35B.

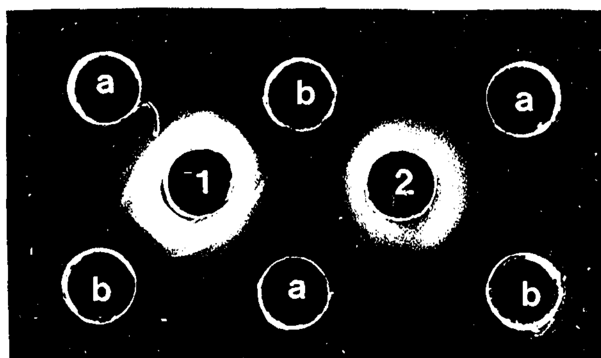


Plate 4. 6 Pattern of an immunodiffusion plate containing unabsorbed and absorbed anti-35B antiserum. Well no. 1 contains unabsorbed anti-35B antiserum, well no. 2 contains cross-absorbed anti-35B antiserum with 2535 antigen, wells denoted 'a' contain heat-treated antigen preparation of *E. salicis* 2535, wells denoted 'b' contain heat-treated antigen preparation of *E. salicis* 35B.

Table 4. 1 Serogroups of *E. salicis* isolates determined by Ouchterlony double diffusion tests.

No. of isolates	isolate	heat- stable antigen
1	447***	+
2	1466***	+
3	2310***	+
4	2317***	+
5	2522***	+
6	**2523*** **	**+**
7	**2525*** **	+
8	2526***	+
9	2529***	+
10	2530***	–
11	2531***	–
12	2532***	+
13	2533***	+
14	2534***	+
15	2535***	+
16	2907***	–
17	2908***	+
18	73**	–
19	94**	+
20	103**	+
21	104**	–
22	118**	+
23	132**	+
24	142**	+
25	143**	+
26	144**	+
27	147**	+
28	149**	+
29	150**	+
30	156**	+
31	173**	+
32	174**	+
33	S1A*	+
34	S4A*	+
35	S5A*	+
36	S7B*	+
37	S8A*	+
38	S9B*	+

39	S10B*	+
40	S11A*	+
41	S12A*	+
42	S13B*	+
43	S14A*	+
44	S14B*	+
45	S15A*	+
46	W1B*	+
47	W2A*	+
48	W3A*	+
49	W4A*	+
50	W6A*	+
51	27B*	+
52	28BD*	+
53	28C*	+
54	29AD*	+
55	30AD*	+
56	30C*	+
57	35B*	–
58	41A1*	–
59	41B2*	+
60	unknown epiphyte $_1$	–
61	unknown epiphyte $_2$	–
62	*E. herbicola* NCPPB 2971	–
63	*E. rhapontici* NCPPB 1578	–

*** from the NCPPB

** from M. de Kam (the Netherlands)

* isolated at UEA (University of East Anglia)

+ : precipitin line

– : no precipitin line

4. 4 Discussion

Most *Erwinia* spp. apart from *E. carotovora* subsp. *atroseptica,* have been reported to be highly toxic for rabbits undergoing immunisation (Lazar, 1971), but no rabbits were lost due to toxic effects in the present study. It has been reported that it was not easy to prepare high titre antiserum to *E. salicis* the homologous titre usually being in the range 1:160 to 1:640 (Slade and Tiffin, 1984). We could obtain 1:1024 titration of both antisera using

thc procedure described in the present study.

Immunodiffusion tests are relatively simple to do and the precipitin lines in the gel are readily visible to the naked eye. But they have the disadvantage that they use much antiserum and a major source of error in tests in agar is to failure to define correctly the conditions needed to obtain the precipitin reaction. Important factors include concentration and diffusion coefficients of reactants, gel pore size, pH, and the incubation temperature. If one of the reactants is in excess, there may be no visible reaction or the line of precipitate may be a broad band close to the well of the more dilute reactant (Crowle, 1960). Hence, great care was taken during the preparation and incubation of immunodiffusion plates represented in this study.

Fifty nine isolates of *E. salicis* were separated into two groups on the basis of presence or absence of heat- stable 'O' antigen in Ouchterlony double diffusion tests with anti-2535 antiserum. If the two antigen wells contained identical antigens, the resulting precipitin lines extended laterally, they interfered, deviated and gradually fused to form a symmetrical arc. The heat-stable 'O' antigens responsible for precipitin band formation could be shown in the majority of the isolates while some English and some Dutch isolates lacked the antigens in the present study. Therefore, no antigenic differences could be detected between English and Dutch isolates of *E. salicis* using the immunodiffusion tests.

Dutch isolate 73 did not react to anti-2535 antiserum supporting Kam's report (1976). Two authentic *E. salicis* isolates NCPPB 2530 and NCPPB 2531 which were recorded as ELISA negative in the competitive assay (see Chapter 2. 3. 4) lacked the heat-stable 'O' antigen, indicating that these two isolates were antigenically different from other isolates tested, although their pathogenicity was confirmed in the identification studies by Wong (1974). In addition, five more isolates, three of which were Dutch isolates, NCPPB 2907, 73, 104, 35B and 41A1 did not have the heat-stable antigen in the immunodiffusion tests but they were ELISA positive in the competitive assay (see Chapter 2. 3. 4). Probably, these 5 isolates contain antigens other than the heat-stable antigen.

Cross-absorbtion tests showed that two isolates used for antiserum production, NCPPB 2535 and 35B, were serologically related to each other to some extent in the immunodiffusion tests. *E. salicis* NCPPB 2535 lost strong and well defined precipitin bands with the cross-absorbed antisera, while a weaker reaction which could be due to insufficient cross-absorbtion of the antisera was observed in both plates containing two different cross-absorbed antisera (see Plates 4. 5-6). Isolate 35B also gave a weaker band with the cross-absorbed anti-35 antiserum indicating that insufficient cross-absorbtion of the two an-

tisera occurred. The reason for the insufficient cross-absorption is not exactly known since both of the reactions took place with excess of antigens. Although it is clear that NCPPB 2535 and 35B are antigenically related, the relation between these two isolates can not be fully explained from cross-absorption tests. These two isolates tested may have common antigenic determinants, epitopes, but they may differ at their molecular size causing precipitin band formations varying in the intensity, number and distance.

These results showed that *E. salicis* is serologically uniform in contrast to other plant pathogenic bacteria e.g. *E. carotovora* (De Boer *et al.*, 1979) and *E. chrysanthemi* (Yakrus and Schaad, 1979). Consequently, these techniques could not be used for distinguishing isolates of *E. salicis* . A new technique, multilocus enzyme electrophoresis typing, was therefore developed in the following chapter (see Chapter 5) in an attempt to develop the technique for further differentiation of *E. salicis* isolates.

CHAPTER V

DEVELOPMENT OF MULTILOCUS ENZYME ELECTROPHORESIS METHOD FOR *E. SALICIS*

5. 1 Introduction

The technique of starch gel electrophoresis of enzymes with specific staining for activity in the gel, the so-called 'zymogram' or 'multilocus enzyme electrophoresis' or 'allozyme electrophoresis' method, was developed by Hunter and Markert (1957) and has found many research applications, e.g. screening studies, comparing relatively large numbers of enzymes among a variety of tissues and organisms and in research in eucaryotic population genetics (Shaw, 1965). Multilocus enzyme electrophoresis has recently been used to estimate the genetic diversity and structure in natural populations of a variety of species of bacteria e.g. *E. coli* (including *Shigella* spp.) (Selander and Levin, 1980; Ochman *et al.*, 1983 and Achtman *et al.*, 1986), *Pseudomonas aeroginosa* (Levin *et al.*, 1984), *Yersinia ruckeri* (Schill *et al.*, 1984), *Gluconabacter* spp. (Yamada and Akita, 1984), *Rhizobium* spp. (Young *et al.*, 1987), *Haemophilus influenza* (Musser *et al.*, 1985) and *Bordetella* spp. (Musser *et al.*, 1986).

Most of the methods have been developed for tissue extracts from mammalian species. A few have been developed for plants and lower animals (Shaw and Prasad, 1970). Standard laboratory methods for studying polymorphic variation in enzymes by gel electrophoresis were described in detail by Harris and Hopkinson (1976). These techniques were adapted with only minor modifications and recommended to many species of bacteria (Selander *et al.*, 1986).

In the present study, I have developed a multilocus enzyme electrophoresis method for *E. salicis* using cellulose acetate gel as a support medium. The method was evaluated at three steps: breakage of bacterial cells, detection of the activity of enzymes in the lysates and, determination of optimal electrophoretic conditions to maximise the resolution of individual bands of activity whilst maximising the separation between different bands. Several methods including sonication, sonication with ballotini beads, breakage by X-press and French pressure cell press were employed to lyse the cells. Lysis was determined visually and microscopically. In general, I adapted the electrophoretic techniques of Richardson *et*

al. (1986) developed for animal systematics and population studies employing cellulose acetate gels to separate and stain the enzymes. In the preliminary studies alkaline phosphatase and in the following studies glucose-6-phosphate dehydrogenase were used to test the enzyme activity of the samples. A total of fifteen enzymes were examined in four *E. salicis* isolates and optimal electrophoretic conditions were determined for each enzyme by testing the variables. Seven enzymes namely, alkaline phosphatase (ALP), glucose-6-phosphate dehydrogenase (G6PD), malate dehydrogenase (MDH), indophenol oxidase (superoxide dismutase) (IPO), glutamate dehydrogenase (GDH), 6-phosphogluconate dehydrogenase (6PGD) and isocitrate dehydrogenase (IDH) were eventually chosen for distinguishing *E. salicis* isolates by multilocus enzyme electrophoresis in the following study (see Chapter 6).

5. 2 Materials and methods

5. 2. 1 Evaluation of lysis methods

5. 2. 1. 1 Preparation of bacterial cultures

E. salicis isolate to be tested was grown on NDA medium for 3 days at 28 °C and was examined visually for purity. Each isolate was then grown in 250 ml of NDB, or for breakage in the French pressure cell press the bacterium was grown in 750 ml of NDB for 2 days at 28 °C on a rotary shaker, to give approximately 10^8–10^9 cells/ ml. Bacterial cultures were harvested by centrifugation of broth cultures at 10 000 rpm for 15 minutes at 4 °C and the resulting pellet was frozen and stored at –20 °C until lysis.

Two different extraction buffers, buffer A and B, were prepared and used at different concentrations to prepare the lysates. In preliminary studies the frozen pellet was resuspended in 5.0 ml of the buffer A (see Table 5. 1) per gram of cells as described by Baptist *et al.* (1969). In later experiment the frozen pellet was resuspended by addition of 2.0 ml of buffer B (see Table 5. 1) per gram of wet cells as described by Selander *et al.* (1986).

5. 2. 1. 2 Use of sonicator for the preparation of lysates

Bacterial cells suspended in the extraction buffer A or B (see Table 5.1) were distrupted by sonication (Dawe Soniprobe, type 7531A, Dawe Instruments, London) for upto 5 minutes at 15 seconds periods with 30-second cooling periods on ice. Foaming was

avoided during sonication. A microtip was used for sonication of bacterial suspension less than 2 ml to be sonicated. In preliminary studies, after each sonication period the bacterial extract was examined visually and microscopically for the breakage.

5. 2. 1. 3 Use of lysozyme in the extraction buffer

Lysozyme (Sigma No. L 6876) was added to the extraction buffer B (see Table 5. 1) at the concentration of 1 mg/ml, 2 mg/ml, 3 mg/ml or 4 mg/ml just before use. Bacterial pellets were suspended by addition of 2 ml of buffer per gram of wet cells and the suspensions were left at room temperature for 10 minutes and then subjected to lysis by sonicator as described above.

5. 2. 1. 4 Use of Ballotini beads for the preparation of lysates

Ballotini beads (grade 12) were added to an equal weight of bacterial suspension in extraction buffer B (see Table 5. 1) as described by Lund (1965) and the suspension was sonicated for 2 minutes as above. The resulting extract was then examined microscopically for breakage.

5. 2. 1. 5 Use of X- press for the preparation of lysates

Bacterial pellet was resuspended in 2 ml of buffer B (see Table 5. 1) per gram of wet cells and the suspension was then frozen at –20 °C overnight. The frozen bacterial suspension was placed in an X- press (AB Biox. Box 235, Nacka 2, Sweden) previously cooled to –20 °C and 5 consecutive pressing was done at –10 °C using a hydraulic press. The extract obtained was examined microscopically for breakage.

5. 2. 1. 6 Use of French pressure-cell press for the preparation of lysates

Bacterial cells obtained from 750 ml of NDB (see 5. 2. 1. 1) were suspended by addition of 2 ml of buffer B (see Table 5. 1) containing 3 mg/ml lysozyme per gram of wet cells. At least 5 ml of bacterial suspension was placed in a French pressure cell press (Slm. Aminco, Slm Instruments, Inc.) and 3 consecutive pressings were done at 1260 psi. The extract was collected in a flask with ice-bath cooling and examined visually and microscopi-

cally after each breakage.

5. 2. 2 Determination of the breakage of cells

First, the initially turbid-cloudy bacterial suspension was examined visually for transparent appearance after treatment. A drop of bacterial lysate was then placed on a microscope slide and examined under the 40x phase-contrast objective of a Zeiss light microscope. When there was only a few intact cells in the field of view the breakage was taken to be successful.

Table 5. 1 Recipes for extraction and electrophoretic buffers (Baptist *et al.*, 1969, Richardson *et al.*, 1986 and Selander *et al.*, 1986).

Code	Buffer	Final molarity of each buffer constituent	Quantities for 1 l of buffer (each was added to 1 l distilled water)
A	Tris-citrate pH 7.0		6.3 g Tris 9.0 g citric acid (diluted 15:1)
B	Tris-EDTA-NADP pH 6.8	10 mM Tris 1mM Na_2EDTA 0.5 mM NADP [a]	1.211 g 0.37 g 0.38 g
C	0.02 M Phosphate pH 7.0	11.6mM Na_2HPO_4 8.4 mM NaH_2PO_4	4.15 g $Na_2HPO_4.12H_2O$ 1.31 g $NaH_2PO_4.2H_2O$
C*	0.02 M Phosphate pH 7.8	Same as buffer C, adjust pH by addition of 4 M NaOH.	

D	0.05 M Tris-maleate pH 7.8	50mM Tris	6.06 g
		20mM Maleic acid [b]	2.32 g
E	0.015 M Tris-EDTA-borate-$MgCl_2$ pH 7.8	15 mM Tris	1.82 g
		5 mM Na_2EDTA	1.86 g
		10 mM $MgCl_2$	0.95 g anhydrous $MgCl_2$
		5.5 mM Boric acid	0.34 g
E*	0.015 M Tris-EDTA-borate-$MgCl_2$ pH 7.0	Same as buffer E, adjust pH by addition of boric acid	
F	0.1 M Tris-EDTA-maleate $MgCl_2$ pH 7.4	100 mM Tris	12.11 g
		1 mM Na_2EDTA	0.37 g
		1 mM $MgCl_2$	0.95 g anhydrous $MgCl_2$
G	0.1 M Tris-maleate pH 7.8	100 mM Tris	12.11 g
		40 mM Maleic acid	4.64 g
H	0.01 M Citrate-phosphate pH 6.4	10 mM Na_2HPO_4	3.58 g $Na_2HPO_4.12H_2O$
		2.5 mM Citric acid	0.53 g
I	0.05 M Tris-maleate pH 7.8	50 mM Tris	6.06 g
		20 mM Maleic acid	2.32 g
J	0.015 M Tris-maleate pH 7.2	15 mM Tris	1.82 g
		7 mM Maleic acid	0.81 g
K	0.1 M Tris-citrate pH 8.0	100 mM Tris	12.1 g
		10 mM Citric acid	3.99 g

[a] Sigma No. N 0505 [b] Aldrich M 15-3

5. 2. 3 Protein estimation of the samples

The method of Smith *et al.* (1985) was employed to determine the protein amount of 4 samples of *E. salicis,* NCPPB 2535, NCPPB 2533, S1A and 35B, in buffer B (see Table 5. 1) containing 3 mg/ml lysozyme prepared by sonication. Bovine Serum albumin (BSA) was used as standard.

Standard bicinchoninic acid (BCA) reagent was made from two reagents, reagent A and B. Reagent A consisted of an aqueous solution of 1 % BCA-Na_2 (Sigma No. D 8409), 2 % Na_2CO_3 . H_2O, 0.16 % Na_2 tartrate (Sigma No.T 6521), 0.4 % NaOH, and 0.95 % $NaHCO_3$. If needed, NaOH (50%) or solid $NaHCO_3$ was used to adjust the pH to 11.25. Reagent B consisted of 4 % $CuSO_4$. $5H_2O$ in distilled water. Standard working reagent (S-WR) was prepared by mixing 100 volume of reagent A with 2 volume of reagent B just before using.

A stock solution of BSA (Sigma No. A-7906) at a concentration of 1000 mg/ml was prepared in distilled water. A set of standard concentrations of BSA in the range of 50-800 µg/ml was prepared diluting the stock solution in extraction buffer B (see Table 5. 1). A standard curve plotting absorbance versus concentration of BSA was then made using the standard assay procedure.

The standard assay procedure consisted of mixing 1 volume sample (standard BSA or unknown) with 20 volume of S-WR (e.g. 150 µl of sample and 3 ml S-WR) in a test tube. After incubation at 37 °C for 30 minutes colour development in the tube was measured at A_{562} . The concentration of protein in *E. salicis* extracts was determined from the plot of concentration versus absorbances obtained for the standard BSA solutions.

5. 2. 4 Storage of samples

After confirming the breakage, the lysate was centrifuged at 16 000 rpm for 30 minutes at 4 °C to remove the cell debris. The supernatant was carefully removed by a pipette and divided into 1 ml portions in eppendorf tubes for long term storage at –80 °C. The supernatant was also stored as a number of individual aliquots of about 5-15 µl, each aliquot in a separate glass capillary tube (Cat. No. 01604, Hawksley and Sons Limited, Marlborough Road, Lancing, West Sussex BN15 8TN) sealed with plasticine, at –20 °C for immediate use.

5. 2. 5 Enzyme reactions tried

Methods for fifteen different enzymes were tested in the development of the technique for *E. salicis*. Table 5. 2 shows the enzymes tested referred to by their E.C. (Enzyme Commission) numbers and abbreviations.

Table 5. 2 Enzymes tested for *E. salicis*.

Alkaline phosphatase (ALP) E.C. 3.1.3.1
Esterases (EST) E.C. 3.1.1.1
Glucose 6-phosphate dehydrogenase (G6PD) E.C. 1.1.1.49
Malate dehydrogenase (MDH) E.C. 1.1.1.37
Malic enzyme (ME) E.C. 1.1.1.40
Indophenol oxidase (superoxide dismutase) (IPO) E.C. 1.15.1.1
Glucose dehydrogenase (hexose 6-phosphate dehydrogenase) (GLDH) E.C. 1.1.1.47
Glutamate dehydrogenase (GDH) E.C. 1.4.1.3
Alcohol dehydrogenase (ADH) E.C. 1.1.1.1
6-Phosphogluconate dehydrogenase (6PGD) E.C. 1.1.1.44
Lactate dehydrogenase (LDH) E.C. 1.1.1.27
Glycerol 3-phosphate dehydrogenase (a GPD) E.C. 1.1.1.8
Isocitrate dehydrogenase (IDH) E.C. 1.1.1.42
Alanine dehydrogenase (ALD) E.C. 1.4.1.1
Leucine aminopeptidase (LAP) E.C. 3.4.1.1

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5. 2. 6 Separation of enzymes

Apparatus for cellulose acetate gel electrophoresis including electrophoresis chamber (Cat. No. 1283), sample applicator (Cat. No. 4090), sample well plate (Cat. No. 4096), aligning base (Cat. No. 4094), paper wicks (Cat. No. 5081), cleaning solution (Cat. No. 5090) and cellulose acetate gels (Titan III, Cat. No. 3024, 96x74 mm) which permitted 12 samples to be processed simultaneously, were obtained commercially (Helena Laboratories,

Beamunt, Texas 77704- 0752).

Four *E. salicis* isolates NCPPB 2535, NCPPB 2533, S1A and 35B were used for development of methods and conditions to separate the 15 enzymes. Electrophoresis was performed at 4 °C in a cold room.

Before loading the samples, the cellulose acetate gel was equilibrated with appropriate electrophoresis buffer which depended on the enzyme (see Table 5. 3). The cellulose acetate gel, plastic coated non-porous side up, was transferred to a 'soaking-tank' containing approximately 200 ml of the appropriate buffer. The gel required at least 15 minutes of full immersion to equilibrate with the buffer and was used within 30 minutes. After equilibration, the gel was taken from the soaking-tank, blotted between two pieces of clean blotting paper and immediately positioned on the aligning base.

Frozen bacterial lysates stored in capillary tubes at –20 °C were handled in the following way. Some adhesive tape was attached, sticky side up, to one surface of a plastic ruler. The tape was taut and adhesive enough to hold the capillary tubes firmly in place. The isolate numbers of samples to be loaded were written directly onto the tape, and the samples were taken from the freezer just before loading, wiped to remove excess moisture and stuck down in the appropriate place. Samples were transferred to wells of the sample well plate from the capillary tubes. An applicator, matching the sample well system, was applied to samples and first blotted on a clean blotting paper. Loading onto the cellulose acetate gel, porous side up, was repeated at 3 times and care was taken not to push the applicator too hard. Origin (point of the application) of the samples was cathodal on the cellulose acetate gel except for alkaline phosphatase (middle). After loading, the cellulose acetate gel, porous side down, was positioned on the wicks. One or two glass microscope slides were put onto the gel to connect the gel with the wick. After each use the applicator was immediately cleaned using 1 % cleaning solution (Helena Laboratories).

The optimal electrophoretic conditions were determined for each of 15 enzymes by testing various buffer systems and other variables e.g. voltage and duration (see Table 5. 3). During electrophoresis a constant voltage was maintained and following electrophoresis the gel was incubated in various enzyme staining solutions.

Table 5. 3 Buffer systems and electrophoretic conditions tested for each enzyme.

Enzyme	Buffer (see Table 5. 1)	Voltage (V)	Time (min.)
ALP	E, E* , I	200, 250, 300	30, 45, 60
			90, 120
EST	E, C, G, H, I,	200, 250	20, 30, 40,
G6PD	C, E	200, 250	20, 30, 45
MDH	E, I, H	200	30, 45
ME	C, C*, E, G, I, J	60, 100, 200	20, 30, 40
IPO	C, H, I	200	30
GLDH	C, E	200	20, 30
GDH	E, F, I	150, 200	30
ADH	C, E, I	200	20, 30
6PGD	C, E	100, 200	20, 30
LDH	C, H, I	200	30
a GPD	C, I	200	20, 30
IDH	H, I	100, 200	20, 25, 30
ALD	I, K	200	20, 30
LAP	I, K	200	25, 30

5. 2. 7 Staining of the gels

At least five minutes before the completion of a run, staining preparations began. Staining buffers (see Table 5. 4) were prepared previously and stored at 4 °C without preservative. Dry components kept under the stated storage regime were weighed out before the power was turned off. Most substrates were added as dry components but a few, malate and lactate, were added as solutions (Table 5. 5 gives the recipes for those substrates) the pH of which was adjusted by the dropwise addition of 1 M sodium hydroxide. Commonly used stain ingredients e.g. NAD (nicotinamide adenine dinucleotide), NADP (nicotinamide adenine dinucleotide phosphate), MTT (dimethylthiazol tetrazolium), PMS (phenazine methosulfate) and $MgCl_2$ (magnesium chloride) were made up as stock solutions (see Table 5. 6)

and they were stable for long periods of time (at least one month).

The stain was made up according to the recipe for the particular enzyme chosen. The recipes suggested by Richardson *et al.* (1986) were followed for staining of all the enzymes except for alanine dehydrogenase (Shaw and Prasad, 1970) and leucine aminopeptidase (Shaw and Prasad, 1970 and Selander *et al.*, 1986). The stain solution including enzyme substrate (dry component or substrate solution), stain buffer, stock solutions and linking enzymes were thoroughly mixed. Dry components was fully dissolved in solution and approximately 4-5 ml of 0.5 % molten agar at 50°C was added to the stain solution. The mixture was shaken gently to prevent air bubbles and poured over the gel placed in a light-proof humid chamber. The staining solution was allowed to solidify on the gel for 10-15 minutes at room temperature. The gel was then incubated at 37 °C for 20 minutes to 1 hour, depending on the enzyme reaction. The gel was checked frequently for the appearance of bands of enzyme activity of the samples. Post-coupling technique was employed to stain the esterases. All ingredients except enzyme dye (Fast blue BB) were mixed and applied to the gel in the normal manner. After incubation at 37 °C for 1 hour, the remaining ingredient dissolved in 2 ml of stain buffer was mixed with the 4-5 ml of molten agar and then applied to the gel. After the reaction was completed the gel was washed gently under the running tap water for 15 minutes to remove stain artifacts and then dried out at room temperature overnight. The dried cellulose acetate gel was covered with transparent plastic film (cling film) labelled and stored at room temperature. Such gels were used directly for recording of data in the following study (see Chapter 6).

If the alkaline phosphatase was present in the sample, Fast blue BB salt chemically reacted with β-Napthyl acid phosphate forming a pinkish-red precipitate where the reaction occurred. The staining technique for dehydrogenases, G6PD, MDH, GDH, 6PGD, IDH, involved hydrogen ion transfers from substrate to NAD or NADP. PMS was then used to transfer the hydrogen ion from the reduced NAD or NADP to MTT (a tetrazolium salt). The soluble, weakly yellow MTT was thereby reduced to insoluble blue-purple formazan, which appeared as a purplish zone on the gel wherever the reaction occurred. Table 5. 7 gives the staining mechanisms of 7 enzymes chosen to develop an enzyme electrophoresis typing method for the study of strain variation of *E. salicis* in the following chapter (see Chapter 6).

Table 5. 4 Recipes for stain buffers (Richardson *et al.*, 1986 and Selander *et al.*, 1986).

Buffer	Recipe
0.1 M Tris-HCl pH 8.6	100 mM Tris; adjust pH to 8.6 with 4M HCl
0.1 M Tris-HCl pH 8.0	100 mM Tris; adjust pH to 8.0 with 4M HCl
0.1 M Tris-HCl pH 7.4	100 mM Tris; adjust pH to 7.4 with 4M HCl
0.1 M Tris-maleate pH 6.0	100 mM Tris; adjust pH to 6.0 with 1 M maleic acid
0.1 M Tris-maleate pH 6.5	100 mM Tris; adjust pH to 6.5 with 1 M maleic acid
Sodium phosphate pH 7.0	mix equal parts of 27,6 g of $NaH_2PO_4 . H_2O$ (monobasic) in 1 liter of water and 53.6 g of $Na_2HPO_4 . 7H_2O$ in 1 liter of water, then dilute the mixture 1:25 water

(All the buffers were stored at 4 °C).

Table 5. 5 Recipes for substrates which were used as solutions (Richardson *et al.*, 1986).

Substrate	Enzyme to be stained	Recipe
Lactate	Lactate dehydrogenase	50 mg/ml L-Lactic acid [1] pH 8.0
Malate	Malate dehydrogenase	50 mg/ml L-Malic acid [2] pH 8.0

(All the solutions were stored at 4 °C).

[1] Sigma No. L 1750

[2] Sigma No. M 1125

Table 5.6 Recipes of the stock solutions (Richardson *et al.*, 1986).

Reagent	Molarity	Quantity	Comments
MTT [1]	14.5 mM	6 mg/ml	MTT does not dissolve easily in water, protect from light
PMS [2]	6.5 mM	2 mg/ml	protect from light
NADP	25 mM	20 mg/ml	
NAD [3]	40 mM	25 mg/ml	
$MgCl_2$	0.2 mM	19 mg/ml	use anhydrous $MgCl_2$ watch for bacterial growth
1M $MgCl_2$	1 mM	95 mg/ml	use anhydrous $MgCl_2$ watch for bacterial growth

[1] Sigma No. M 2128 [2] Sigma No. P 9625 [3] Sigma No. N 7004

5.3 Results

Many methods were tried to disrupt the cells of *E. salicis*. After a successful treatment the solution appeared transparent and only a few intact cells could be seen in the preparation by the microscopy.

A great deal of care was taken during sonication, not to overheat the sample. After a total of 5 minutes sonication in 5 ml of buffer A (see Table 5. 1) per gram of wet cells or in 2 ml of buffer B (see Table 5.1) per gram of wet cells, no further breakage was detected. Lysates prepared by sonication were first tested for alkaline phosphatase activity. More concentrated samples in buffer B (see Table 5. 1) gave dark pink bands of alkaline phosphatase activity while diluted samples in buffer A (see Table 5. 1) gave very weak bands. Three mg/ml lysozyme in the extraction buffer B (see Table 5. 1) improved the breakage and activity bands were stronger than before. Therefore buffer B (see Table 5. 1) containing 3 mg/ml lysozyme was chosen as an extraction buffer in the preparation of further samples.

Sonication did not give consistent breakage, however. Some constitutive dehydrogenases e.g. glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase did not appear on the gel although the activity of alkaline phosphatase had already been detected as proof of breakage in preliminary studies. Therefore, in the following studies glucose-6-phosphate dehydrogenase, more labile than alkaline phosphatase, was used to confirm the breakage and enzymic activity of the samples.

No breakage could be obtained in the sample sonicated with ballotini beads for 2 minutes. Therefore, no attempt was made to detect alkaline phosphatase activity.

After 5 consecutive pressings with X-press, in which the extracts remained frozen, 50-60 % breakage was judged by microscopy. The extract was also tested for alkaline phosphatase activity but no band could be obtained, indicating that breakage was not sufficient.

French pressure cell press required at least 5 ml of concentrated bacterial suspension therefore individual cultures were grown in 750 ml of NDB. After 3 pressings at 1260 psi, complete breakage was obtained. The extract was tested not only for alkaline phosphatase activity but also for glucose-6-phosphate dehydrogenase activity and gave positive reaction for both indicating that breakage of the cells had occurred without causing the loss of activity of some dehydrogenases.

A total of fifteen enzymes were tested in four samples (lysates) of *E. salicis*, NCPPB 2535, NCPPB 2533, S1A and 35B, and activity of 11 out of 15 enzymes could be detected as pink (for alkaline phosphatase) or blue-purple bands (for dehydrogenases) on the gel.

Alkaline phosphatase: Activity of alkaline phosphatase in four samples was observed as dark pink bands very close to the application point (middle of the gel for this particular enzyme) using two electrophoresis buffers, buffer E and I (see Table 5. 1), at pH 7.8. The pH of the buffer E (0.015 M Tris-EDTA-Borate-$MgCl_2$ pH 7.8) was brought to 7.0 by addition of some more boric acid (buffer E*) and as a result the activity bands could further migrate to cathodal side of the gel after 1 hour electrophoresis. Warping and distortion in the movement of the bands were observed at 250 V and the gel was burnt at 300 V for 90 minutes electrophoresis. If the duration was longer than 60 minutes at 200 V activity bands smeared on the gel indicating imperfect resolution. The optimal electrophoretic conditions were obtained by using buffer E* (0.015 M Tris-EDTA-Borate-$MgCl_2$ pH 7.0) with 1 hour electrophoresis at 200 V at 4 °C. Table 5. 7 gives the optimal electrophoretic conditions and stain recipe of alkaline phosphatase for *E. salicis*.

Esterases: Several buffers, C, E, G, H, and I (see Table 5. 1), were tried in four

E. salicis isolates but no enzyme activity could be detected applying different combinations of several electrophoretic conditions (see Table 5. 3) or the post-coupling technique suggested by Richardson *et al.* (1986). Therefore, this enzyme was not used further in this study.

Glucose-6-phosphate dehydrogenase: Activity bands of glucose-6-phosphate dehydrogenase appeared quickly on the gel. Bands were sharper in buffer C (0.02 M Phosphate pH 7.0) than in buffer E at pH 7.8 (see Table 5. 1) and migration was anodal. If the voltage or duration was increased, 30 or 45 minutes at 250 V, the activity bands ran off the end of the gel. Twenty minutes of duration at 200 V at 4 °C gave the optimal electrophoretic conditions for glucose-6-phosphate dehydrogenase and after 20 minutes of incubation in stain ingredients sharp bands appeared making the gel easily scorable. The optimal electrophoretic conditions and stain recipe of the enzyme is shown in Table 5.7.

Malate dehydrogenase: Activity bands of malate dehydrogenase were detected in four *E. salicis* isolates tested. More than one band, 2 or three, for each isolate occurred on the gel indicating the variation between isolates and optimal separation of bands was achieved at 200 V for 30 minutes duration using buffer I (0.05 M Tris-maleate pH 7.8). Staining of the gel for malate dehydrogenase activity required 30 minutes at room temperature (see Table 5.7).

Malic enzyme: Several buffers, C, E, G, I and J (see Table 5.1), were tested to provide the optimal separation of malic enzyme on cellulose acetate gel. Although the activity of the enzyme was detected in four *E. salicis* isolates tested the problem of warping and imperfect resolution could not be avoided even though combinations of voltage and duration were tested (see Table 5. 3). Activity bands of the enzyme could be best located using buffer C* (0.02 M Phosphate pH 7.8) at 100 V with 30 minutes electrophoresis but, since the bands obtained were not consistent enough to be used as a differentiation criteria, malic enzyme was not included in the typing scheme in the following study (see Chapter 6).

Indophenol dehydrogenase: Activity bands of indophenol oxidase appeared as colourless bands on a blue-purple background on any gel stained with reaction mixtures containing MTT and PMS following incubation at 37 °C for more than 45 minutes. Optimal separation was obtained using buffer C with electrophoresis at 200 V for 30 minutes as it was shown in Table 5. 7. To increase the background colour intensity the gel was exposed to u.v. light for 1 hour. It was also observed that scoring the mobility of the bands could be best done just before the washing the gel to remove stain ingredients.

Glucose dehydrogenase: No enzyme activity could be obtained for glucose dehydrogenase using two buffers having different pH values with any electrophoretic condition

tested (see Table 5. 3). If the staining was allowed to continue for longer than 45 minutes formazan precipitation occurred on the gel as a blue-purple background as was observed for indophenol oxidase.

Glutamate dehydrogenase: The activity bands of glutamate dehydrogenase in four isolates of *E. salicis* migrated from the cathode to anode and appeared weakly for each isolate. Optimal separation of glutamate dehydrogenase and stain of activity bands were achieved using buffer F (0.1 M Tris-EDTA-maleate-$MgCl_2$ pH 7.4) with electrophoresis at 200 V for 30 minutes and applying the suggested recipe by Richardson *et al.* (1986). (Table 5. 7 gives the optimal conditions and stain recipe for the enzyme). Staining quality could not be improved by increasing the amount of dry substrate (glutamate) to 30-40 mg but, since the bands were sharp and scorable with careful examination on the light box, glutamate dehydrogenase was included in the typing scheme in the following study (see Chapter 6).

Alcohol dehydrogenase: Very faint bands presumably belonging to alcohol dehydrogenase were obtained in the gels electrophoresed at 200 V for 20 or 30 minutes using three different buffers, C, E and I (see Table 5. 1). Addition of 10 mg of NAD to the soaking buffer, as suggested by Richardson *et al.* (1986) did not enhance the enzyme activity. Longer incubation (more than 45 minutes) caused blue-purple formazan precipitation on the gel making the activity bands unscorable. Therefore, alcohol dehydrogenase was not used for *E. salicis* typing.

6-Phosphogluconate dehydrogenase: 6-Phosphogluconate dehydrogenase had the highest activity of the enzymes tested. Mobility variants of the enzyme in the four samples occurred as very dark bands. Smearing of the bands of the enzyme was removed using buffer C (0.02 M Phosphate pH 7.0) and sharper bands were obtained with electrophoresis at 200 V for 20 minutes (see Table 5. 7 gives the optimal electrophoretic conditions and stain recipe). The bands of 6-phosphogluconate dehydrogenase ran off the anodal end of the gel if the duration of the electrophoresis was longer than 30 minutes.

Lactate dehydrogenase: Faint, smeared bands were observed on the gel stained for lactate dehydrogenase activity. Different electrophoresis buffers having different pH values (see Table 5. 3) did not remove the smearing and longer incubation (more than 45 minutes) at 37 °C caused blue-purple formazan precipitation on the gel. Hence, lactate dehydrogenase could not be used for electrophoresis typing of *E. salicis* (see Chapter 6).

Glycerol-3-phosphate dehydrogenase: Activity of glycerol-3-phosphate dehydrogenase was detected in four samples of *E. salicis* as smeared, faint bands. Use of different pH values in two different buffers (see Table 5. 3) did eliminate imperfect resolution but stain-

ing intensity of the bands could not be enhanced. It was presumed that bands differed in their mobility from cathode to anode but, because of the bands were very faint and smeared, this enzyme was not used further.

Isocitrate dehydrogenase: Activity of isocitrate dehydrogenase in four samples of *E. salicis* was easy to assay but bands were smeared. Optimal resolution was obtained using buffer I (0.05 M Tris-maleate pH 7.8) with electrophoresis at 200 V for 25 minutes at 4 °C. The migration was anodal and staining reaction was completed in 20 minutes at room temperature (see Table 5. 7).

Alanine dehydrogenase: Activity of Alanine dehydrogenase could not be detected using the electrophoretic conditions (see Table 5. 3) and the stain recipe suggested by Selander *et al.* (1986).

Leucine aminopeptidase: Recipe of Selander *et al.* (1986) was applied with modification of stain ingredients. Fast blue BB salt was used instead of Black K salt and no activity bands of leucine aminopeptidase was observed in the gel electrophoresed in two different buffers, buffer I and K (see Table 5. 1) at 200 V for 25 or 30 minutes.

Storage conditions, at – 20 °C and at - 80 °C, did not affect the enzyme activity of the samples and both preparations were still active even after 1 year of storage.

Protein amounts of 4 samples tested were between 6.5–7.5 mg/ml and no difference was observed in the intensity of staining of the activity bands.

Table 5. 7 Optimal electrophoretic conditions and stain recipes of the seven enzymes with staining mechanisms. (4-5 ml of 0.5 % molten agar is mixed with the stain in gredients and quantities are given for the whole cellulose acetate gel 96 x 74 mm).

1. Alkaline phosphatase (ALP) :

A. Electrophoretic conditions

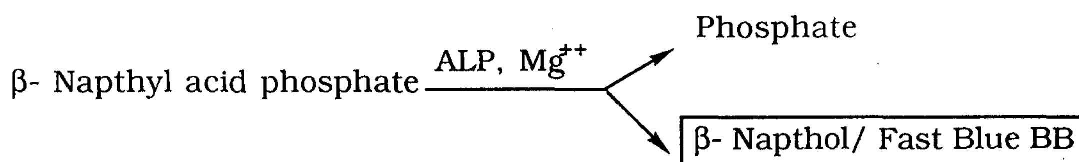
Buffer⁺ : E*

Position of origin : Middle

Duration of run : 1 hour (200 V at 4 °C)

B. Staining

Mechanism :



Dry components :	β- Napthyl acid phosphate [1]	10 mg
	Fast blue BB salt [2]	8 mg
Stain buffer * :	0.1 M Tris- HCl pH 8.6	2 ml
Stock solution** :	$MgCl_2$	1 ml
Comments :	Incubate the gel at 37 °C for 1 hour.	

2. Glucose-6-phosphate dehydrogenase (G6PD) :

A. Electrophoretic conditions

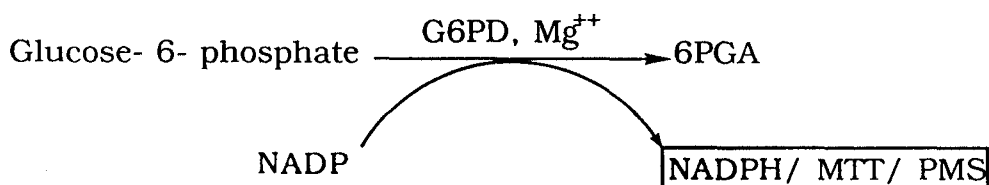
Buffer⁺ : C

Position of origin : Cathode

Duration of run : 20 minutes (200 V at 4 °C)

B. Staining

Mechanism :



Dry component :	Glucose 6- phosphate[3]	6 mg
Stain buffer* :	0.1 M Tris- HCl pH 8.0	2 ml
Stock solutions** :	NADP, 1 M $MgCl_2$, MTT, PMS	0.1 ml each

Comments : Incubate the gel at room temperature for 20 min.

3. Malate dehydrogenase (MDH) :

A. Electrophoretic conditions

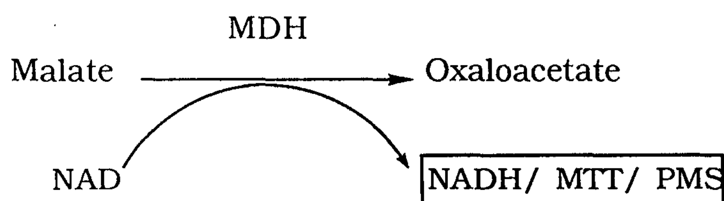
Buffer +: I

Position of origin : Cathode

Duration of run : 30 minutes (200 V at 4 °C)

B. Staining

Mechanism :



Stain buffer* :	0.1 M Tris- HCl pH 8.0	2 ml
Substrate solution*** :	Malate	0.2 ml
Stock solutions** :	NAD, MTT, PMS	0.1 ml each

Comments : Incubate the gel at 37 °C for 45 min.

4. Indophenol oxidase (IPO) :

A. Electrophoretic conditions

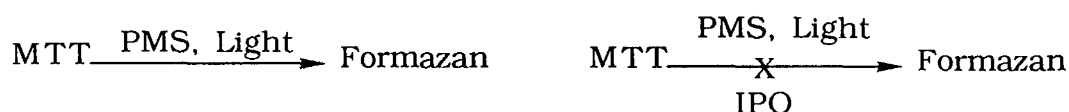
Buffer⁺ : C

Position of origin : Cathode

Duration of run : 30 minutes (200 V at 4 °C)

B. Staining

Mechanism :



Stain buffer* :	0.1 M Tris- HCl pH 8.0	2 ml
Stock solutions** :	MTT, PMS	0.1 ml each

Comments : Leave the stain on the gel for 1 hour allowing exposure to strong sunlight or u.v. light.

5. Glutamate dehydrogenase (GDH) :

A. Electrophoretic conditions

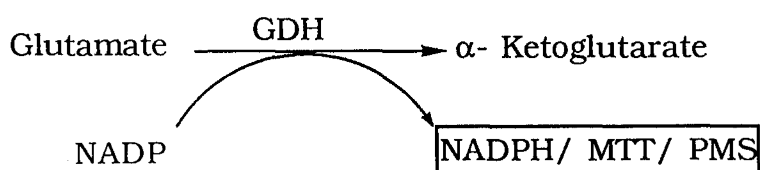
Buffer⁺ : F

Position of origin : Cathode

Duration of run : 30 minutes (200 V at 4 °C)

B. Staining

Mechanism :



Dry component :	(Mono) Sodium Glutamate [4]	20 mg
Stain buffer* :	0.1 M Tris- HCl pH 8.0	2 ml

Stock solutions** : NADP, MTT, PMS 0.1 ml each

Comment : Incubate the gel at 37 °C for 1 hour.

6. 6-Phosphogluconate dehydrogenase (6PGD) :

A. Electrophoretic conditions

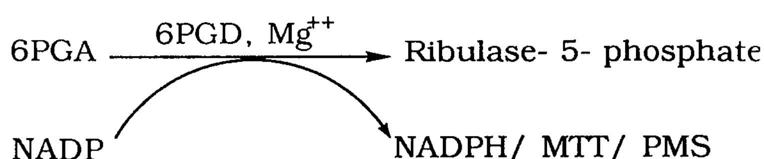
Buffer +: C

Position of origin : Cathode

Duration of run : 20 minutes (200 V at 4 °C)

B. Staining

Mechanism :



Dry component :	6-phosphogluconic acid [5]	5 mg
Stain buffer* :	0.1 M Tris- HCl pH 8.0	2 ml
Stock solutions** :	NADP, MTT, PMS, $MgCl_2$	0.1 ml each

Comment : Incubate the gel at room temperature for 30 min.

7. Isocitrate dehydrogenase (IDH) :

A. Electrophoretic conditions

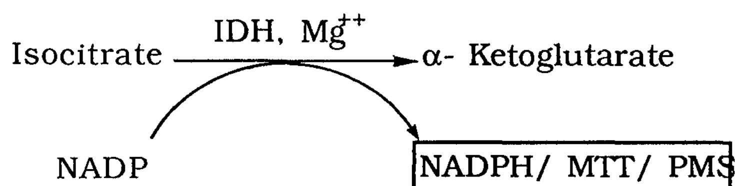
Buffer+: I

Position of origin : Cathode

Duration of run : 25 minutes (200 V at 4 °C)

B. Staining

Mechanism :



Dry component :	DL-Isocitric acid [6]	10 mg
Stain buffer* :	0.1 M Tris- HCl pH 8.0	2 ml
Stock solutions** :	NADP, $MgCl_2$, MTT, PMS	0.1 ml each

Comment : Incubate the gel at room temperature for 30 min.

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[1] Sigma No. N-7375 [2] Sigma No. F 3378 [3] Sigma No. G-7250

[4] Cambrian Chemicals Ltd. AA28

[5] Sigma No. P-7627 [6] Sigma No. I-1252

* see Table 5. 4 ** see Table 5. 6 *** see Table 5. 5

+ see Table 5. 1

5. 4 Discussion

Lysozyme hydrolyses the peptidoglycan layer of Gram-positive bacteria causing lysis and death. Addition of 3 mg/ml lysozyme to the extraction buffer B (see Table 5. 1) aided lysis of *E. salicis* as it did for *Rhizobium* spp. (Young, 1985), both of which are Gram-negative bacteria.

Several methods of lysis e.g. sonication (Selander *et al.*, 1986), glass beads (Williams and Bowden, 1968), X-press (Lund, 1965) and, freezing and thawing (Braude *et al.*, 1983) have been suggested to lyse the bacterial cells. Sonication is especially useful for cells that are difficult to disrupt. Many bacteria require a short time of sonication e.g. *E. coli, Shigella* spp. and *Salmonella* spp. require only 1 minute (Bowman *et al.*, 1967), representatives of plant pathogenic bacteria (*Agrobacterium* spp. , *Corynebacterium* spp. *Erwinia carotovora*, *Pseudomonas phaseolicola* and *Xanthomonas fragariae*) require 3 minutes (El-Sharkawy and Huisingh, 1971a), *Rhizobium melliloti* requires 45 seconds

(Eardley *et al.*, 1990) and *Mycobacterium* spp. requires 3 minutes (Wasem *et al.*, 1991).

In our study, sonication took 5 minutes to break *E. salicis* cells and there was inadequate recovery of all enzymes either because of insufficient breakage or denaturation during sonication.. The use of the French pressure cell press was more laborious than the sonicator and it required at least 5 ml of concentrated bacterial suspension. Therefore the isolate had to be grown in 750 ml of NDB which was 3 times more than that required for sonication. However, the French pressure cell press was chosen to break the *E. salicis* cells in the following study (see Chapter 6) because it gave good recovery of enzyme activity.

In general, multilocus enzyme electrophoresis does not require precise quantification during sample preparation. Only in a few studies (El-Sharkawy and Huisingh, 1971a, b and Goullet, 1980) protein determination of the samples was done. The present study showed that the intensity of the staining reaction on the gel surface was similar in all the samples tested. Therefore as long as the samples receive the identical treatment there is no need to estimate the protein amount of the samples.

A variety of support media e.g. starch (Shaw and Prasad, 1970), acrylamide and agarose (Harris and Hopkinson, 1976) and cellulose acetate (Preston *et al.*, 1965; Richardson *et al.*, 1986) have been suggested for use in enzyme electrophoresis. In the present study, I preferred to use cellulose acetate gel to develop a multilocus enzyme electrophoresis typing method for *E. salicis* considering the number of isolates to be tested (86) in the following study (see Chapter 6). Because cellulose acetate gel:

— required a very small volume of stain (2-3 ml) and sample (2-3 μl)

— preparation time for loading was short (20- 30 minutes for soaking)

— run time of electrophoresis was short (20 minutes to 1 hour)

— stain time was short (20 minutes to 1 hour)

— handling was very easy.

To be chosen for typing in the multilocus enzyme electrophoresis method, an enzyme should obviously be present in the sample at sufficient concentration to score the activity bands and the electrophoretic resolution (i.e. the sharpness of the bands) should be adequate to confidently distinguish two electromorphs.

In the present study 7 enzymes were chosen, alkaline phosphatase, glucose-6-phosphate dehydrogenase, malate dehydrogenase, indophenol dehydrogenase, glutamate dehydrogenase, 6-phosphogluconate dehydrogenase and isocitrate dehydrogenase which fulfilled these criteria. Use of more concentrated sample or different staining recipes could be employed to re-test the activity or to improve the resolution and staining intensity of re-

maining 8 enzymes in future studies.

In conclusion, the first requirement to develop a reliable multilocus enzyme electrophoresis method for distinguishing isolates of *E. salicis* has been achieved. In the following study (see Chapter 6) I employ the method and interpret the geographical distribution of these strains.

CHAPTER VI

APPLICATION OF MULTILOCUS ENZYME ELECTROPHORESIS TYPING FOR DIFFERENTIATION OF *E. SALICIS* ISOLATES

6. 1 Introduction

Many methods including serotyping, biotyping and bacteriophage typing are used to detect phenotypic variation between isolates in bacterial systematics and epidemiology. Multilocus enzyme electrophoresis detects the variation in an enzyme locus among isolates of a species, providing information on genetic structure. Since most enzymes examined are constitutive, all isolates of a species may be typed by the method. Besides providing a framework for determining the overall genetic relationships this method also provides a more discriminating technique to study epidemiology of disease. Multilocus enzyme electrophoresis method has been widely used to assess the genetic structures of natural populations of several bacterial species and recently has been applied to reveal the epidemiology of several species of clinical bacteria. Examples of applications of multilocus enzyme electrophoresis typing for both aims are given in Chapters 1. 10. 6 and 5. 1.

In the study presented in this thesis, conventional methods to distinguish between isolates of *E. salicis* have included biotyping, bacteriophage typing and serotyping. Biotyping of *E. salicis* was based on galactose utilisation and two biotypes of the bacterium were identified distinguishing English and Dutch isolates. Bacteriophage typing was more discrimiating in distinguishing between isolates and 5 lysotypes were defined, one of which included only the Dutch isolates of the bacterium, confirming the biotyping. However, the method did not differentiate between Dutch isolates of *E. salicis*. Serotyping of the bacterium using polyclonal antiserum was of limited value for tracing the distribution of *E. salicis* isolates since only two groups were separated based on the presence or absence of a heat-stable 'O' antigen. Therefore, for the proposed epidemiological study of *E. salicis*, a more discriminating method was required to distinguish between clones of *E. salicis*.

In the present study, multilocus enzyme electrophoresis typing was applied to a total of 86 isolates of *E. salicis* and mobility variants of 7 enzymes, alkaline phosphatase, indophenol oxidase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, malate dehydrogenase, glutamate dehydrogenase and isocitrate dehydrogenase, detect-

ed by electrophoresis were used as genetic markers to distinguish the strains of the bacterium.

In a further study, 8 cultures of *E. salicis* isolated from 3 adjacent trees in Wiltshire in 1991 were analysed to study the distribution of ETs in single trees and in adjacent trees.

6. 2 Materials and methods

6. 2. 1 Bacterial cultures

A total of 86 isolates were typed with the multilocus enzyme electrophoresis method. Characteristics of 78 isolates of *E. salicis* including 17 authentic isolates obtained from NCPPB, 15 isolates obtained from the Netherlands and 46 cultures isolated from diseased trees in England between 1989 and 1990 are given in detail in Table 2. 2 (see Chapter 2).

In a further study, 8 isolates of *E. salicis* obtained in 1991 from 3 neighbouring trees in Wiltshire, in a severely diseased plantation, were tested. Three cultures, W2-2A, W2-4A and W2-4B, were isolated from separate branches of tree no. 2, four cultures, W3-1A, W3-1B, W3-2B and W3-7A, were isolated from separate branches of tree no. 3 and only one culture, W4-5B, could be isolated from tree no. 4.

6. 2. 2 Growth of bacteria and electrophoresis of enzymes

The techniques of multilocus enzyme electrophoresis of *E. salicis* have been given in previous chapter (see Chapter 5).

Twelve lysates could be processed simultaneously on a single gel and the last well contained the control lysate (NCPPB 2529). Lysates were electrophoresed on cellulose acetate gels and stained for 7 metabolic enzymes by the methods described (see Chapter 5). The enzymes studied were alkaline phosphatase, indophenol oxidase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, malate dehydrogenase, glutamate dehydrogenase and isocitrate dehydrogenase. The buffer systems for electrophoresis and staining recipes are given in Table 5. 7 (see Chapter 5).

If the isolate did not show activity for any of 7 enzymes tested, the isolate was regrown, re-broken and re-electrophoresed 2 more times. If, however there was only a slight visible difference between the mobility of bands, the order of extracts on the gels was al-

tered to enable better comparison of mobility. If variation was detected between the enzyme activities of isolates indicating mobility difference, the concentrated sample was diluted to 1/2 and 1/5 and the electrophoresis was repeated.

6. 2. 3 Scoring the gels

Comparisons of the mobilities of enzymes from different isolates were made visually against one another on a light box. For each enzyme, distinctive mobility variants (electromorphs) were numbered in order of decreasing rate of anodal migration and were equated with alleles at the corresponding structural gene locus. The absence of enzyme activity after re-testing 2 more times was scored as a null character state and allele.

6. 2. 4 Statistical analysis

Two computer programmes to analyse genetic diversity and relationships among bacterial strains characterised by multilocus enzyme electrophoresis were obtained from Thomas S. Whittam, Department of Biology, Pennsylvania State University, University Park, PA 16802 (814)–863–1970.

The first programme, ETDIV, found and listed the electrophoretic types in a collection of 78 *E. salicis* isolates with multilocus enzyme profiles. The second programme, ETCLUS, used the output file created by the first programme and found a dendogram based on the average linkage algorithm. Genetic distance was measured as the proportion of mismatched loci between pairs of electrotypes. Null alleles that were scored as '0' were not used in the calculation of pairwise distances.

6. 3 Results

6. 3. 1 Enzyme activities and loci

Five out of seven of the enzymes tested exhibited a single electrophetic band for 86 *E. salicis* isolates except two enzymes. All the isolates seemed to have two electrophoretic bands with glutamate dehydrogenase but only the darkest band was considered in the interpretation since the second band was too faint to score. Malate dehydrogenase exhibited upto three bands depending on the isolate tested.

Two electromorphs were identified for alkaline phosphatase. All isolates of *E. salicis* had the same electromorph, except the two isolates 90-97 and 90-98. Two isolates, NCPPB 2530 and NCPPB 2531, were scored as null since they did not have alkaline phosphatase activity. Plate 6. 1 shows a typical gel pattern of alkaline phosphatase.

Three electromorphs were identified for indophenol oxidase. One of the electromorphs was exhibited by two isolates, NCPPB 2530 and NCPPB 2531, another by the three isolates, S7B, 90-97 and 90-98, and the third electromorph was observed in all other isolates. Very faint gel pattern of indophenol oxidase can be seen in Plate 6. 4.

The majority of the isolates of *E. salicis* had the same electromorph for glucose-6-phosphate dehydrogenase. Nine isolates, NCPPB 2530, NCPPB 2531, 103, S7B, S15A, 41A1, 90-21, 90-97 and 90-98, shared 4 different electromorphs. Plate 6. 2 shows a gel pattern of glucose-6-phosphate dehydrogenase.

More variation was observed in the mobility of bands of 6-phosphogluconate dehydrogenase and 7 electrophoretic variants (electromorphs) were identified. Plate 6. 3 shows one of gel pattern of 6-phosphogluconate dehydrogenase.

Glutamate dehydrogenase resolved as two bands of varying intensity after electrophoresis. The faint band was ignored and the majority of the isolates of *E. salicis* had the same electromorph. Five isolates, NCPPB 2530, NCPPB 2531, S7B, 90-97 and 90-98, were assigned to be a different electromorph. A typical gel pattern of glutamate dehydrogenase together with indophenol oxidase is shown in Plate 6. 4.

The most variation was observed with malate dehydrogenase. Differences in the number and mobility of the bands were equated with alleles at the corresponding structural gene locus. Plate 6. 5 shows a typical gel pattern of malate dehydrogenase.

Five electromorphs were identified for isocitrate dehydrogenase with the *E. salicis* isolates tested and the majority of the isolates had the same electromorph. Two isolates, NCPPB 2530 and NCPPB 2531, were null and 12 isolates shared four electromorphs. Plate 6. 6 shows a typical gel pattern of isocitrate dehydrogenase.

In the collection of 86 isolates as a whole, the 7 enzyme loci assayed were polymorphic for alleles encoding electrophoretically detectable variants. A total of 23 electrotypes (ETs) were identified most of which differed from one another at only one or a few loci. Table 6. 1 shows the 23 electrotypes of *E. salicis,* including the reference isolate.

Of the 23 electrotypes, 12 were represented with multiple isolates of *E. salicis.* Table 6. 2 shows the electrotypes with multiple isolates. Eleven electrotypes, ETs 4, 5, 6, 9, 13, 14, 15, 17, 18, 19 and 20, were represented by only one isolate of *E. salicis* (see Table

6. 1).

Fourteen out of 23 electrotypes, ETs 4, 5, 6, 7, 14, 15, 16, 17, 18, 19, 20, 21, 22 and 23, included only the English isolates of *E. salicis* while 3 electrotypes, ETs 9, 12 and 13, were represented by 5 Dutch isolates of the bacterium. Six electrotypes, ETs 1, 2, 3, 8, 10 and 11, were represented by isolates from both England and from the Netherlands and contained 50 out of 78 isolates tested. No evidence of a general genetic difference between isolates from England and from the Netherlands was evident.

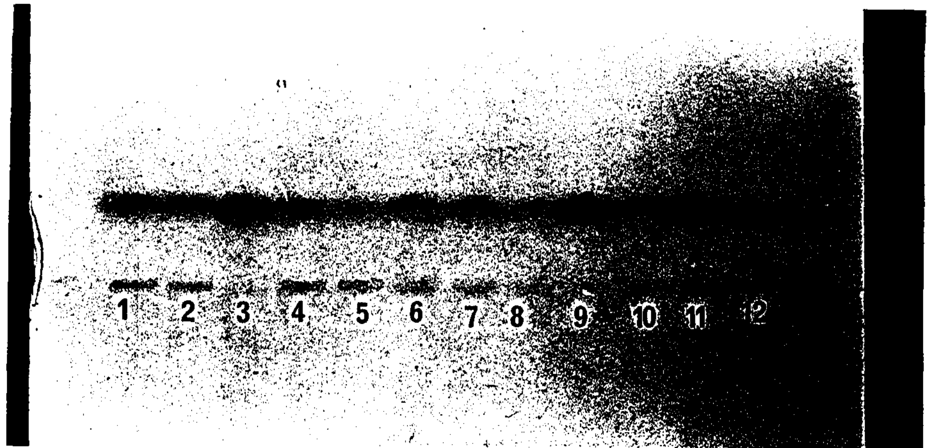


Plate 6. 1 A typical electrophoretic banding pattern of alkaline phosphatase enzyme on cellulose acetate gel. All the isolates had the same pattern as the control isolate (well no. 12). Isolates in the wells; no. 1: 30AD, no. 2: 30C, no. 3: 35B, no. 4: 41A1, no. 5: 41B2, no. 6: 90-1, no. 7: 90-8, no. 8: 90-18, no. 9: 90-21, no. 10: 90-23, no. 11: 90-30 and no. 12: NCPPB 2529.
A: anode, C: cathode, O: application point.

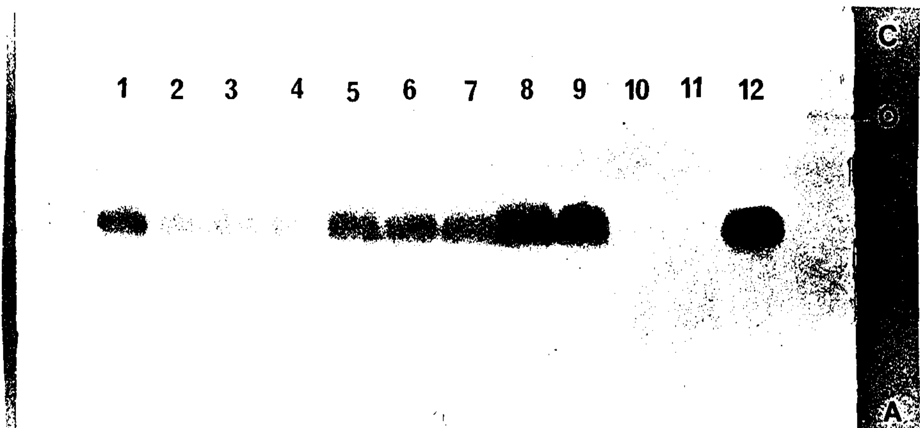


Plate 6. 2 A typical electrophoretic banding pattern of glucose-6-phosphate dehydroge nase enzyme on cellulose acetate gel. Except wells no. 10 and no. 11 all the isolates had the same pattern as control isolate on the gel. Isolates in the wells; no. 1: 90-36, no. 2: 90-38, no. 3: 90-39, no. 4: 90-40, no. 5: 90-43, no. 6: 90-44, no. 7; 90-45, no. 8: 90-52, no. 9: 90-65, no. 10: 90-97, no. 11:90-98, no. 12: NCPPB 2529.
A: anode, C: cathode, O: application point.

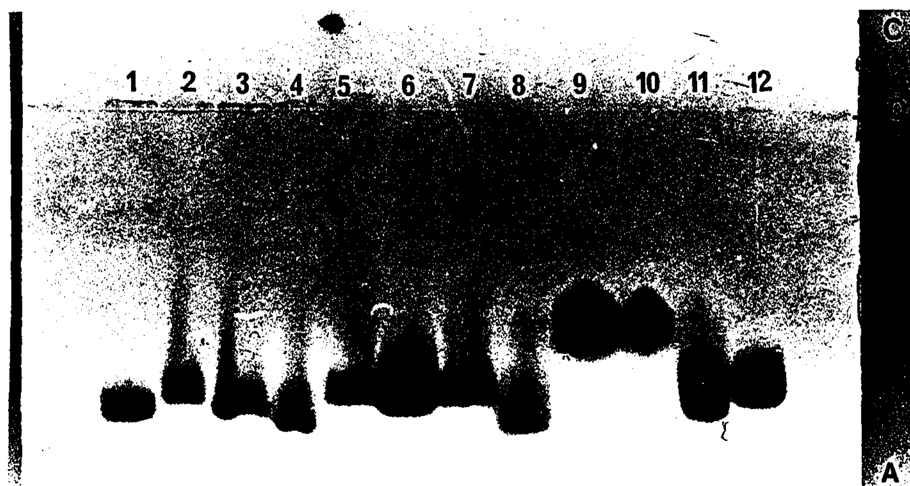


Plate 6. 3 The banding pattern of 6-phosphogluconate dehydrogenase enzyme on cellu lose acetate gel. Four electrophoretically distinguishable allozymes are evident on this gel. Wells no. 4 and no. 8 had the same pattern while no. 1, no. 3, no. 6, and no. 11 had another pattern. The third pattern belonged to no. 2, no. 5, no. 7, and no 12 (control isolate). No. 9 and no. 10 were in a different pattern. Isolates in the wells; no. 1: 447, no. 2: 1466, no. 3: 2310, no. 4: 2317, no. 5: 2522, no. 6: 2523, no. 7: 2525, no. 8: 2526, no. 9: 2530, no. 10: 2531, no. 11: 2532, no. 12: NCPPB 2529.
A: anode, C: cathode, O: application point.

Plate 6. 4 A typical electrophoretic banding pattern of glutamate dehydrogenase enzyme on cellulose acetate gel. All the isolates had same pattern as the control isolate (well no. 12). A very faint banding pattern of indophenol oxidase is observed above the glutamate dehydrogenase bands. Isolates in the wells;
no. 1: W1B, no. 2: W2A, no. 3: W3A, no. 4: W4A, no. 5: W6A, no. 6: 27B, no. 7: 28BD, no. 8: 28C, no. 9: 29AD, no. 10: 30AD, no. 11: 30C, no. 12: NCPPB 2529.
A: anode, C: cathode, O: application point.

Plate 6. 5 A typical electrophoretic banding pattern of malate dehydrogenase enzyme on cellulose acetate gel. Three electrophoretically distinguishable allozymes are ev ident on this gel and all the isolates had the same pattern except 3 isolates which were in wells no. 10, no. 11 and no. 12 (control isolate). Isolates in the wells; no. 1: 90-36, no. 2: 90-38, no. 3: 90-39, no. 4: 90-40, no.5: 90-43, no. 6: 90-44, no. 7: 90-45, no. 8: 90-52, no. 9: 90-65, no. 10:90-97, no. 11:90-98, no. 12: NCPPB 2529.

A: anode, C: cathode, O: application point

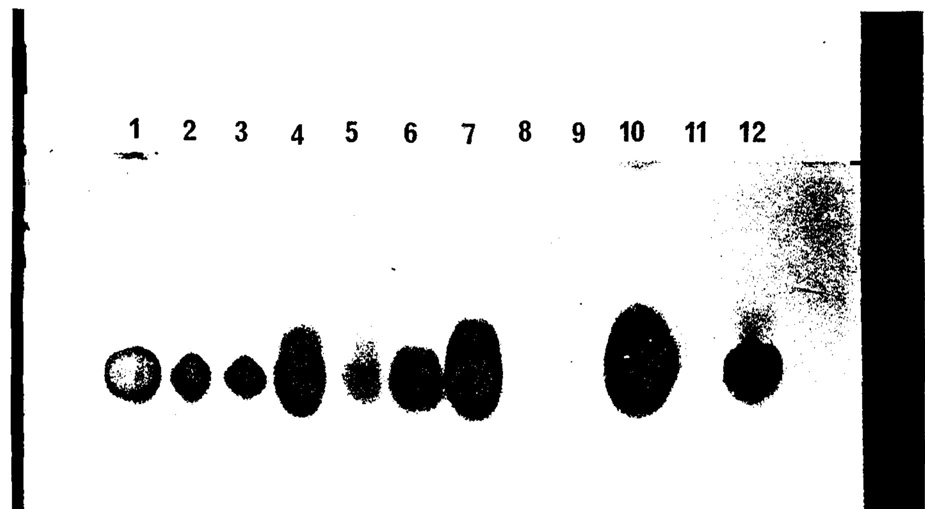


Plate 6. 6 A typical electrophoretic banding pattern of isocitrate dehydrogenase enzyme on cellulose acetate gel. All the isolates had the same pattern as the control iso late (well no. 12). Isolates in the wells; no. 1: 2533, no. 2: 2534, no. 3: 2535, no. 4: 2907, no.5: 2908, no. 6: 73, no. 7: 94, no. 8: 2530, no. 9: 2531, no. 10: 118, no. 11: Blank, no. 12: NCPPB 2529.

A: anode, C: cathode, O: application point

Table 6. 1 Electromorph profiles of 23 ETs of *E. salicis*.

ET	No. of isolates	Reference isolate	Electromorphs at the following enzyme loci [a]						
			ALP	IPO	G6PD	6PGD	GDH	MDH	IDH
1	11	447	2	1	4	2	1	2	3
2	13	1466	2	1	4	3	1	2	3
3	11	2317	2	1	4	1	1	2	3
4	1	2523	2	1	4	2	1	3	1
5	1	2526	2	1	4	1	1	5	3
6	1	2529	2	1	4	3	1	6	3
7	2	2530	0	2	2	5	2	1	0
8	5	2532	2	1	4	2	1	6	3
9	1	103	2	1	5	2	1	7	3
10	4	104	2	1	4	1	1	7	3
11	6	132	2	1	4	2	1	7	3
12	3	142	2	1	4	3	1	7	3
13	1	150	2	1	4	4	1	2	3
14	1	S7B	2	3	3	6	2	4	4
15	1	S8A	2	1	4	4	1	7	3
16	2	S12A	2	1	4	4	1	8	3
17	1	S14B	2	1	4	3	1	8	3
18	1	S15A	2	1	5	3	1	2	3
19	1	41A1	2	1	5	2	1	6	3
20	1	90-21	2	1	5	3	1	7	3
21	3	90-38	2	1	4	1	1	2	3
22	5	90-43	2	1	4	3	1	2	5
23	2	90-97	1	3	1	7	2	1	2

[a] ALP: alkaline phosphatase, IPO: indophenol oxidase, G6PD: glucose-6-phosphate dehydrogenase, 6PGD: 6-phosphogluconate dehydrogenase, GDH: glutamate dehydrogenase, MDH: malate dehydrogenase and IDH: isocitrate dehydrogenase.

Table 6. 2 Electrotypes with multiple isolates

ET	No. of isolates	Isolates
1	11	447, 2310, 2908, 94, S9B, S13B, W3A, 27B, 30AD, 30C, 41B2
2	13	1466, 2522, 2525, 143, 144, 147, 173, S10B, S11A, W1B, W4A, 90-1, 90-36
3	11	2317, 2907, 73, 174, S1A, S5A, W2A, W6A, 28C, 29AD, 90-113
7	2	2530, 2531
8	5	2532, 2533, 2534, 2535, 118
10	4	104, S4A, S14A, 90-31
11	6	132, 28BD, 90-8, 90-18, 90-23, 90-30
12	3	142, 149, 156
16	2	S12A, 35B
21	3	90-38, 90-39, 90-40
22	5	90-43, 90-44, 90-45, 90-52, 90-65
23	2	90-97, 90-98

6. 3. 2 Geographical distribution of electrotypes in England

Forty six isolates of *E. salicis,* numbered from 33 to 78 in Table 2. 2 (see Chapter 2) collected from different geographical locations in England, represented 15 out of the 23 electrotypes obtained by the study. Each of the 15 electrotypes was coded by a colour or a letter (see Table 6. 4) and the locations of these electrotypes was plotted. Figure 6. 1 shows the geographical distribution of these 46 English isolates of *E. salicis.*

Identical electrotypes were recovered from diseased trees at widely separated geographical locations indicating widespread distribution of clones of *E. salicis.* Electrotypes

1, 2, 3, and 11 were especially prevalent among the electrotypes and no clustering of electrotypes was observed in any location.

In Wiltshire, in a severely diseased plantation, 3 different electrotypes, ETs 1, 2, and 3, were identified from 5 isolates of *E. salicis*, W1B, W2A, W3A, W4A and W6A, obtained from 5 adjacent trees (see Figure 6. 1).

In Beccles, a single unique electrotype, ET 21, was identified representing 3 isolates of *E. salicis*, 90-38, 90-39 and 90-40, obtained from a diseased *Salix alba* tree (tree no. 14 shown in Figure 7. 3. 1 in Chapter 7). Again in Beccles 3 individual cultures, 90-43, 90-44, 90-45, isolated from the same tree (tree no. 13 shown in Figure 7. 3. 1 in Chapter 7) were found to belong to ET 22 confirming that in these instances one single tree carried only one type of electrotype.

In addition to above findings, two isolates, 30AD and 30C, collected from the same *S. alba* var. *caerulea* tree in Bungay were in a single electrotype, ET 1.

Not only single electrotypes but also, different electrotypes were recovered from the single trees in some locations. *E. salicis* isolates S14A and S14B collected from a single tree located in Long Melford were represented by two different electrotypes, ETs 10 and 17. Two isolates, 28BD and 28C collected from a single tree located in Bungay were in two different electrotypes, ETs 11 and 3; *E. salicis* isolates 41A1 and 41B2 collected from the same tree in Epping also gave different electrotypes which were ETs 19 and 1. Not only some isolates of *E. salicis* collected during the course of this study but also, some NCPPB isolates of the bacterium gave different electrotypes although it was recorded that they were isolated from single trees. *E. salicis* NCPPB 2522 and 2523 were represented by two different elecrotypes, ETs 2 and 4 respectively. *E. salicis* NCPPB 2529 and 2530 gave two different electrotypes, ETs 6 and 7, together with isolates NCPPB 2531 and 2532, ETs 7 and 8.

6. 3. 3 Electrotype analysis of isolates from Wiltshire

E. salicis isolates W1B, W2A, W3A, W4A and W6A were collected from five different *S. alba* var. *caerulea* trees adjacent to each other in Wiltshire in 1989 and these isolates were represented in 3 electrotypes differing only at one enzyme locus, 6-phosphogluconate dehydrogenase, indicating that neighbouring trees carried different electrotypes of *E. salicis*.

In a further study, in 1991, multiple isolations were made from the same location and 8 isolates could be recovered from three of five neighbouring trees. These isolates to-

gether with 1989 isolates of these 3 trees, W2A, W3A and W4A, were subjected to enzyme electrophoresis typing with 7 enzymes.

Three cultures, W2-2A, W2-4A and W2-4B, isolated from tree no. 2 gave the electrophoretic profile of electrotype 1 (see Table 6. 1) together with 1989 isolate W2A. Four cultures, W3-1A, W3-1B, W3-2B and W3-7A, isolated from the neighbouring tree no. 3 were identified as electrotype 3 (see Table 6. 1) together with 1989 isolate of W3A. Only one isolate, W4-5B, could be recovered from tree no. 4 and this isolate was found to be electrotype 2 (see Table 6. 1), the same as that of W4A, obtained from the same tree in 1989.

The above results supported the view that some trees carry only one type of electrotype, as was found in Beccles and in Bungay. These results indicate that in Wiltshire, the disease has not spread from tree to tree since different electrotypes were found in the adjacent trees.

Electrotypes of isolates W2A and W3A was found to have altered during storage. The electrotype for these two bacteria was altered in a single band when the cultures were recovered from storage for two years at -80 °C in glycerol. The original enzyme extract which had also been stored, was unchanged over this period. This appears to have been an isolated incident however and other cultures e.g. W4A, control isolate NCPPB 2529 and S7B gave the same electrophoretic profiles, ETs 2, 6 and 14 respectively shown in Table 6. 1, on recovery after similar storage at different times indicating the reproducibility of the multilocus enzyme electrophoresis analysis .

Table 6. 4 Fifteen electrotypes belonged to 46 English isolates of *E. salicis*.

ET *	No. of isolates	Isolate	Code
1	7	S9B, S13B, W3A, 27B, 30AD, 30C, 41B2	●
2	6	S10B, S11A, W1B, W4A, 90-1, 90-36	●
3	7	S1A, S5A, W2A, W6A, 28C, 29AD, 90-113	●
10	3	S4A, S14A, 90-31,	●
11	5	28BD, 90-8, 90-18, 90-23, 90-30	◉
14	1	S7B	ⓐ
15	1	S8A	ⓑ
16	2	S12A, 35B	O
17	1	S14B	ⓒ
18	1	S15A	ⓓ
19	1	41A1	ⓔ
20	1	90-21	ⓕ
21	3	90-38, 90-39, 90-40	O
22	5	90-43, 90-44, 90-45, 90-2, 90-65	●
23	2	90-97, 90-98	⊘

* Table 6. 1 shows the electrophoretic profiles of electrotypes.

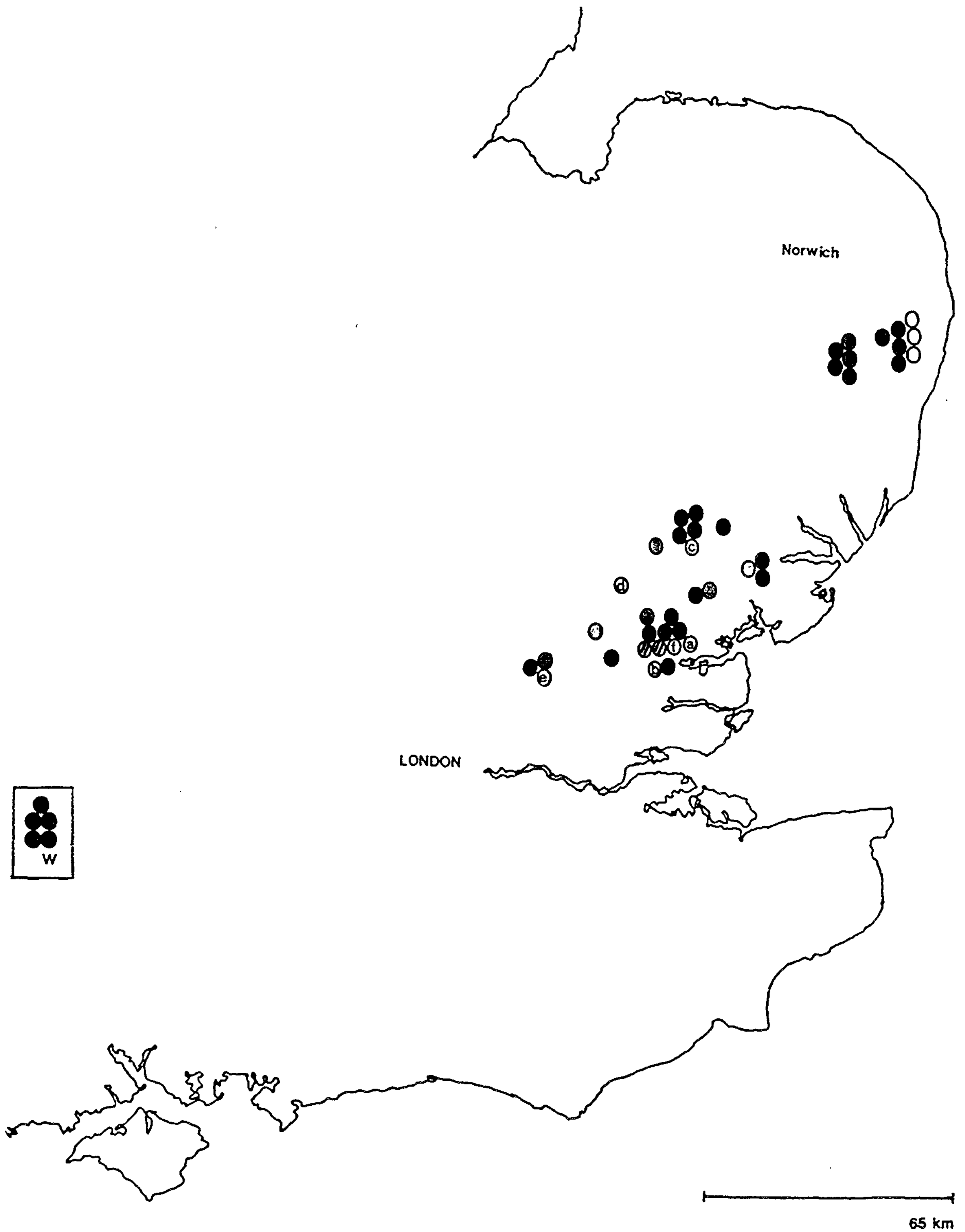


Figure 6. 1 Geographical distribution of ETs of some English isolates of *E. salicis* in East Anglia and in Wiltshire (W: Wiltshire).

6. 3. 4 Genetic relationships of electrotypes

The dendogram in Figure 6. 2 summarises estimates of the genetic relationships of the 23 electrotypes representing 78 isolates of *E. salicis*.

Because a variety of dendograms can be generated with different measures of genetic distance or methods of clustering (Selander *et al.*, 1985), the dendogram in Figure 6. 2 should be regarded as a hypothesis rather than a definitive scheme of genetic relationships.

Cluster analysis of the 23 electrotypes revealed two primary divisions (A and B in Figure 6. 2). Division A included 20 of the 23 electrotypes identified; these 20 electrotypes were represented by the 73 of the 78 isolates examined. Included in subdivision 1 of division A, were the 2 electrotypes, ET 21 and ET 22. This subdivision represented five Beccles isolates and two isolates obtained from different locations in England. Subdivision 2 of division A included 18 electrotypes which was represented by the majority of the isolates including 65 English and Dutch isolates of *E. salicis*. The most frequently observed multilocus genotypes, ETs 1, 2 and 3, belonged to this subdivision.

Division B included three electrotypes, ET 23, ET 7 and ET 14, representing 5 English isolates (see Figure 6. 2) three of which, S7B, 90-97 and 90-98, were isolated during course of the study.

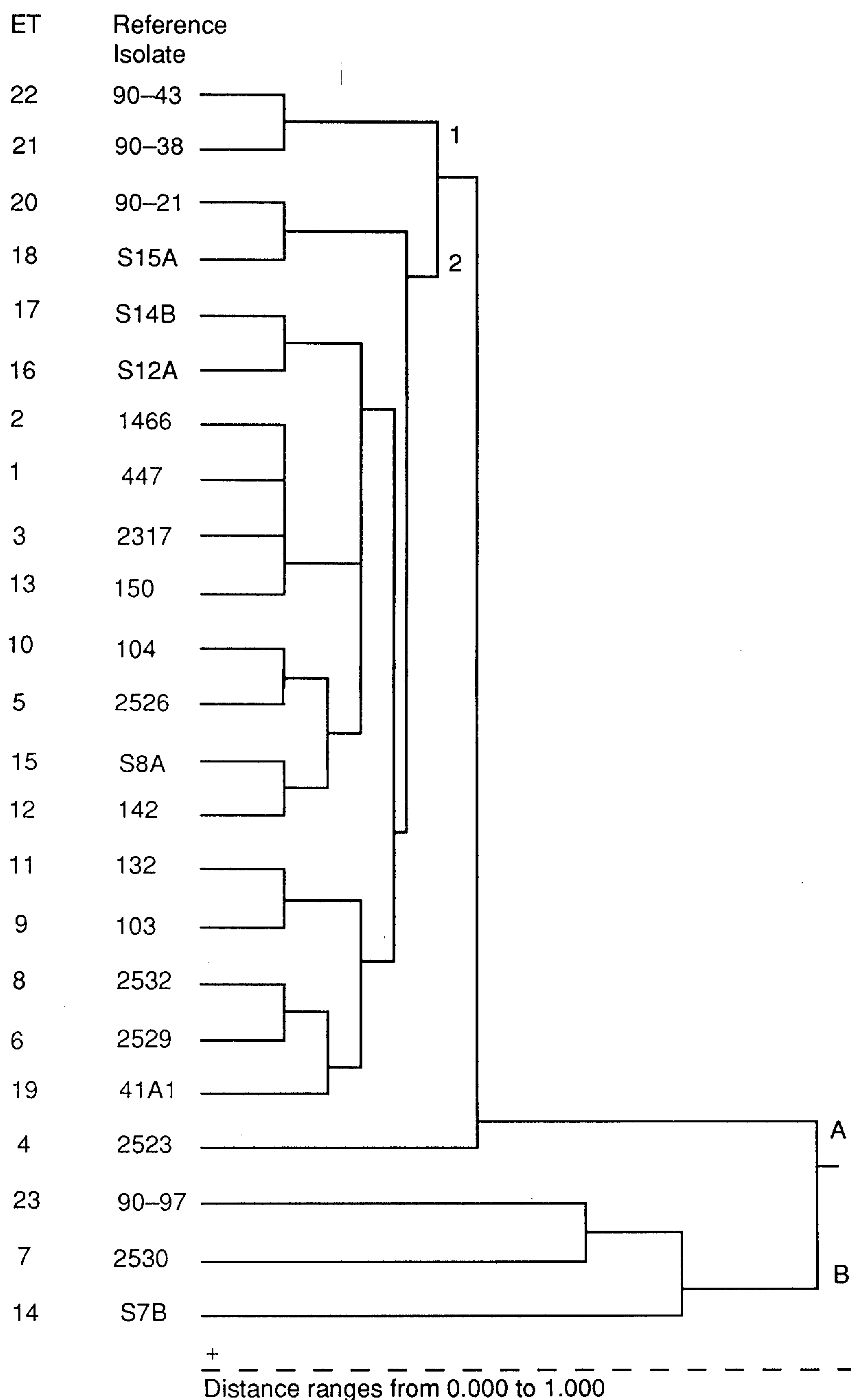


Figure 6. 2 Genetic relationships among 23 ETs of *E. salicis* estimated on the basis of electrophoretically detectable allelic variation at seven enzyme loci. A repre sentative isolate is listed for each electrotype.

6. 4 Discussion

The purpose of this work was not to study the genetic structure of *E. salicis,* but rather to identify the strains and then investigate their distribution. The study of genetic diversity and genetic structure in natural populations of some bacteria required more enzymes e.g. *Legionella pneumophila* required 22 enzymes (Edelstein *et al.*, 1986), *Yersinia ruckeri* and *Bordetella* spp. required 15 enzymes (Schill *et al.*, 1984 and Musser *et al.*, 1986) and *E. coli* required 12 enzymes (Ochman and Selander, 1984). It has been suggested that for epidemiological studies the analysis of only a few polymorphic loci may be sufficient depending on the number and relationships of the strains involved in particular epidemiological situations (Selander *et al.* , 1986). For this purpose, the analysis of only seven polymorphic loci has been sufficient in the present study.

The analysis of electrophoretic variation at seven enzymes revealed extensive allozyme variation in *E. salicis.* Distribution of electromorphs in English and Dutch isolates were quite similar for most loci, several genotypes were closely related or identical at all seven enzymes in both groups of isolates. Therefore, English and Dutch isolates of *E. salicis* were indistinguishable by multilocus enzyme electrophoresis typing in contrast to biotyping and bacteriophage typing.

A particularly attractive feature of enzyme polymorphisms is that the electrophoretic variants (allozymes) may be selectively neutral or nearly so and, therefore minimally subject to evolutionary convergence (Whittam *et al.*, 1983; Ochman and Selander, 1984 and Hartl and Dykhuizen, 1984). On this assumption, the considerable single-locus genetic diversity in *E. salicis* suggests that some electrotypes assigned to this species have persisted for periods of time sufficiently long to have spread over large geographic areas and , through mutation, to have produced clusters of closely related strains.

It has been found that serotypes of *E. coli* are unreliable indicates of clonal identity and they may be genetically dissimilar (Ochman and Selander, 1984; Caugant *et al.* , 1985). Caugant *et al.* (1985) concluded that the intensity of natural selection for cell surface antigens must be substantially greater than that for the electromorph variants of enzymes and the enzymes assayed were primarily intracellular components for which structural variations were unlikely to have significant effects on the ecological performance of *E. coli* in contrast to surface antigens of the bacterium. Although there is no comparison of bacteriophage typing results with enzyme electrophoresis typing, I assume that the lysotypes of *E. salicis* are not reliable indicators of genetic relatedness since both typing methods detect phenotypic

variation which may be under extreme selection pressure.

It is clear that multilocus enzyme electrophoresis typing and bacteriophage typing can not be used interchangeably for differentiation of *E. salicis* isolates in our study since the lysotypes (see Table 3. 3 in Chapter 3) of *E. salicis* were inconsistent with the electrotypes identified (see Table 6. 1). Lysotypes α_1, α_2, α_3 and β were randomly distributed among the division A electrotypes (see Figure 6. 2). Only one exception was that the bacteriophage-untypable isolates, NCPPB 2530, NCPPB 2531, S7B, 90-97 and 90-98, were found to be in a distinct division, division B, by the multilocus enzyme electrophoresis typing method, adding weight to the argument that these isolates should be regarded as subspecies of *E. salicis*. However, larger samples of isolates are required to reach a conclusion.

The existence of a single unique electrotype, ET 21, representing three Beccles isolates from a single tree, *S. alba*, is particularly interesting and warrants detailed investigation of the site.

A hypothesis that long distance dispersal is achieved primarily by propagating materials (sets) is consistent with results of the present study, and with the finding that *E. salicis* has been detected in symptomless 1-4 year old sets and in the symptomless stools producing these sets (Turner *et al.,* 1992). Evidence supporting the above hypothesis is that the current outbreak of watermark disease in a single plantation of *S. alba* var. *caerulea* in Wiltshire (Turner *et al.*, 1991). It is known that the trees in Wiltshire were grown from sets obtained from Essex. These sets probably carried different electrotypes of *E. salicis* and the three electrotypes occurred commonly in isolates of *E. salicis* from watermark diseased willows growing in Essex. Although my results indicate that watermark disease is apparently not transmitted from one tree to another over short distance, the possibility that rain and wind are agents of its dispersal can not be dismissed because Van der Zweep and Kam (1982) detected the bacterium in vegetation surrounding a diseased tree.

The prevalence of some electrotypes, ETs 1, 2, 3 and 11, may be due to several factors. Bacteria exist in the natural environment primarily as clones and there is little exchange of genetic material between clones (Selander and Levin, 1980; Caugant *et al.*, 1981 and Young, 1989). The prevalence of any one clone at a particular point in time will therefore reflects its overall adaptation to the environment; any one clone will eventually decline and it will be replaced by another better able to thrive in that environment. Individual clones may persist for considerable periods of time, changing only as they accumulate mutations of adaptive significance, or undergo rare genetic recombination.

The reason why the electrotypes of two Wiltshire isolates collected in 1989 was un-reproducible in a further analysis in 1991 is not known. One very weak explanation might be that genetic alterations (mutation or loss of plasmid) occurred during the storage of the isolates. Another reason might be that 1989 stocks were mixed populations of the isolates.

Further study of the distribution of electrotypes from a larger selection of symptomless mature trees, from symptomless sets and from symptomless stools may answer the questions and enhance our knowledge of the distribution of strains of *E. salicis*. To address these questions the first requirement is to isolate the bacterium. A study of immunoisolation of *E. salicis* from symptomless trees is presented in the following chapter (see Chapter 7).

Chapter VII

IMMUNOISOLATION OF *ERWINIA SALICIS* FROM *SALIX ALBA*

7. 1 General introduction

When *E. salicis* antigens were first detected in symtomless sets and trees (Turner *et al.*, 1992), it was recognised that the significance of this observation to the epidemiology of watermark depended upon the proof that these were associated with infection by viable cells of *E. salicis*. A priority for the present study was therefore to develop methods for the isolation of viable cells of *E. salicis* from this population, in the presence of large numbers of other, contaminating bacteria. The isolation of *E. salicis* from diseased symptomless wood has therefore formed an important part of the study presented in this thesis. Two methods, immunofluorescence colony staining and immunomagnetic separation have offered good prospects for isolation of low numbers of bacteria present in the complex natural sources (e.g. Chapter 1.11).

Immunofluorescence colony staining (IFC) was first described by Van Vuurde (1987) and experiments showed that colonies of phytopathogenic bacteria grown in agar media can be stained with homologous antibodies labelled with fluorescein isothiocyanate, and that the pathogen even could be re-isolated after staining and immunofluorescence observation. The method also has the advantage that cross-reacting colonies could be used for research on antiserum specificity. The method was successfully applied for detection of low levels of *Erwinia chrysanthemi* and *E. carotovora* subsp. *atroseptica* in cattle manure slurry (Van Vuurde and Roozen, 1990). The technique also enabled the detection of *Xanthomonas campestris* pv. *begoniae* in washings of begonia leaves (Van Vuurde, 1990 a) and *Pseudomonas syringae* pv. *phaseolicola* and *Clavibacter michiganensis* subsp. *michiganensis* in infected seeds with large numbers of saprophytes (Franken and Van Vuurde, 1990).

Immunomagnetic separation (IMS) has been shown to be a very effective tool for the separation and isolation of specific cells from blood (Nustad *et al.*, 1988). The technique was also applied for isolation and identification K88 antigen-positive ($K88^+$) *Escherichia coli* from a mixed culture of five different O serogroups of *E. coli* (Lund *et al.*, 1988) and the sensitivity of the test was found to be $4x10^3$ cfu/ml. Immunomagnetic separation with immunobeads was used to detect *Listeria monocytogenes* in foods at less than

1×10^2 bacterial cells/ml in pure cultures and less than 2×10^2 bacterial cells/ml could be detected in enriched foods (Skjerve *et al.*, 1990). *Salmonella* serogroup C_1 strains (O:6,7), e.g. *S. choleraesuis* and *S. paratyphi* C, were detected in blood and stool samples by using the immunomagnetic separation technique (Luk and Lindberg, 1991). The sensitivity of the method was found to be 10^3-10^4 bacterial cells/ml and it was recorded that the presence of blood (10% v/v) or stool (1% w/v) components did not interfere with the immunomagnetic assay performance. The method was also applied for isolation of *Salmonella livingstone* from pure cultures, mixed cultures and food samples (Vermunt *et al.*, 1992). Although *Aeromonas hydrophila* (strain G5) and, to a lesser extent, *Enterobacter agglomerans* (strain G2) showed considerable cross-reaction with the antibody coated beads, it was concluded that immunomagnetic separation of *Salmonella* spp. offered good prospects for concentrating salmonella cells from heterogeneous bacterial suspensions of food samples . The only application of the method with plant pathogenic bacteria has been to isolate *Xanthomonas campestris* pv. *pelargonii* isolated from mixed populations and from contaminated leaf surfaces (Jones and Van Vuurde, 1990).

In the present investigation both IFC and IMS techniques were used in an attempt to isolate *E. salicis* from symptomless infected willow wood.

7. 2 Immunofluorescence Colony Staining (IFC)

7. 2. 1 Introduction

In the present study, IFC was evaluated for *E. salicis* using pure and mixed cultures of the bacterium and then applied to symptomless plant samples obtained from different branches of an infected but symptomless tree. In a further study, plant samples were collected from different locations of an symptomless infected tree by hand drilling. As a control, the samples were also assayed for *E. salicis* antigen by the double antibody sandwich (DAS) ELISA technique. The plant samples used for IFC staining were also plated out by the conventional 'streaking' method, to compare the efficiency of these two techniques for isolation of *E. salicis*. The specificity of the FITC-coupled anti-*E. salicis* antiserum was also confirmed by immunofluorescence (IF) cell staining of *E. salicis* in pure and mixed cultures.

7. 2. 2 Materials and methods

7. 2. 2. 1 ELISA test

A double antibody sandwich (DAS) ELISA test (Davis, J. pers. comm.) was employed. Gamma globulin (Ig G) was purified from anti-2535 antiserumby first precipitating the protein with sodium sulphate and then passing the dissolved protein through a DEAE-Sephacel column preequilibrated with 17.5 mM phosphate buffer pH 6.5 (see 7. 2. 2. 2. 2 for procedure). Unabsorbed fractions with the highest A_{278} nm were pooled and the protein content of the suspension was determined by the BCA method of Smith *et al.* (1985) as described in Chapter 5. 2. 3.

Coating of each well of the flat bottom 96 well microtitration plate (Nunc, Nunc-immunoplate maxisorp) was done by addition of 250 μl of Ig G containing 10 μg/ml protein in coating buffer (0.01 M phosphate buffer pH 8.0) (see appendix for the recipe). After incubation for 3 hours at 37 °C, the plate was washed three times with PBST (0.01 M phosphate buffered saline containing 0.5 % Tween 20, pH 7.4) (see appendix for recipe) using a semiautomatic plate washer. Unreacted sites were blocked by addition of 300 μl of 1 % bovine serum albumin (BSA) (Sigma No. A 7906) in coating buffer. After incubation for two and a half hours at 37 °C, the plate was again washed three times with distilled water. The coated plates were air-dried overnight and stored over silica-gel until use.

Conjugation of peroxidase with the Ig G was carried out by the simplified $NaIO_4$ method of Tijssen (1985 a) using peroxidase (Sigma No. P 8125).

After coating the plates with the Ig G fraction of rabbit antiserum as above, 200 μl of the plant sample or pure culture suspended in 1 ml of 0.01 M PBS without Tween 20 pH 7.4 at a concentration of 10^8-10^9 cells/ml was added to the wells in duplicate. Bacterial suspensions of *E. salicis* NCPPB 2535 in PBS pH 7.4 were added to three wells in at ten-fold dilutions to give 10^7, 10^6, 10^5 cells/ml. After incubation for 3 hours at 37 °C, the plate was washed five times with PBST and 200 μl of 1: 5000 dilution of the antiserum conjugated with peroxidase was applied to each well. After further incubation for 3 hours at 37 °C, the plate was again washed five times with PBST and 200 μl of peroxidase substrate solution [8 mg of 2-2'-Azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (Sigma No. A-1888) in 20 ml

of 100 mM phosphate-citrate buffer pH 7.4 containing 2 µl of 30 % H_2O_2] (see appendix for the recipe of 100 mM phosphate-citrate buffer) was added. After incubation for 2 hours at room temperature, the optical density was read at 414 nm on a plate-reader (Titertek, Multiscan).

7. 2. 2. 2 Preparation of fluorescein isothiocyanate conjugated anti-*E. salicis* antiserum (FITC-As)

7. 2. 2. 2. 1 Production of a specific antiserum

The antiserum was raised against *E. salicis* NCPPB 2535 and antigen preparation, antiserum production, separation and preservation of the antiserum and determination of the titre of the sera have been described (Chapter 2. 2. 6).

7. 2. 2. 2. 2 Purification of immunoglobulin (Ig) G for conjugation to FITC

The methods of Tijssen (1985 b) were employed to purify the gamma globulin of the antiserum and the purification was achieved at two steps:

a. DEAE-Sephacel column:

A DEAE-Sephacel column was prepared by using 2.5 ml of DEAE-Sephacel (Pharmacia Code No. 17-0500-01, in 20 % ethanol) per ml of antiserum to be purified. Prior to use the column was equilibrated with 17.5 mM phosphate buffer pH 6.5 (see appendix for the recipe) by several washings. Anti-*E. salicis* NCPPB 2535 antiserum was diluted in an equal volume of 35 mM phosphate buffer pH 6.5 (see appendix for the recipe) and then applied to the column at room temperature. When all the serum has passed into the column, 17.5 mM phosphate buffer pH 6.5 was carefully applied and 2 ml of fractions were collected. All fractions from the protein peak consisting of Ig G with A_{278} more than 0.8 were pooled and then dialysed against several changes of 0.5 M phosphate buffer pH 8.0 (see appendix for the recipe) at 4 °C overnight.

b. Sodium sulphate precipitation:

The volume of the protein solution was measured after dialysis and sodium sulphate (Na_2SO_4) was added very slowly with gently agitation to give 18 % w/v. After incubation for 1 hour at room temperature, the turbid suspension was centrifuged at 8000 rpm for 15 minutes. The supernatant was discarded and the pellet was dissolved in 0.5 M phos-

phate pH 8.0 at a volume equivalent to 40 % the volume of the original protein solution. After addition of sodium sulphate to give 12 % w/v very slowly, and following incubation at room temperature for 30 minutes, the turbid suspension was again centrifuged at 8000 rpm for 15 minutes and the pellet was resuspended in a small volume, equivalent to one fifth the volume of original protein solution, of 0.1 M carbonate-bicarbonate buffer pH 9.5 (see appendix for the recipe). The protein concentration of the solution was determined by the BCA method of Smith *et al.* (1985) as described in Chapter 5. 2. 3.

7. 2. 2. 2. 3 Conjugation of Ig G preparation with FITC

Conjugation of the gamma globulin fraction of the anti-2535 antiserumwith fluorescein isothiocyanate (FITC) was carried out by the method of Allan and Kelman (1977) with minor modifications. The protein content of Ig G solution was adjusted to contain 10 mg/ml (1 %) by addition of 0.1 M carbonate-bicarbonate buffer pH 9.5. A standard solution, containing 2 mg/ml FITC (Sigma No. F 7250) in 0.1 M carbonate-bicarbonate buffer pH 9.5 was prepared. The standard FITC solution was added to Ig G solution, at a volume of one tenth the volume of the Ig G solution, in 5 µl aliquots very slowly by gentle agitation. The Ig G solution containing FITC was brought to pH 9.5 by addition of 0.1 N NaOH. The reaction continued in a light proof box at room temperature overnight .

7. 2. 2. 2. 4 Separation of free FITC after conjugation

The method of Chantler and Mc Illmurray (1987) was used to separate the free FITC after conjugation, with a minor modification. The conjugate suspension was dialysed against several changes of 0.01 M phosphate buffered saline (PBS) pH 7.4 (see appendix for the procedure) for 3 days at 4 °C. The dialysate was then centrifuged at 3000 rpm for 3 minutes to remove particulate material.

7. 2. 2. 2. 5 Evaluation and the storage of the conjugate

The efficacy of the conjugation was determined by measuring the optical density of the conjugate after dialysis at 280 nm and 495 nm as described by Chantler (1982). A ratio of A_{495} / A_{280} between 0.6 and 0.9 was accepted to be appropriate for the detection of *E. salicis* colonies in the preparations.

The conjugate was stored in 25μl aliquots in Eppendorf tubes at – 20 °C. No preservative was used in the storage of the conjugate.

7. 2. 2. 3 Immunofluorescence (IF) cell staining of *E. salicis* in pure and mixed cultures

The basic procedure of Stead (1987) was employed for the direct immunofluorescence cell staining of *E. salicis* with some minor modifications. The assay was carried out with pure and mixed cultures of *E. salicis* :

a. Pure cultures: *E. salicis* NCPPB 2535 was grown on NDA at 28 °C for 3 days and the bacterial growth was resuspended in 0.05 M phosphate buffer pH 7.0 to give approximately 10^8-10^9 cells/ml (A_{550}=0.5).

b. Mixed cultures: *Pseudomonas syringae* pv. *atropurpurea* was grown on nutrient agar at 28 °C, overnight. The bacterial growth was resuspended in 0.05 M phosphate buffer pH 7.0 to give approximately 10^8-10^9 cells/ml (A_{550}=0.5). *E. salicis* suspension prepared as above was two-fold diluted in the phosphate buffer and the dilution was mixed with the bacterial suspension of *Ps. syringae* pv. *atropurpurea* at the ratios of 1:6, 1:7, 1:8, 1:9 and 1:10.

Thirty μl of the pure or mixed bacterial suspensions of *E. salicis* was put to 6 mm diameter window of the multi-spot slide cleaned in ethanol before use and allowed to air dry in a laminar flow cabinet. After fixing by gently heating in a Bunsen flame, the preparation was washed in 0.01M phosphate buffer pH 7.2 (see appendix for the recipe) for 3 minutes. The excess moisture was taken up with absorbent paper. Thirty μl of FITC-As diluted in PBS pH 7.4 at a ratio of 1:30 was added to each window and incubated in a light proof humid chamber at 28 °C for 30 minutes. The preparation was washed in 0.01 M phosphate buffer, each one taking 3 minutes, in the dark with gently shaking. After placing between the filter papers, the preparation was allowed to air dry at room temperature and then examined microscopically for the presence of bright-green fluorescence cells.

7. 2. 2. 4 Source and maintenance of plant material

Eleven cuttings, some having the symptoms of watermark disease, numbered from 1 to 11 in Table 7. 2. 1, were collected from different branches of a diseased symptomless *S. alba* (tree no. 3 shown in Figure 7. 3. 1) located in Beccles in 1991 by Dr. J. G. Turner.

In a further study three drill samples, 13c (1), 13c (2) and 13c (3) in Table 7. 2. 1, were obtained from a symptomless diseased *S. alba* (tree no. 13c shown in Figure 7. 3. 1) located in Beccles in 1992 by J. Bowen and myself by hand drilling (Characteristics of the samples are given in Table 7. 2. 1). Samples were placed in polythene bags and stored at –20 °C until use.

7. 2. 2. 5 Preparation of plant samples

For the cuttings, the bark of the wood was peeled using sterilised forceps. The end was then cut across and chopped into thin sections with a pair of sterilised secateurs. Approximately 5 g of chopped wood was collected in a beaker and 40 ml of 0.01 M phosphate buffered saline pH 7.4 without Tween-20 was added. The drill samples were also weighed. Approximately 40 ml of the buffer was added to each sample, which weighed in the range 2-4 g. The beaker containing buffer and wood was placed under vacuum in a desiccator for 2-3 minutes. After incubation at 4 °C overnight, the suspension was filtered through the muslin and then centrifuged at 10 000 rpm for 20 minutes. The supernatant was carefully removed and the pellet was resuspended in a small volume of PBS pH 7.4. After further centrifugation at 14 000 rpm for 5 minutes, the pellet was resuspended in 1.5 ml of PBS pH 7.4 and 0.5 ml of the sample was saved for ELISA test described in this study. The remaining sample was used both for streaking onto agar plate and for the preparation of a dilution series of plant samples for IFC staining.

7. 2. 2. 6 Isolation of *E. salicis* by conventional methods

A loopful of plant sample was streaked onto a NDA plate and the plate was incubated at 28 °C. The plates were examined daily and single colonies were subcultured and then assayed for *E.salicis* by ELISA.

7. 2. 2. 7 Preparation of dilutions of pure and mixed cultures of *E. salicis* and plant samples for IFC staining

Three types of samples were examined by IFC staining.

(a) Pure cultures of *E. salicis* :

E. salicis NCPPB 2535 was grown in NDB for two days at 28 °C on a rotary shak-

er. The optical density of the culture was adjusted to A_{550}= 0.2 to give 4 x 10^8 cells/ml by addition of sterile NDB. Ten-fold dilution series of the culture were prepared upto 10^{-7} dilution.

(b) Mixtures of *E. salicis* and another bacterium :

To prepare the mixed cultures, *E. salicis* NCPPB 2535 was grown as above and the optical density of the culture was adjusted to A_{550}=0.2 to give 4 x 10^8 cells/ml. An unknown epiphyte (S1C) isolated from watermark diseased tree was also grown in NDB for two days at 28 °C and the optical density of the culture was adjusted to A_{550}=0.2 to give 1.7 x 10^8 cells/ml. These two cultures were mixed at the ratios of 1:1, 1:10, 1:100 and 1:1000 and a ten-fold dilution series of these mixtures was then prepared in 4.5 ml of PBS pH 7.4.

(c) Extracts of plant material :

Ten-fold dilution series of the plant samples were prepared in PBS pH 7.4 by addition of 0.5 ml of plant sample to 4.5 ml of buffer to give a final volume of 5 ml in each dilution; the last dilution was 10^{-10}.

7. 2. 2. 8 Preparation of agar plates for IFC staining

Methods of Van Vuurde (1990 b) were used with minor modifications to prepare agar plates and staining of the plates with FITC-As. Molten NDA was prepared in 4 ml aliquots in Bijoux bottles and was kept molten at 45 °C until use. One ml of the dilution series of the pure culture or mixed cultures of *E. salicis* , or the plant samples prepared as above was added to 4 ml of the molten NDA to give 5 ml final volume and mixed vigorously. The mixture was then poured into a polystyrene petri dish, 5 cm diameter. The plate was swirled gently to give a homogeneous distribution of the bacteria in the agar. The plates were incubated at 28 °C for two days (until pinhead size colonies were formed).

The plates containing upto 10^3-10^4 colonies were chosen and the agar was dried into a thin film by blowing warm air (40 °C) over the agar surface for 6-8 hours. The dried agar film with colonies was stored in a container with silica gel at 4 °C.

7. 2. 2. 9 Determination of the working dilution of the FITC-As for IFC staining

1:50, 1:100, 1:150 and 1:200 dilution series of FITC-As were prepared in phosphate buffered saline (PBS) pH 7.4. Plates of pure cultures of *E. salicis* containing 10^3-10^4 colonies were incubated with 2.5 ml of each of dilution series of the FITC-As. After incubation in a light proof box at room temperature overnight , the non-bound conjugate was removed by three 5 minutes washes in PBS pH 7.4. The excess PBS was removed with a pipette and the plate was examined by a fluorescence microscope. The dilution which gave bright green fluorescence of colonies of *E. salicis* NCPPB 2535 with negligible or no background staining was chosen as optimal working dilution.

7. 2. 2. 10 IFC staining of the plates

The plates containing up to 10^3-10^4 colonies were chosen for immunofluorescence colony staining. Twenty five μl of the conjugate (FITC-As) was diluted in PBS pH 7.4 to give a final volume of 2.5 ml. The hundred-fold diluted (2.5 ml) conjugate was added to the petri dish containing dried agar film, and incubated in a light proof box, at room temperature overnight. The non-bound conjugate was removed by three 5 minute washes in PBS pH 7.4 and the agar film was examined by u.v. microscopy.

7. 2. 2. 11 Microscopy

Following staining, all immunofluorescence preparations were examined using Nikon epi-fluorescence microscope, excitation 450-490 nm, emission > 520 nm. The microscope magnification was x 400 for the IF cell staining preparations and x63 for the IFC staining preparations. Photographs of IF cell staining preparations were taken with Kodak Gold 400 film while the photagraphs of IFC staining preparations were taken with Kodak Tmax 400 film.

7. 2. 3 Results

7. 2. 3. 1 Specificity of the FITC-As

Specificity of the anti-2535 antiserumwas previously evaluated by means of the competitive ELISA and agglutination tests (Chapter 2) and the immunodiffusion test (Chapter 4). These observations indicated that the antiserum was highly specific for *E. sali cis*. In the present study, additional evidence for the specificity of the fluorescein isothio-cyanate coupled anti-*E. salicis* antiserum (FITC-As) was obtained by IF cell staining of *E. salicis*. In the mixed cultures of *E. salicis* containing *Ps. syringae* pv. *atropurpurea* only the *E. salicis* cells stained brightly-green while the other bacterium did not stain confirming the specificity of the FITC-As (see Plate 7. 2. 1a-b).

7. 2. 3. 2 IFC staining of pure cultures of *E. salicis*

The ratio of A_{495} / A_{280} of the FITC-As solution following dialysis was 0.93, indicating the success of conjugation reaction. The optimal working dilution which gave intense specific staining of the colonies of *E. salicis* was determined with 1:100 diluted FITC-As. The background staining was negligible at this dilution.

E. salicis colonies in the agar reached pin-head size after two days incubation at 28 °C and the colonies appeared as bright-green fluorescence with a fluorescent halo (see Plate 7. 2. 2).

7. 2. 3. 3 IFC staining of mixed cultures of *E. salicis*

Sensitivity of the FITC-As for detection of *E. salicis* in mixed cultures was evaluated by preparing dried-agar films with suspensions of *E. salicis* mixed with populations of the epiphyte, S1C, at different ratios. With this procedure, *E. salicis* could be detected in plates containing 40 cells/ml of *E. salicis* mixed with 17 cells/ml of S1C (ratio 1: 0.425), when the sample contained 40 cells/ml mixed with 1.7×10^2 cells of S1C per millilitre (ratio 1: 4.25) and 4 x 10 cells/ml mixed with 1.7×10^3 cells of S1C per millilitre (ratio 1: 42.5). No *E. salicis* colony could be detected in the other plates containing the ratio 1: 425 *E. salicis* to the unknown epiphyte. A microscope field showing *E. salicis* colonies mixed with unknown epiphyte, S1C, in a plate after two days incubation is shown in Plate 7. 2. 3 a.

<u>Plate 7. 2. 1a,b</u> *E. salicis* mixed with *Ps. syringae* pv. *atropurpurea*. The photograph in the plate (a) was taken with fluorescence filters while the plate (b) shows the same microscope field with the light microscopy without flu orescence filters. Arrows in plate (b) show the fluorescence stained cells of *E. salicis* in plate (a). (Microscope magnification is x 400).

Colonies of the S1C were larger than *E. salicis* colonies with this incubation period and they stained yellowish-brown in the microscope field. The colonies of S1C sometimes masked the *E. salicis* colonies (see Plate 7. 2. 3 b).

7. 2. 3. 4 Assay of plant samples

7. 2. 3. 4. 1 ELISA assay

Colour development in the wells of microtiter plate indicated a positive reaction. Presence of the specific antigen from the sample allowed antibody to react with the antigen, and a colour was produced following the addition of the peroxidase enzyme substrate. All the plant samples numbered from 1 to 13 were ELISA positive and had an OD $_{414}$ value more than 0.150. Samples 13a, 13b and 13c had a value more than 0.161 (see Table 7. 2. 1).

7. 2. 3. 4. 2 IFC staining assay

Immunofluorescence positive *E. salicis* colonies were predominant in the plates containing plant samples numbered 1, 3, 4, 5, 7, 8, 9, 10 and 11 (Table 7. 2. 1) diluted to 10^{-5} to and 10^{-6}. Contaminants did not appear in some plates and in some plates a few contaminant colonies were observed (Plate 7. 2. 4). The presence of low numbers of contaminants in the plates prepared from plant samples did not affect the colony size of *E. salicis.*

No immunofluorescence positive *E. salicis* colony could be detected in the plates prepared from symptomless plant samples, 2, 6, 13c (1), 13c (2) and 13c (3) (Table 7. 2. 1). Only the contaminant colonies were observed in the plates having up to 10^{-3} and 10^{-4} diluted plant samples.

The immunofluorescence colony staining technique was also compared with conventional plating method. Nine of the 14 plant samples having the symptoms of watermark disease (Table 7. 2. 1) gave *E. salicis* colonies predominantly on the streaked plates after 2-3 days incubation at 28 °C. No *E. salicis* colony was observed in the plates streaked out from plant samples 2, 6, 13c (1), 13c (2) and 13c (3). The identity of the pure cultures was confirmed by the ELISA test.

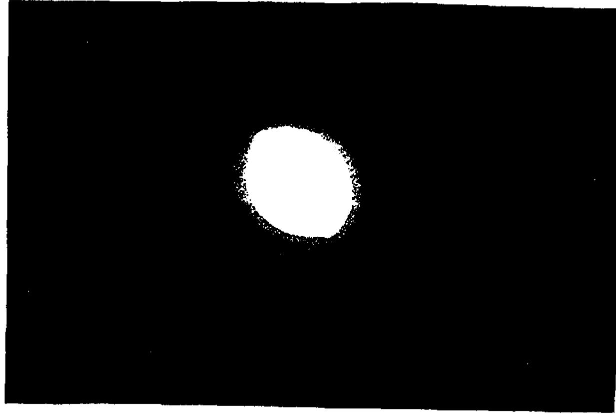
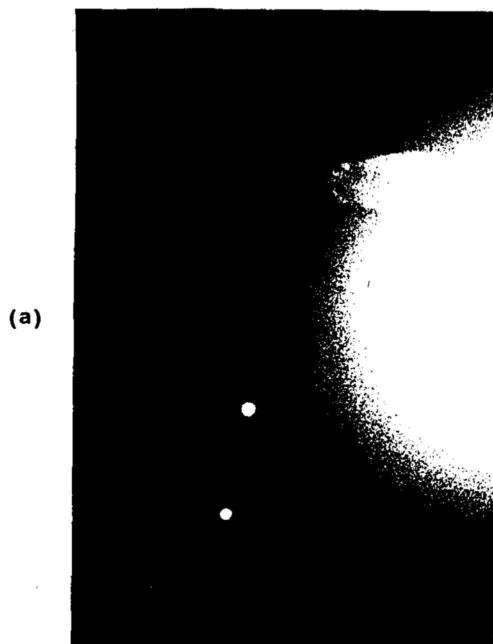


Plate 7. 2. 2 Immunofluorescence stained colony of *E. salicis* in pure culture. FITC-As dilution is 1:100. (Microscope magnification is x 63).



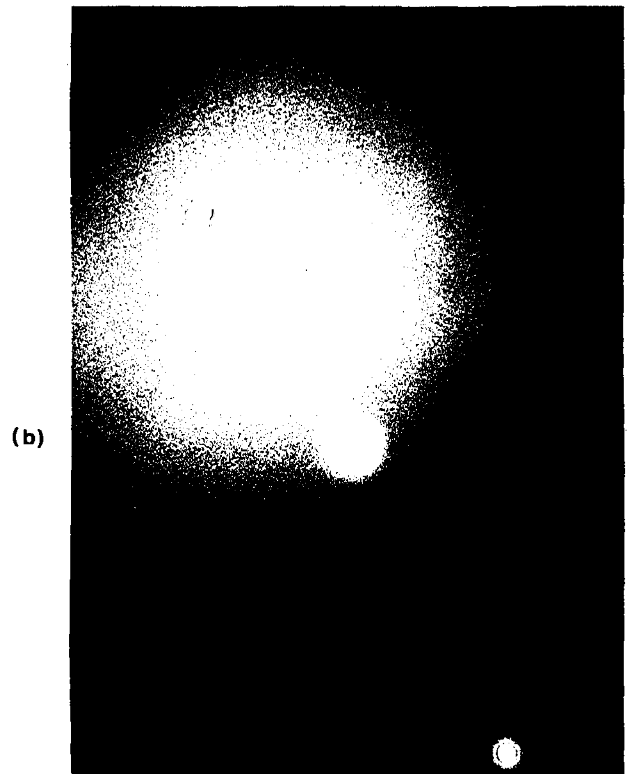


Plate 7. 2. 3 a, b Immunofluorescence stained colonies of *E. salicis* among large colonies of unknown epiphyte, S1C. FITC-As dilution is 1:100. (Microscope magnification is x63).

Table 7. 2. 1 Characteristics of plant samples tested with immunofluorescence colony staining, ELISA and conventional 'streaking' methods.

Plant sample no.	Symptoms	DAS ELISA	streaked colony	IF (+) colony
1	slightly stained, older wood	+	+	+
2	no stain, younger wood	+	–	–
3	discolouration, younger wood	+	+	+
4	discolouration, younger wood	+	+	+
5	young twigs from sample 3 and 4	+	+	+
6	no stain, 2-3 years old wood	+	–	–
7	stain in the young wood	+	+	+
8	slightly stained, 2-3 years old wood	+	+	+
9	stain, 2-3 years old wood	+	+	+
10	stain, 2-3 years old wood	+	+	+
11	young twig with ooze	+	+	+
13c (1)	symptomless tree	+	–	–
13c (2)	symptomless tree	+	–	–
13c (3)	symptomless tree	+	–	–

+ : positive

_ : negative

Plate 7. 2. 4 Immunofluorescence stained colony of *E. salicis* next to a saprophyte colony in plant sample extract. Fading of the fluorescein dye due to late photographing is observed with *E. salicis* colony. FITC-As dilution is 1:100. (Microscope magnification is x63).

7. 2. 4 Discussion

The advantage of the immunofluorescence colony staining (IFC) technique over the conventional isolation methods is that the low numbers of the target bacterium could be detected as fluorescence stained distinct colonies which could be subcultured for further study (Van Vuurde, 1987).

Detection of low numbers (10^2 cfu/ml) of *Erwinia chrysanthemi* and *E. carotovora* subsp.*atroseptica* in the extract of potato peel and cattle manure slurry was achieved by using IFC technique (Van Vuurde, 1990 a and Van Vuurde and Roozen, 1990).

The IFC technique used in this study for the isolation of low numbers of *E. salicis* failed to detect the bacterium in the samples from symptomless plants which were positive by ELISA. The reason for the failure is not known. Although the detection limit of the technique in the present study seems to be high, it is probably not enough to detect low numbers

of *E. salicis* in the presence of high ratio of rapidly-growing saprophytes from the symptomless plant samples. One possibility might be that DAS ELISA detects antigens of both live and death cells of the bacterium and if the bacterium present in the plant sample was dead, no colony of *E. salicis* would grow in the plates. Another reason might be that ELISA detects not only bacterial cells but also soluble antigens of *E. salicis* (Kam, 1982 a, b). Therefore, symptomless plant sample might have given a positive ELISA result due to soluble antigens of the bacterium originally present in the diseased part of the plant.

In the present study, the IFC technique was not superior to the conventional streaking methods for isolation of *E. salicis*. Presumably this is because most of the plant materials contained high numbers of *E. salicis* which gave nearly pure cultures on the streaked plates.

E. salicis colonies in the mixed culture plates (Plate 7. 2. 3a,b) appeared smaller than the colonies of the bacterium in the pure culture plates (Plate 7. 2. 2) and in the plant sample plates (Plate 7. 2. 4). The possibility is that the large numbers of fast growing unknown epiphyte, S1C, exhausted the nutrients in the media before the slow growing *E. salicis* produced normal-sized colonies.

It has been reported that direct staining methods have greater specificity than indirect methods (Stead, 1987). Van Vuurde (1990 a) reported that indirect IFC gave less specific results for *Erwinia chrysanthemi* and resulted in a higher background. Use of indirect immunofluorescence staining technique is therefore not likely to increase the detection of *E. salicis* in the symptomless plant sample. Hence, a new technique based on serology and magnetic beads is presented in the following section.

7. 3 Immunomagnetic Separation (IMS)

7. 3. 1 Introduction

In the present study, IMS was evaluated for trapping and isolating *E. salicis* from symptomless plant samples. The sensitivity of the method was tested by incubating different numbers of IMP with low numbers (1.8×10^3 and 1.8×10^4 cells/ml) of pure cultures of *E. salicis*. A reconstruction assay was carried out to examine the performance of immunomagnetic separation in the presence of plant material and then the technique was applied to symptomless infected plant material. Identification of isolates obtained by the IMS technique was confirmed with the bacteriophage sensitivity assay. The technique was also compared by DAS ELISA for sensitivity.

7. 3. 2 Materials and Methods

7. 3. 2. 1 Coating immunomagnetic particles with anti-*E. salicis* antiserum (IMP-As)

7. 3. 2. 1. 1 Production of a specific antiserum

The antiserum was raised against *E. salicis* NCPPB 2535 and antigen preparation, antiserum production , separation and preservation of the antiserum and determination of the titre of the sera have been described (Chapter 2. 2. 6).

7. 3. 2. 1. 2 Purification of Ig G for attachment to immunomagnetic particles

The methods of Tijsen (1985 b) were employed to purify the gamma globulin of the antiserum as described in Chapter 7. 2. 2. 2. 2.

7. 3. 2. 1. 3 Attachment of anti-*E. salicis* antiserum to IMP

Superparamagnetic, monosized, polystyrene beads with a diameter of 2.8 µm (Dynabeads M-280) with covalently linked sheep anti-rabbit Ig G antibodies containing

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6-7 x 10^8 beads/ml (10 mg/ml) in PBS pH 7.4 with 0.1 % BSA were obtained from Dynal A/S (P.O. Box 158 Skoyen N-0212 Oslo 2, Norway).

The immunomagnetic particles (IMP) were coated as prescribed by the manufacturer except that filter sterilised PBS pH 7.4 with 0.1 % bovine serum albumin (BSA) (Sigma No. A-7906) was used in all experiments. Dynabeads M-280 were suspended by vortexing and a magnetic bar was placed against the wall of the Eppendorf tube for trapping IMP and the fluid was pipetted off. The tube was removed from the magnetic bar and an excess of PBS- 0.1 % BSA was added. The procedure was repeated twice and the washed IMP were suspended in the volume of PBS-0.1 % BSA originally pipetted from the vial. IMP were then suspended by shaking and incubated with 10 μg Ig G per mg of beads at 4 °C, overnight by sufficient shaking to avoid settling of IMP. After washing for 4 times, 30 minutes each time at 4 °C, the IMP were suspended in PBS-0.1 % BSA and stored at 4 °C. No preservative was used in the storage of IMP. Before use, the IMP were washed three times at 4 °C, 10 minutes per wash, when stored for more than two weeks. The coating procedure was checked by agglutination reaction.

7. 3. 2. 1. 4 Agglutination reaction with IMP-As

Fifteen μl of coated IMP (approximately 10^7 IMP per ml) in PBS pH 7.4 without 0.1 % BSA were mixed with an equal volume of *E. salicis* NCPPB 2535 grown in NDB. Control experiments included only uncoated IMP and an *E. salicis* culture. The slides were tilted and agglutination was checked within 10 minutes using x100 objective of Nikon light microscope and the photographs were taken with Kodak Vericolor III film.

7. 3. 2. 2 Isolation of *E. salicis* from pure cultures with IMP-As

E. salicis NCPPB 2535 was grown in NDB at 28 °C on a rotary shaker. The optical density of the culture was adjusted to A_{550}=0.12 to give 1.8 x 10^8 cells/ml. Ten-fold dilution series of this stock suspension were prepared in PBS-0.1 % BSA to give 1.8 x 10^3 and 1.8 x 10^4 cells/ml and 20 μl of each of bacterial dilutions was spotted on NDA plate before IMS treatment to compare with IMS treated samples. After incubation at 28 °C for 3 days the colonies were counted.

One hundred µl of each of bacterial dilutions was mixed with antibody coated IMP to give a final concentration of 10^6 and 10^7 beads/ml. During overnight incubation of IMP with bacterial cultures at 4 °C, the mixture was shaken to avoid settling of IMP. After incubation, the particles were separated from the cell suspension using a magnetic bar placed against the wall of the Eppendorf tube. The residual liquid was removed and four washing steps in PBS- 0.1% BSA, each one taking 10 minutes at 4 °C, were carried out. The particles were then suspended in the original volume of bacterial culture using PBS- 0.1%BSA. Two ten-fold dilutions of each suspensions were prepared in PBS-0.1 % BSA.

To measure the recovery of the cells after IMS treatment, 20 µl of each of bacterial suspension was spotted on NDA plate. After incubation at 28 °C for 3 days, the colonies were counted.

7. 3. 2. 3 Isolation of *E. salicis* from artificially inoculated wood samples with IMP-As ('Reconstruction assay')

Drill samples of healty willow wood was obtained from a *Salix alba* var. *caerulea* located in the University of East Anglia in 1992. 1.8 x 10^3, 1.8 x 10^4, 1.8 x 10^5 and 1.8 x 10^6 cells/ml concentrations of *E. salicis* were prepared in PBS-0.1 % BSA as described above (Chapter 7. 3. 2. 2) and 1 ml of each of bacterial suspension was mixed with 1 g of wood soaked in 10 ml of PBS pH 7.4. After incubation at 4 °C for 1 hour, the suspension was filtered through muslin and then centrifuged at 10 000 rpm for 20 minutes. The supernatant was carefully removed and the pellet was resuspended in a small volume of PBS pH 7.4. After further centrifugation at 14 000 rpm for 5 minutes, the pellet was resuspended in 1 ml of PBS pH 7. 4 and 100 µl of sample was mixed with coated IMP to give a final concentration of 10^7 beads/ml. After overnight incubation at 4 °C, the mixture was washed and plated as described with the pure cultures. The plate was incubated at 28 °C for 3 days and colonies were counted.

7. 3. 2. 4 Assay of plant samples

7. 3. 2. 4. 1 Source and maintenance of plant material

Multiple samples were collected from four different *S. alba* trees located in Beccles in 1992 by hand drilling by J. Bowen and myself. A diagram of the site is given in Figure 7. 3. 1. Two samples, 3 and 3a, were obtained from a severely diseased tree (tree no.3). Samples 10a, 10b, 10c, 10d and 10e were obtained from tree no.10. Samples 13a (a), 13a (b) and 13a (c) belonged to tree no.13a and samples 13b (a), 13b (b), 13b (c), 13b (d) and 13b (e) were obtained from tree no. 13b. Tree 10, 13a and 13b displayed no apparent symptom of watermark. Plant samples were placed in polythene bags and stored at – 20 °C until use.

7. 3. 2. 4. 2 Preparation of plant samples

Forty ml of PBS pH 7. 4 was added to the plant samples and after incubation at 4 °C for 5-6 hours, the suspension was filtered through muslin and then processed as described with the reconstruction assay. The final pellet was resuspended in 1 ml of PBS pH 7. 4 and 0.5 ml of sample was saved for ELISA test. Fifty µl of sample was streaked onto a NDA plate before IMS treatment and incubated at 28 °C for 3 days. One hundred µl of sample was incubated with IMP-As for isolation of *E. salicis*.

7. 3. 2. 4. 3 DAS ELISA test of plant samples

ELISA test of plant samples was performed as described in Chapter 7. 2. 2. 1.

7. 3. 2. 4. 4 Isolation of *E. salicis* from infected plant samples with IMP-As

One hundred µl of sample was mixed with IMP-As to give a final concentration of 10^7 beads/ml and after overnight incubation at 4 °C with shaking, the sample was washed in PBS-0.1% BSA four times as described with the pure cultures of *E. salicis*. In a further study, 5 samples belonged to tree no.13b were washed at six steps to decrease the non-specific binding. IMS treated sample was streaked onto NDA plate and incubated at 28 °C for 3

days until the *E. salicis* colonies appeared.

7. 3. 2. 4. 5 Identification of *E. salicis* isolates by bacteriophage sensitivity assay

Five bacteriophages, L, 1467, T3, C and M, were used in the assay. The details of bacteriophage sensitivity assay is given in Chapter 3. 2. 2. 6. Briefly, the test isolate was grown in NDB at 28 °C overnight and after preparing bacterial lawn of the culture on NDA plate, 20 µl of each of undiluted bacteriophage suspensions was spotted. The plates were incubated at 28 °C for 24 hours and bacteriophage sensitivity was recorded as the presence of confluent lysis on the spotting.

7. 3. 3 Results

7. 3. 3. 1 Optimum conditions for the immunomagnetic separation (IMS) of *E. salicis*

To determine the optimum concentration of immunomagnetic particles for binding *E. salicis*, two concentrations of IMP were tested. Cell concentrations of *E. salicis* were varied from 1.8 x 10^3 to 1.8 x 10^4 cfu/ml. Table 7. 3. 1 shows that the highest recovery was obtained by using 10^7 IMP/ml. Low cell concentration (1.8 x 10^3 cfu/ml) gave only slightly lower recovery (63 %) than from a higher cell concentration (1.8x10^4 cfu/ml; recovery 68 %) (see Table 7. 3. 1). Further experiments were carried out with a concentration of 10^7 IMP/ml only.

In agglutination experiments with coated IMP, *E. salicis* reacted very strongly (see Plate 7. 3. 1), indicating that 10 µg Ig G per mg of IMP was suitable for optimal binding. Control experiments showed that uncoated IMP did not bind to *E. salicis* (see Plate 7. 3. 2).

Table 7. 3. 1 Percentages of *E. salicis* recovered from suspensions with different cell concentrations and coated immunomagnetic particles (IMP).

Concentration of IMP (particles/ml)	Initial bacterial concentration (cfu/ml)	Recovery (%)
10^7	1.8×10^3	63
10^7	1.8×10^4	68
10^6	1.8×10^3	11
10^6	1.8×10^4	33

7. 3. 3. 2 Immunomagnetic separation of *E. salicis* from artificially inoculated wood samples ('Reconstruction assay')

To assess the potential usefulness of the immunomagnetic separation technique for isolation of *E. salicis* from symptomless infected wood, I examined its performance in the presence of plant material. For this, 1 g of willow wood chips was inoculated with 1 ml of different cell concentrations of *E. salicis* varying from 1.8×10^3 to 1.8×10^6 cfu/ml. After immunomagnetic separation of the resulting suspension with 10^7 IMP/ml an increase was observed in the percent recovery of *E. salicis,* in relation to the initial concentration of *E. salicis* (see Table 7. 3. 2). Increasing the number of *E. salicis* per g of wood raised the bound fraction of bacteria from 8.3 % (1.8×10^3 cells/g of wood) to 23 % (1.8×10^6 cells/g of wood) (see Table 7. 3. 2). These results also indicated that the recovery of *E. salicis* was affected in the presence of wood. In the presence of the wood sample, the recovery of*E. salicis* decreased from 63 % (see Table 7. 3. 1) to 8.3 % (see Table 7. 3. 2) for 1.8×10^3 cfu/ml and from 68 % (see Table 7. 3. 1) to 15 % (see Table 7. 3. 2) for 1.8×10^4cfu/ml.

Plate 7. 3. 1 Microscopic photograph of *E. salicis* attached to IMP-As. (Microscope magnification is x 1000).

Plate 7. 3. 2 Microscopic photograph of *E. salicis* mixed with uncoted IMP (negative control). (Microscope magnification is x 400).

Table 7. 3. 2 Percentages of *E. salicis* recovered from suspensions of artificially inocu lated wood samples (IMP concentration is 10^7 particles/ml).

Bacterial concentration (added cells/g of wood)	Recovery (%)
1.8×10^3	8.3
1.8×10^4	15
1.8×10^5	21
1.8×10^6	23

7. 3. 3. 3 Isolation of *E. salicis* from infected plant samples with immunomagnetic sep aration

The IMS technique isolated *E. salicis* from heavily diseased willow wood (sample nos. 3 and 3a) (see Table 7. 3. 3). Primarily contaminants with possibly a few tiny colonies of *E. salicis* appeared on the plates streaked before IMS treatment. A striking decrease was observed in the number of contaminant cells in the plates prepared from the IMS treated samples 3 and 3a, giving nearly pure cultures of *E. salicis*.

Thirteen test samples shown in Table 7. 3. 3 were treated with IMS technique and *E. salicis* could be isolated from samples 10a, 10d and 13a (a). No *E. salicis* could be isolated from five samples of tree 13b which were washed 6 times instead of 4 after IMS treatment. The proportion of suspected *E. salicis* colonies was increased after IMS treatment. Plates 7. 3. 3 and 7. 3. 4 shows the two streaked plates which were prepared before and after IMS treatment of the sample 10d. The number of suspect colonies of *E. salicis* was high in the IMS treated plate of sample 10d (see Plate 7. 3. 4) while only two suspect colonies grew on the IMS treated plates of each of samples 10a and 13a (a).

After single cell purificaton of suspect colonies, the culture was tested with the bacteriophage sensitivity assay to test their identity as *E. salicis*. Six cultures were grown from 6 suspect colonies isolated from 3 plant samples (two for each) with IMS technique and they were sensitive to bacteriophages L, 1467, C and M. These 6 cultures did not react to bacte

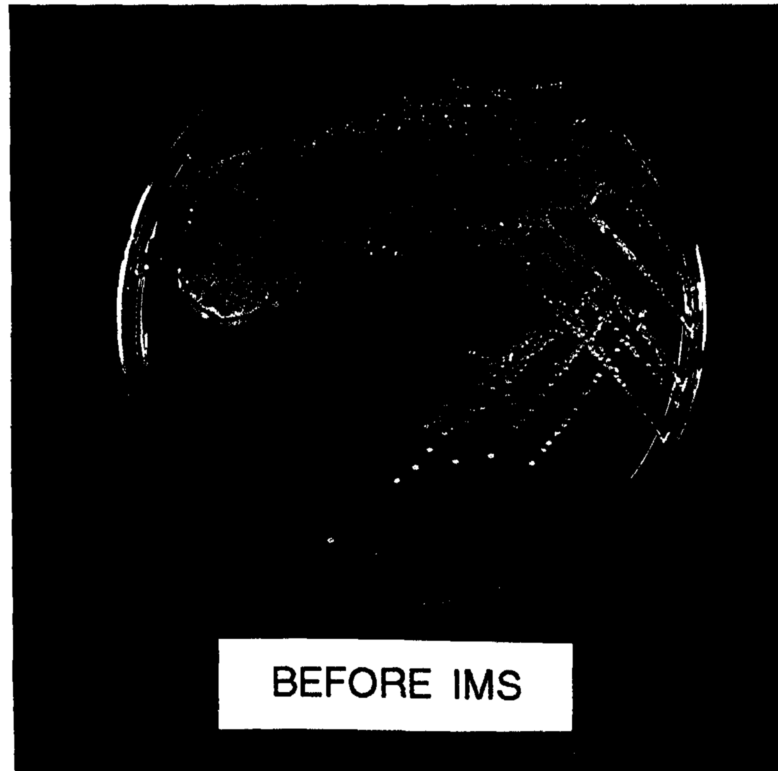


Plate 7. 3. 3 A plate prepared before immunomagnetic separation of symptomless sample 10d on NDA. Arrows indicates two tiny suspected *E. salicis* colonies.

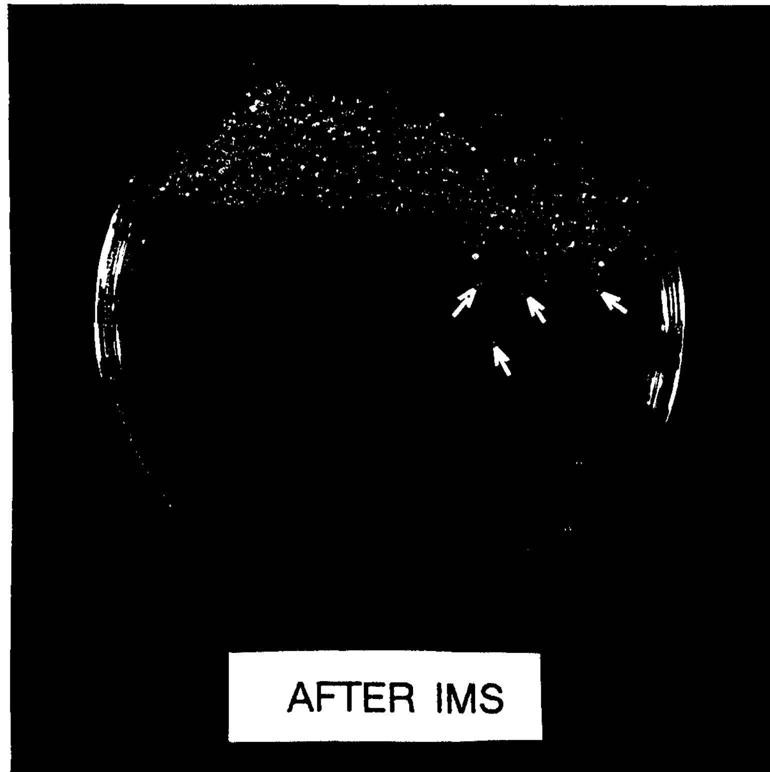


Plate 7. 3. 4 A plate prepared after immunomagnetic separation of symptomless sample 10 d on NDA. Large number of suspect colonies of *E. salicis* (indicated by arrows) after IMS, although non-*E. salicis* colonies were also observed on the plate.

riophage T3 as those typical English isolates in lysotype α_1 (see Chapter 3. 3. 3. 2). The bacteriophage sensitivity assay confirmed the identification of *E. salicis* isolates obtained by IMS treatment of the plant samples.

Table 7. 3. 3 Detection of *E. salicis* in plant samples by DAS ELISA, and isolation of *E. salicis* from these samples before, and following, IMS.

Sample	Isolation before IMS	Isolation after IMS	Detection by DAS ELISA
3	+	+	+
3a	+	+	+
10a	–	+	–
10b	–	–	–
10c	–	–	–
10d	+	+	+
10e	–	–	–
13a (a)	–	+	–
13a (b)	–	–	–
13a (d)	–	–	–
13b (a)	–	–	–
13b (b)	–	–	+
13b (c)	–	–	+
13b (d)	–	–	–
13b (e)	–	–	–

+ : positive

– : negative

7. 3. 3. 4 DAS ELISA assay of plant samples at different intervals

The Beccles site (see Figure 7. 3. 1) was first visited in 1989 and one drill sample was collected from each of 19 *S. alba* trees and these samples were assayed for *E. salicis* antigen by double antibody sandwich (DAS) ELISA technique by J. Davis (pers. comm.). Tree nos. 9, 14 and 16 had the external symptoms of watermark disease and ELISA test confirmed their infection giving positive reactions. The ELISA test also detected *E. salicis* antigens in 9 trees, tree nos. 1, 8, 10, 12, 13a, 13b, 13c, 15, 17, 18 and 19, which had no symptoms of the disease, indicating possible symptomless infections (see Table 7. 3. 4).

The site was then visited in 1991 and it was observed that heavily diseased trees 9, 14 and 16 had been felled. Multiple drill samples were obtained from tree nos. 8, 10, 12 and 13. After testing by DAS ELISA test, only one sample of tree nos. 10 and 13a were recorded as ELISA positive and no *E. salicis* antigen was detected in any of the multiple samples from tree nos. 8, 13b and 13c. Table 7. 3. 4 shows the ELISA test reactions of samples belonged the trees in Beccles at different times.

The Beccles site was visited in 1992 and multiple drill samples were obtained from tree nos. 10, 13a and 13b. After employing DAS ELISA, colour development in the wells of the microtiter plate indicated a positive reaction. ELISA reactions of the 15 plant samples collected in 1992 are shown in Table 7. 3. 3. Two control samples, 3a and 3, having the symptoms of the watermark disease and three test samples obtained from symptomless infected willow wood, 10a, 13b (b) and 13b (c), had an OD_{414} value of more than 0.165 indicating a positive reaction. The remaining 10 test samples (see Table 7. 3. 3) were recorded as ELISA negative.

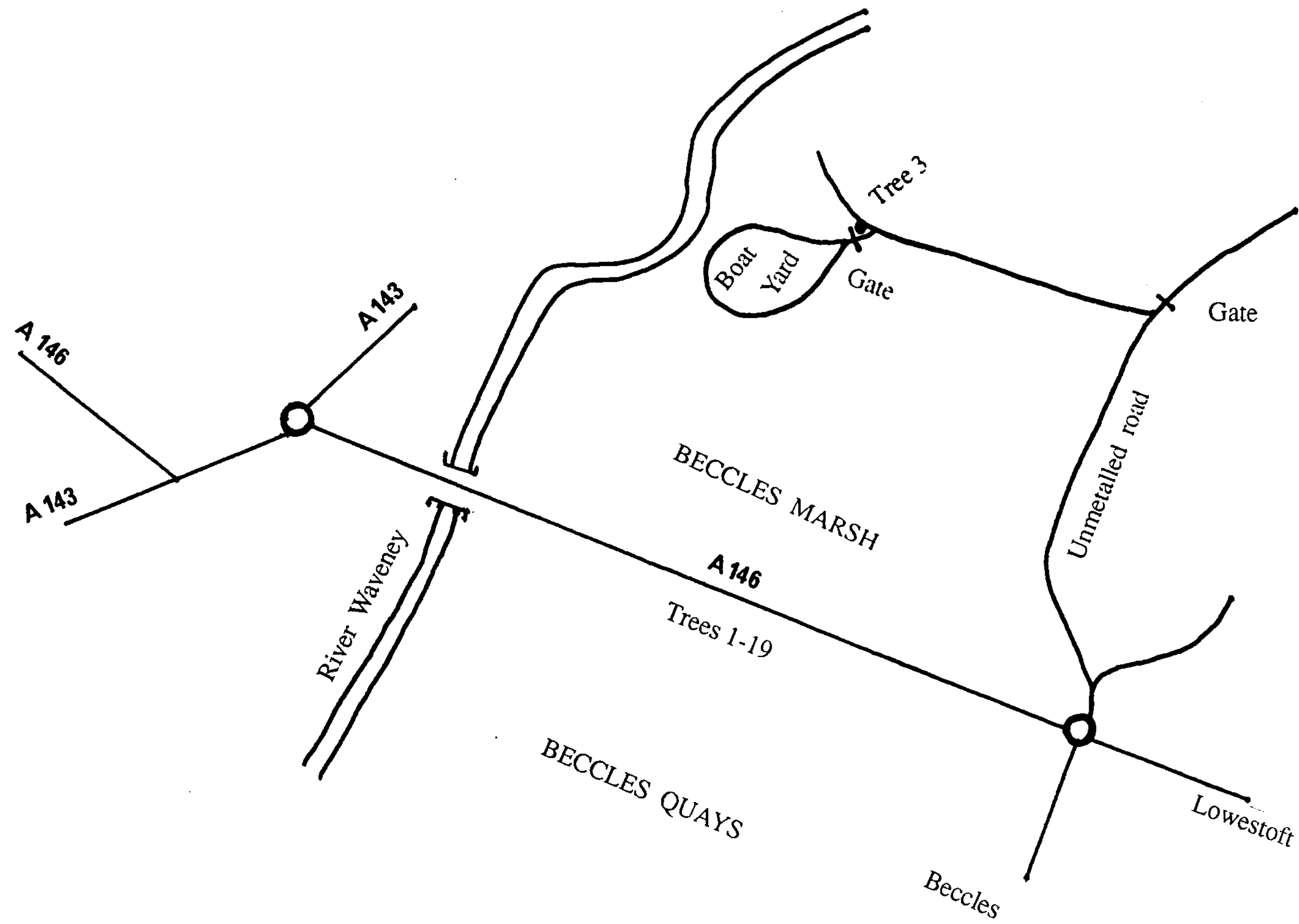


Figure 7. 3. 1 A diagram of the Beccles site.
(Unmetalled road was lied with willows, planted more than 20 years ago).

Table 7. 3. 4 DAS ELISA reactions of plant samples obtained from Beccles at differ ent times.

Tree no.	ELISA reactions 1989	1991	1992
1	+	N. T.	N. T.
8	+	–	N. T.
9	+	FELLED	
10	+	+	+ (see Table 7. 3. 3)
12	+	–	N. T.
13a	+	+	– (see Table 7. 3. 3)
13b	+	–	+ (see Table 7. 3. 3)
13c	+	–	N. T.
14	+	FELLED	
15	+	N. T.	N. T.
16	+	FELLED	
17	+	N. T.	N. T.
18	+	N. T.	N. T.
19	+	N. T.	N. T.

+ : positive reaction

– : negative reaction

N. T. : not tested

7. 3. 4 Discussion

The aim of the IMS is the selective trapping of low numbers of *E. salicis* from a mixture of organisms in the symptomless infected willow wood. The IMS technique showed that the recovery of *E. salicis* was dependend on the concentration of IMP in the test

suspension. Higher concentration of IMP increased the recovery of *E. salicis*. A low cell concentration gave lower recovery than high cell concentration, as was found previously for K88$^+$ *E. coli.* (Lund *et al.*, 1988), *L. monocytogenes* (Skjerve *et al.*, 1990) and *Salmonella* spp. (Vermunt *et al.*, 1992).

Skjerve *et al*. (1990) and Vermunt *et al.* (1992) reported that the prolonged incubation period gave higher recoveries of the target bacteria. They also found that a prolonged incubation time increased the non-specific binding. In the present study, the duration of incubation for IMS of *E. salicis* was not studied but overnight incubation of suspension at 4 °C probably increased the chance of trapping of the target bacterium and the non-specific binding was observed. The non-specific binding that may be due to adhesion of bacteria to the surface of the polystyrene beads was also reported for several microorganisms e.g. *Staphylococcus aureus* and *Aeromonas hydrophila* (Vermunt *et al.*, 1992).

IMS technique performed by culturing the reconstituted *E. salicis*, gave lower number of bacteria than added. This may be explained by the fact that bacteria attached to one or a few particles gave rise to only one colony on the plate. Furthermore, some particles and bacteria may have been lost in the washing procedure, thereby reducing the number of colonies recovered.

The ratio between non-suspect and suspect colonies was decreased after IMS treatment of 15 plant samples and higher recovery of *E. salicis* was obtained particularly from heavily diseased plant samples. The most significant result was that the isolation of low numbers of *E. salicis* from three test samples having no symptoms of the disease.

No *E. salicis* colony could be isolated from samples 13b (b) and 13b (c), although they gave a ELISA positive reaction. The reason for this is not known but, it might be that low numbers of the bacterium was washed away due to increased washing steps of samples after IMS treatment. Another explanations might be that the samples contained dead cells of the bacterium or soluble antigens of the bacterium were detected by the DAS ELISA tests.

No quantitative study IMS from plant samples was made in the present investigation but, because IMS technique used in the present study could isolate the target bacterium from not only ELISA positive symptomless infected sample, 10d, but also from the two ELISA negative samples, 10a and 13a (a), it is likely that the IMS technique is more sensitive than DAS ELISA for the detection and isolation of low numbers of *E. salicis* in symptomless infected wood. Therefore, the IMS technique promises to be a sensitive and specific approach for the detection and isolation of *E. salicis* in the symptomless plant material.

ELISA reactions of the multiple plant samples belonged to a single tree in Beccles

site was different for each sample indicating that the infected tissue is not homogeneous in the tree. Hence, the necessity of testing many samples belonged to a single tree is obvious.

One important point in the immunoisolation of *E. salicis* from the symptomless infected wood is that the use of hand driller to obtain plant samples since the electrical driller may kill the cells of the bacterium due to heat formation during the process.

In summary, I have developed an immunoisolation technique, based on polyclonal antibodies and magnetic monodisperse polymer particles, for *E. salicis* cells in suspensions of symptomless plant material. The main advantages of IMS are speed, sensitivity, specificity and no, or very little, sophisticated equipment is necessary. The success with IMS technique for isolation of *E. salicis* in mixed populations of plant samples indicate that this procedure may be used in the further studies of immunoisolation. Further work may improve the sensitivity of the technique.

CHAPTER VIII

GENERAL DISCUSSION

In the work described in this thesis, experiments were done to improve our understanding of the epidemiology of watermark disease in two ways. The first aimed to describe the spread of the bacterium by distinguishing isolates of *E. salicis* and by examining the geographical distribution of these strains. The second approach was to test for the presence of latent infection.

One of the greatest challenges has been to reliably identify the causal agent, *E. salicis*. No pathogenicity test was included in the study because these require trees 4-5 years old (Day, 1924 and Dowson, 1937), they are not reliable [the success of artificial infection is very small, ca 10 %, (Wong, 1974)] and the time for the symptoms to appear may be more than 1 year. To circumvent this, I have compared the cultures isolated during the course of this study with the authentic isolates of *E. salicis* using a variety of bacteriological tests. Many of the cultures used in this study were obtained from the NCPPB and from the Netherlands and their pathogenicity have been already proven. The remainder of the isolates were collected from diseased wood from Wiltshire and from different locations in East Anglia. The cultures could be easily isolated in early summer but, it became progressively more difficult to isolate bacteria with cultural characteristics corresponding to *E. salicis* from samples taken in the late summer, due to rapidly-growing mucoid colonies of a variety of 'saprophytic bacteria'. This suggests that the population of living *E. salicis* in watermark diseased wood rises during the early part of the season and then declines in late summer.

Identification of *E. salicis* isolates was confirmed by two important tests one of which was competitive ELISA, employing a polyclonal antiserum raised against *E. salicis* NCPPB 2535. Seventy three of 78 isolates believed to be *E. salicis* reacted positively with the antiserum with five exceptions. Two 'authentic' isolates of *E. salicis*, NCPPB 2530 and NCPPB 2531, were ELISA negative and three cultures, S7B, 90-97 and 90-98, isolated during the course of the study gave an intermediate reaction. These five cultures were found also to be untypable with the five bacteriophages, L, 1467, T3, C and M, three of which isolated in the present investigation. The *E. salicis* isolates NCPPB 2530, NCPPB 2531 and S7B were also found in an atypical phenotype with the API 20E test. The API 20E phenotype of the other isolates tested were remarkably uniform and mainly differed from each

other in their reaction to inositol and melibiose. Sometimes it was difficult to score these two reactions. However, the API 20E phenotype of NCPPB 2530, NCPPB 2531 and S7B differed from the 'norm' in other characters which were invariate for the remaining cultures. I have doubt about the identity of these 5 isolates, NCPPB 2530, NCPPB 2531, S7B, 90-97 and 90-98, as *E. salicis*.

Bacteriophage sensitivity tests have been extremely useful for the confirmation of identity of the cultures presumed to be *E. salicis* although insufficient lysotypes (α_1, α_2, α_3 and β) were discovered for the purpose of an epidemiological study. The most important discovery of the bacteriophage typing scheme was that the Dutch and English isolates of *E. salicis* could be reliably distinguished, a result confirmed by the galactose utilisation tests. It is not known whether the Dutch and English isolates also differ in their virulence.

Immunodiffusion tests carried out with anti-2535 antiserum revealed that *E. salicis* is a remarkably homogeneous species. Most of the cultures tested had an heat-stable 'O' antigen while only a few of them did not have. By contrast to these results, application of the method to other plant pathogenic *Erwinia* sp.has revealed extensive variation. For example, 18 serogroups were identified in *E. carotovora* (De Boer *et al.*, 1979).

However, multilocus enzyme electrophoresis typing has been extremely useful in the differentiation of isolates in the present study and a large number–23–of electrotypes of 78 isolates of *E. salicis* could be obtained, indicating strain variation of the bacterium. Although the electrotypes of *E. salicis* were inconsistent with the lysotypes identified by the bacteriophage sensitivity assay, the fact that 3 adjacent watermark diseased trees in Wiltshire were each infected with a different electrotypes of *E. salicis* has allowed the conclusion that the disease had not been transmitted from one tree to another in this plantation. Apparently, these trees had been infected from different sources carrying different electrotypes of the bacterium. Current work (Turner *et al.*, 1992), using double antibody sandwich ELISA, has shown that *E. salicis* was present in symptomless 1-4 year old sets and symptomless stools producing these sets. Therefore, it is likely that watermark can be transmitted through planting of infected sets.

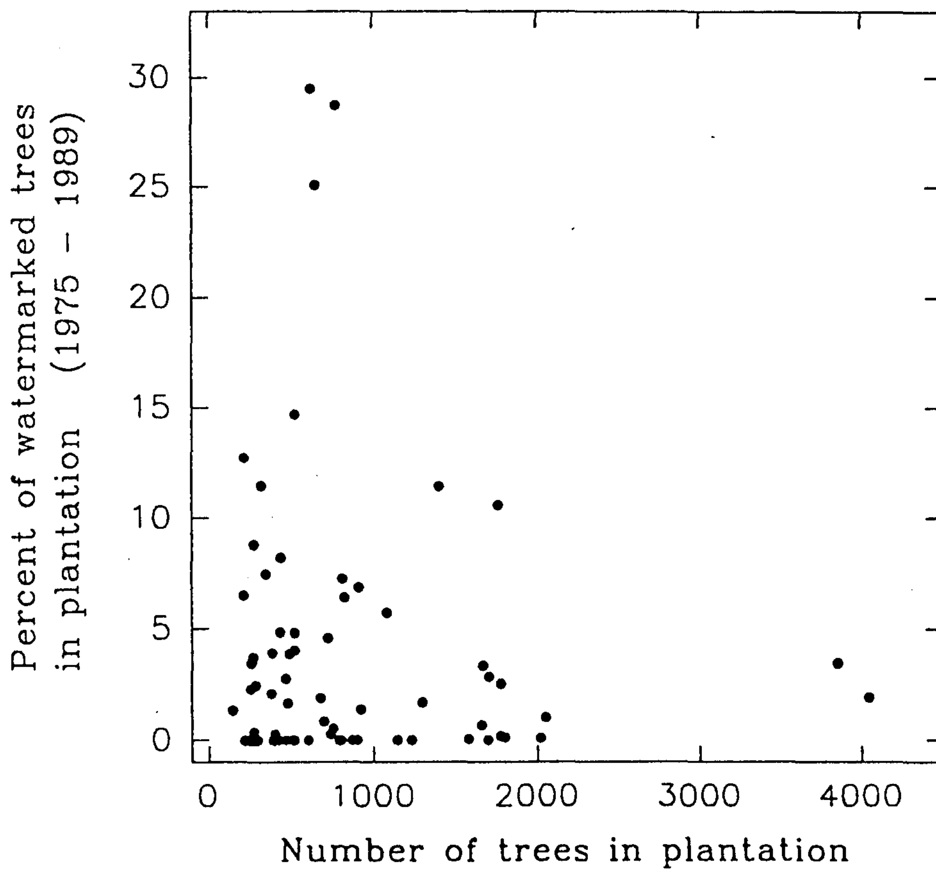
Symptomless or 'latent' infections have been defined as the presence of bacteria in plant tissues without effecting any gross change in form or function and may imply the low numbers of bacterium (Hayward, 1974). Symptomless infections have been reported for many plant pathogenic bacteria e.g., *Erwinia carotovora* var. *carotovora* and *E. carotovora* var. *atroseptica* (Perombelon, 1973).

It has been reported that watermark disease symptoms have not been seen in trees younger than 4-5 years old (Day, 1924 and Dowson 1937) and only occasionally in trees younger than 7 or 8 years old (Wong, 1974), indicating the long incubation period of watermark disease. Symptomless infection of watermark disease was first reported by Wong and Preece (1973) and Wong (1974) and they isolated *E. salicis* from the symptomless trees. Adegeye (1975) also found that *E. salicis* was subsequently present in all the artificially inoculated cuttings which were cultured but its presence did not lead to the development of visible symptoms in most of these cuttings. In a study, Gremmen and Kam (1975) isolated *E. salicis* from symptomless trees and they recommended the use of 1-2 year old cuttings as a propagation material. They thought that these young cuttings are not yet infected by *E. salicis*. Kam (1983) examined 373 one-year old *Salix alba* cuttings collected both from healthy and diseased stools and found that *E. salicis* was present in only one cutting. Although many road plantations in the Netherlands grown from one-year old cuttings are seriously attacked by the watermark disease, he concluded that the transmission of watermark disease by means of the propagating material was negligible. However, the present study goes further toward providing proof of transmission in propagating material than any of the previous studies.

The ultimate evidence of the potential infectivity of symptomless propagating material was that I have been able to isolate cultures of *E. salicis* from symptomless *Salix alba* trees with the immunoisolation method. Commercially available magnetic beads were used for the immunomagnetic separation (IMS) of the bacterium from symptomless infected wood samples. Although there is only one report of the application of the method in plant pathology (Jones and Van Vuurde, 1990) with *Xanthomonas campestris* pv. *pelargonii,* it shows great promise with *E. salicis* in the present investigation. Immunofluorescence colony staining (IFC) technique, another immunoisolation method, was found insufficiently sensitive for the purpose, due to rapidly-growing saprophytic colonies in the plates.

Based on the above conclusions, symptompless infected propagating material could be an agent over long-distance dispersal of *E. salicis*. It appears to have been responsible for the outbreak of watermark in Wiltshire. Also, the planting of infected sets is the probable cause of the isolated and apparently random occurrences of diseased trees in otherwise healthy plantations. The figure below, compiled from Essex County Council Records, shows that the majority of estates have less than 5 % infection over a 14 year period. In these estates, the infection tends to be isolated and random and could come from planting of infected material. However, severe watermark outbreaks are highly localised to individual

estates where losses can be as high as 25-30 %. This latter observation raises the question about the source of the disease in the heavily infected estates. The source of the disease is unlikely to result from the planting of infected sets alone, because records show that they did not originate from a single source. It is more likely that there may be a localised source of infection e.g. an old diseased willow tree, shedding bacteria into the locality. Alternatively, it is possible that these estates are unsuited for willow production and weakened trees are predisposed to infection.



Future work on the epidemiology of watermark should address the following two questions:

1. What is the proportion of infected symptomless trees in estates where infection levels are high and how does this compare with estates where watermark rarely occurs?
2. Are infected symptomless trees themselves infectious?

My study has only began to address the problem of the epidemiology of watermark disease of willows. Most of the work has been devoted to the development techniques e.g. electrotyping and immunoisolation together with more reliable methods for the identification of *E. salicis*. These methods can now be applied to large scale studies to examine the extent of tree-to-tree transmission in the short-distance spread of the disease. For example, using a combination of ELISA and immunoisolation techniques, the presence of *E. salicis* could be studied in soil, other vegetation, leaf surfaces, roots etc. in the heavily infected plantations and in plantations where the disease is absent. A selective medium which will prevent the growth of saprophytic bacteria in the isolation plates still remains as a necessity in the future studies.

In summary, the present investigation has provided two important contributions to the study of the epidemiology of watermark disease. Firstly, I have been able to provide evidence that infection may arise from the planting of infected sets and secondly, the occurrence of symptomless infection in trees has been proven.

APPENDIX

1. Phosphate buffered saline with Tween-20 (PBST) pH 7.4

NaCl	8 g
KH_2PO_4	0.2 g
$Na_2HPO_4 . 12 H_2O$	2.9 g
KCl	0.2 g
Tween-20	0.5 ml
Distilled water	1 l

2. Phosphate buffered saline (PBS) pH 7.4

NaCl	8 g
KH_2PO_4	0.2 g
$Na_2HPO_4 . 12 H_2O$	2.9 g
KCl	0.2 g
Distilled water	1 l

3. 0.01 M Phosphate (coating buffer for ELISA) pH 8.0

Na_2HPO_4	6.72 g
$NaH_2PO_4 . 2H_20$	0.41 g
Distilled water	1 l

4. 0.05 M Phosphate buffer pH 7.0

Na_2HPO_4	4.26 g
KH_2PO_4	2.72 g
Distilled water	1 l

5. 0.01 M Phosphate buffer pH 7.2

$Na_2HPO_4 . 12H_2O$	2.7 g
$NaH_2PO_4 . 2H_2O$	0.4 g
Distilled water	1 l

6. 0.1 M Carbonate-bicarbonate buffer pH 9.5

$NaHCO_3$	8.4 g/100 ml
Na_2CO_3	5.3 g/50ml

56.8 ml of $NaHCO_3$ is added to 14.4 ml of Na_2CO_3 and the mixture is made upto 1 l by addition of distilled water.

7. 17.5 mM Phosphate buffer pH 6.5

Na_2HPO_4	0.496 g/200 ml
$NaH_2PO_4 . 2H_2O$	1.092 g/400 ml

340 ml of $NaH_2PO_4 . 2H_2O$ is added to 160 ml of Na_2HPO_4 and the mixture is made upto 1 l by addition of distilled water.

8. 35 mM Phosphate buffer pH 6.5

Na_2HPO_4	0.496 g/200 ml
$NaH_2PO_4 . 2H_2O$	1.092 g/400 ml

340 ml of $NaH_2PO_4 . 2H_2O$ is added to 160 ml of Na_2HPO_4.

9. 0.5 M Phosphate buffer pH 8.0

Na_2HPO_4	7.098 g/100 ml
KH_2PO_4	0.680 g/10 ml

64.6 ml of Na_2HPO_4 is added to 6.22 ml of KH_2PO_4 and the mixture is made upto 1 l by

addition of distilled water.

10. 100 mM Phosphate-citrate buffer pH 7.4

Citric acid	19.21 g/l
Na_2HPO_4	28.98 g/l

6.14 ml of citrate buffer is mixed with 3.86 ml of phosphate buffer just before use and the mixture is made upto 20 ml by addition of distilled water.

11. 0.01 M Phosphate buffered saline (PBS) pH 7.2

NaCl	8.0 g
$Na_2HPO_4 \cdot 12H_2O$	2.7 g
$NaH_2PO_4 \cdot 2H_20$	0.4 g
Distilled water	1 l

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