Project title:	**Hedera: biology and epidemiology of *Xanthomonas* leaf spot**
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TABLE OF CONTENTS

PRACTICAL SECTION FOR GROWERS

Objectives and background

Bacterial leaf spot of ivy (*Hedera* spp.), caused by *Xanthomonas hortorum* pv. *hederae* was identified as the second most prevalent bacterial disease during a survey of hardy nursery stock (HNS 71) carried out on behalf of HDC during 1996/97. The pathogen was found to be present at seven of the eight nurseries visited and on a range of *Hedera* spp. growing both under protection and in the open and at all stages of production – rooted cuttings, liners and finals. Primary symptoms of the disease are irregular, dark, watersoaked spots/areas on the leaves. Severe symptoms can result in defoliation or even plant death. Plants become unsaleable due to a poor appearance resulting from the leaf spots or due to lack of foliage and dieback. The problems experienced by some growers have been so severe that they have ceased production of ivies.

Copper sprays have been used by some growers in an attempt to control the disease, but apparently with little success. In the absence of chemical control agents, control measures should be based on disease avoidance and/or disease resistance. However, there is almost no information on this disease available in the scientific literature, in particular, there is no information on pathogenic variation (i.e. races), host resistance, epidemiology (i.e. sources of infection, survival of the pathogen, mechanism of spread) and diagnosis. Hence it is impossible to target control measures. There is therefore a clear need to gain some understanding of the basic biology and mechanisms of infection for this disease, to determine the existence of any race/cultivar specific interactions, to develop diagnostic reagents and typing methods and thence determine the primary sources of the pathogen.

The commercial objective of this project is to use bacterial leaf spot of ivy as a model patho-system to provide basic information on the biology and epidemiology of bacterial diseases of HNS which can be used in the development of an effective integrated control strategy. It is anticipated that the principles developed could also be applied to a wider range of bacterial diseases of HNS. The choice of this particular crop/pathogen system is based not only of its high degree of importance to the industry but also because it represents an ideal model system for carrying out research in terms of availability of plant material, range and variation of species/varieties, ease and convenience of inoculation and symptoms production.

During the first year of the project:

- A collection of isolates of the pathogen was established
- Isolates were characterised by means of traditional bacteriological tests
- Molecular (DNA) fingerprinting techniques were developed and optimised
- A routine pathogenicity test was developed
- Sources of different ivy species/cultivars were identified.

The primary targets for the second year were:

- Complete DNA fingerprinting
- Establish a routine detection method
- Establish a representative collection of ivy cultivars
- Screen the collection for resistance to a range of pathogen isolates

- Determine if there is any pathogenic variability amongst different strains of the pathogen
- Begin epidemiological studies on commercial nurseries.

Summary of results

- DNA fingerprinting divided isolates of the pathogen into three major groups, indicating that there could be three distinct strains of *Xhh.*
- All of the recent isolates of *Xhh* from the previous HDC study (HNS 71) and this study from nurseries in the UK were assigned to two of the three groups.
- Two selective media, Brilliant Cresol Blue Cellobiose (BCBC) and Tween, were developed from information gathered during carbon utilisation and antibiotic sensitivity tests in the first year, and from selective media recipes for other *Xanthomonas* species.
- An antiserum specific to *Xhh* was produced for detection and confirmation of the identity of isolates from selective media plates.
- Twenty ivy cultivars were selected and propagated for use in resistance studies.
- Most cultivars were fully susceptible to all isolates of the pathogen.
- A few species/cultivars were less susceptible to all isolates
- There was no evidence of the existence of pathogen races, i.e. all isolates gave a similar response on all cultivars.

Action points for growers

- Bacterial leaf spot is caused by *Xanthomonas hortorum* pv. *hederae* (*Xhh*)
- Three different genetic strains of the pathogen *Xhh* have been identified using RAPD-PCR, but only two of these have been found recently on commercial nurseries in the UK
- Selective media have been developed and antiserum produced for use in on-nursery epidemiology studies
- *Hedera maderensis* subsp. *iberica* was the least susceptible to all isolates of the pathogen.
- There is no evidence for races within *Xhh*.

Practical and financial benefits

The project will provide basic information which will be of use in developing a (non-chemical) control/management strategy for this disease based on disease avoidance and will identify resistant varieties which could either be used directly or as a source of resistance in a future breeding programme. Growers will be able to use the information to develop better production practices and in their choice of varieties to be grown. This project will add considerably to the knowledge base of bacterial diseases of HNS and although targeted at a specific pathogen/crop combination, it is likely that some of the principles developed from this work would be applicable to other bacterial diseases of HNS.

SCIENCE SECTION

Introduction

A survey of bacterial diseases of hardy nursery stock (HNS 71) was carried out on behalf of HDC during 1996/97. The aim of HNS 71 was to identify the most important/widespread bacterial diseases which would form the targets for future work, and make most effective use of resources. A bacterial leaf spot on ivy (*Hedera* spp.) apparently caused by *Xanthomonas hortorum* pv. *hederae* was found to be widespread on seven of the eight nurseries visited, and on a range of different *Hedera* spp. growing both under protection and in the open. In addition to the primary symptom of irregular, dark, watersoaked spots/areas on the leaves, considerable defoliation or even plant death may occur. Plants become unsaleable due to a poor appearance resulting from the leaf spots or due to lack of foliage and dieback. Plant losses have also occurred after sale. The problems experienced by some growers have been so severe that they are considering ceasing production of ivies. Letters were also written to HDC indicating the seriousness of the problem and the urgent need for work. Symptoms have been seen at all stages of production – rooted cuttings, liners, finals.

This disease was first observed by Lindau in Germany (1894), although no attempt was made to isolate or confirm pathogenicity. Arnaud, (1920) reported a disease displaying similar symptoms in France and named the causal bacterium, *Bacterium hederae* n.sp. Pathogenicity was not confirmed until 1921 by Killian (1921) who also noted reactions on several media. Burkholder and Gutterman (1932) renamed the pathogen *Phytomonas hederae* believing the organism responsible was the same in each of the recorded cases.

In 1939, the genus *Xanthomonas* was proposed by Dowson (1939), leading to the renaming of the organism as *Xanthomonas hederae*. Young *et al.* (1978) revised the nomenclature and classification of all plant pathogenic bacteria and re-named the bacterium as *Xanthomonas campestris* pv *hederae*. Subsequent research by Vauterin *et al.* (1995) examined DNA homology and nutritional profiles of the species and pathovars within the genus *Xanthomonas* and lead to a complete revision of the genus with the ivy pathogen renamed as *Xanthomonas hortorum* pv *hederae.*

Copper sprays have been used by some growers in an attempt to control the disease, but apparently with little success. In the absence of chemical control agents control measures should be based on disease avoidance and/or disease resistance. However, there is almost no information on this disease available in the scientific literature, in particular, there is no information on pathogenic variation (i.e. races), host resistance, the potential for vascular spread (systemic infection), epidemiology (i.e. sources of infection, survival of the pathogen, mechanism of spread), diagnosis. Hence it is impossible to target control measures. There is therefore a clear need to gain some understanding of the basic biology and mechanism of infection for this disease, to determine the existence of any race/cultivar specific interactions, to develop diagnostic reagents and typing methods and thence to determine the primary sources of the pathogen.

The commercial objective of this project is to use bacterial leaf spot of ivy as a model patho-system to provide basic information on the biology and epidemiology of bacterial diseases of HNS which can be used in the development of an effective integrated control strategy. It is anticipated that the principles developed could also be applied to a wider range of bacterial diseases of HNS. The choice of this particular crop/pathogen system is based not

only of its high degree of importance to the industry but also because it represents an ideal model system for carrying out research in terms of availability of plant material, range and variation of species/varieties, ease and convenience of inoculation and symptoms production.

During the first year of the project a collection of isolates of the pathogen was established, isolates were characterised by means of traditional bacteriological tests, DNA fingerprinting techniques were developed and optimised, a routine pathogenicity test was developed, and sources of different ivy species/cultivars were identified.

This report covers work done in the second year of the project. The primary targets for the second year were:

- Complete DNA fingerprinting
- Establish a routine detection method
- Establish a representative collection of ivy cultivars
- Screen the collection for resistance to a range of pathogens.
- Determine if there is any pathogenic variability amongst different strains of the pathogen
- Begin epidemiological studies on commercial nurseries.

Materials and Methods

DNA fingerprinting

DNA isolation and quantification. Thirty-three isolates of *Xhh* were selected on the basis of geographic origin and host of origin (Table 1). Two isolates of *Xhh* from *Schefflera arboricola* were also included in this study and isolates of *Xanthomonas campestris* pv. *campestris*, *Xanthomonas hortorum* pv. *pelargonii* and *Pseudomonas syringae* pv. *syringae* were included as standards.

Isolates were grown on King's Medium B (King *et al*., 1954) (to obviate problems with excessive polysaccharide production on YDC) for 2 days at 30°C. Total Genomic DNA was extracted from 1 ml of a suspension containing approx. 10^8 cells/ml with the Qiamp DNeasy tissue kit (Qiagen, Crawley, West Sussex, UK) following the manufacturer's instructions. The concentration of DNA in extracts was estimated using the mini-gel method of Sambrook *et al*. (1989).

RAPD-PCR. The isolates were tested under the optimised conditions and with the set of ten RAPD primers which were identified in the first year. PCR reactions were performed in a total volume of 25 µl using the optimised master mix and cycling conditions (Tables 2 and 3). DNA amplification was carried out in a Hybaid Omnigene thermal cycler (Hybaid Limited, Ashford, Middlesex, UK). A negative control containing no DNA was included in all reactions. PCR products were separated on a 1.4% agarose gel made with 0.5x TBE (Tris borate-EDTA) buffer and molecular biology grade agarose (Amersco, Ohio, USA) stained with ethidium bromide. Size markers (1 Kb plus DNA ladder, Gibco) were loaded into the lanes at both ends of the gels. Gels were electrophoresed at 100 V for 1.5 hours in a 0.5 x TBE buffer. All reactions were performed at least twice. Gels were observed using a dual intensity transilluminator (Ultra violet products, Cambridge, UK) and photographed using a Polaroid 4+ camera (Polaroid, Cambridge, UK).

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Data analysis. The molecular weight of each band was estimated by visual comparison with the size markers included in each gel. Each isolate was then scored for the presence/absence of all possible bands. A similarity matrix was constructed for all pair-wise combinations of isolates using Genstat statistical analysis software and the Jacard similarity coefficient (Payne *et al*., 1993). Hierarchical cluster analysis was used to generate a dendrogram from the similarity matrix by the average linkage method in Genstat (Payne *et al*., 1993).

Selective media

Evaluation of selective media for other Xanthomonas species. Four selective media for *Xanthomonas* species were selected for evaluation on the basis of results of the carbon utilisation and antibiotic sensitivity tests: NSCAA for *Xanthomonas campestris* pv. *campestris* (Randhawa and Schaad, 1984), D-5 for *Xanthomonas hortorum* pv. *carotae* (Kuan *et al*., 1985), Tween for *Xanthomonas vesticatora* (McGuire, 1986) and CS for *Xanthomonas campestris* pv. *dieffenbachiae* (Norman and Alvarez, 1989). The growth of seventy-four bacterial isolates from ivy including fifty-four isolates of *Xhh* and twenty non-pathogenic (non-*Xhh*) isolates was examined on each of the media. A suspension of each isolate containing approximately 10^5 cfu/ml was prepared in SDW. Plates of each medium were inoculated with 5 µl of each bacterial suspension using a multi-point inoculator (Denley instruments, Surrey, UK). Plates were allowed to dry and incubated at 30°C for 3 d. Results were recorded as either growth (1) or no growth (0).

Optimisation of Tween medium. The effect of varying the concentration of the different antibiotics in the original medium on the growth of the 74 isolates from ivy was examined as above. Chlorothalonil was used as a replacement for cycloheximide as it was being discontinued. Plates were recorded for presence/absence of growth after 3 and 4 d in comparison to control plates containing no antibiotics.

Brilliant cresol blue cellobiose (BCBC). The recipe for BCBC medium was formulated from recipes of other selective media for *Xanthomonas* species and from data obtained from carbon utilisation and antibiotic sensitivity tests. The effects of varying concentrations of antibiotics including Brilliant cresol blue, cephalexin, chlorothalonil, 5-fluorouracil and tobramycin on the growth of the 74 isolates from ivy was examined as above.

The effect of pH on recovery of isolates on BCBC medium was examined by plating ten-fold dilution series of *Xhh* isolates 7183, 7733 and 7744 onto plates of YDC and BCBC adjusted to pH 6.8, 7.0 and 7.2. An aliquot (100 µl) of each dilution was pipetted onto the surface of the plates and spread with a bent glass rod. Plates were incubated at 30°C and the number of colonies growing on each medium was recorded after 3 and 4 d.

Recovery of Xhh on Tween and BCBC media. *Xhh* isolates 7183, 7733 and 7744 were suspended in sterile distilled water and then adjusted to an optical density of 0.4 at 620 nm in a Spectronic spectrophotometer (Milton Roy Company) by dilution. A tenfold dilution series was then prepared and 100 µl of each dilution pipetted onto the surface of plates of YDC, BCBC and Tween media and spread with a bent glass rod. Plates were incubated at 30°C and the number of colonies growing on each medium was recorded after 2 and 3 d (YDC) or 3 and 4 d (BCBC and Tween)

Serology

Agglutination with antisera raised to other Xanthomonas *species*. Eighty-two bacterial isolates, including fifty-four isolates of *Xhh*, twenty non-*Xhh* isolates from ivy and eight control isolates were tested for cross-reactivity with eight antisera from the HRI antiserum collection and which had previously been raised to different *Xanthomonas* species. The control isolates were those to which each antiserum had been prepared. A *Staphylococcus aureus* conjugate was made and tested following the protocol given by Lyons and Taylor (1990). The conjugated antisera (7 µl) were pipetted into wells on a multi-well slide. Isolates were tested by touching a single colony of the bacterium on a plate with a wooden toothpick and gently mixing the toothpick with the antisera. A positive reaction was characterised by granular clumping of the stained cell suspension within a few seconds of mixing. Isolates were scored as positive (+) or no reaction (-).

Antiserum for Xhh. An antiserum was raised against whole cells of *Xhh* isolate 7183 using standard methods. The antiserum was then tested in slide agglutination against 82 isolates as above and in indirect ELISA (see below).

ELISA. Antigens for ELISA were prepared from YDC plates of isolates which had been grown for 48 h at 30°C. The plates were flooded with PBSA (phosphate buffered saline containing 0.05% azide) and the cells harvested by gently scraping the plate surface with a glass rod to form a cell suspension. The suspensions were then adjusted to an optical density of 0.4 at 620 nm by dilution with PBST (phosphate buffered saline containing thermosal) using a Spectronic spectrophotometer (Milton Roy Company, Northampton, UK).

Wells of ELISA plates were coated with 100 µl of antigen overnight at 4°C, washed, blocked for 1 h at room temperature with 200 µl of blocking buffer, washed then incubated for 45 min at 37°C with 100 µl of diluted antiserum (1:10200), washed then incubated for 45 min with goat anti-rabbit conjugated antiserum, then washed again before finally adding substrate and incubating in the dark. Absorbance at 405 nm was then read in a plate reader after 30 and 60 min.

Resistance and pathogenic variability

Selection of isolates and cultivars. Ten isolates of *Xhh* were chosen (Table 11) to represent the three DNA fingerprinting groups, and the range of different geographic sites and cultivars of origin. Ivy species and cultivars were chosen on the basis of inclusion in the host resistance studies of Osbourne and Chase (1985) and to represent those most commonly grown in the UK.

Plants. Stock plants of the selected species/cultivars were obtained from Fibrex nurseries (Warwickshire). Inter-nodal cuttings were taken from these plants and rooted in peat blocks under a low polythene tunnel on a bench within a glasshouse. After 6 weeks the cuttings were gradually hardened off, potted on into 7 cm pots of Levington M2 compost and then grown on for a further 8 months before inoculation.

Inoculation. Four plants of each cultivar were tested with each isolate. Two methods of inoculation were used: pin inoculation and spray inoculation. The two youngest leaves on each of two plants were inoculated using an insect pin by scraping single colonies from agar plates with the pin and stabbing the underside of the leaves at five points. The two youngest leaves on each of the other two plants were spray inoculated with a visibly turbid bacterial

suspension of each isolate on the underside of the leaf with a DeVibliss atomiser connected to an air compressor. All plants were maintained in a mist chamber at 100% RH and a temperature of 18-20°C for 24 h before and after inoculation. Following inoculation and misting, plants were maintained in a glasshouse with minimum temperatures of 18/12°C (day/night) and venting at 20/14°C (day/night). Plants were observed regularly for the appearance of symptoms. Pin inoculated plants were recorded by measuring the size of each lesion and the number of inoculation points showing symptoms. The spray-inoculated plants were recorded by counting the number of lesions.

Results

DNA fingerprinting

All isolates tested produced bands with the ten primers tested. All reactions were performed at least twice and bands were only scored if observed in every reaction. The number of bands produced and the level of discrimination varied with the different primers tested. A total of 155 possible fragment lengths were produced by the ten primers. All the isolates of *Xhh* from ivy gave the same pattern with primers 2, 4, 10, 13, 18 and 19, but gave varying patterns with primers 3, 5, 11 and 12.

The dendrogram produced from the similarity matrix is shown in Fig.1. All isolates of *Xhh* from ivy formed one major cluster (average similarity >76 %) which could be divided into three sub-groups at the 80% similarity level. The majority of isolates (21 of 31 *Xhh* isolates from ivy) were contained within a single group (Group 1). Isolates obtained from the UK as part of this study and previous studies at HRI (HNS 71: Roberts, 1997) were all contained in Groups 1 and 2, whereas isolates from the UK, USA and Denmark obtained from the NCPPB were all contained in Group 3.

Selective media

Evaluation of selective media for other Xanthomonas species. The growth of *Xhh* and other non-pathogenic isolates from ivy on the four media originally developed for other *Xanthomonas* spp./pvs is shown in Table 5. Growth of *Xhh* and inhibition of non-pathogenic isolates was most successful on the Tween medium: all fifty-four isolates of *Xhh* tested grew and only seven of the twenty non-pathogenic isolates grew. This medium was therefore selected for further development, although, *Xhh* colonies were not surrounded by zones of crystals which the medium was originally designed to show.

Optimisation of Tween medium. Isolates of *Xhh* were able to grow on Tween medium with the inclusion of cephalexin to 50 mg/l, chlorothalonil to 20 mg/l, 5-fluorouracil to 6 mg/l, tobramycin to 0.6 mg/l and tween to 5 g/l (Table 6). The antibiotics were then combined at the optimum concentrations to assess whether inhibition occurred due to their interaction. All isolates of *Xhh* were able to grow on Tween medium containing cephalexin (15 mg/l), chlorthalonil (20 mg/l), 5-fluorouracil (6 mg/l) and tobramycin (0.6 mg/l)(see Appendix I). Only one non-*Xhh* isolate was able to grow on this medium. This isolate, 7735C produced yellow non-mucoid growth on the medium and is easily differentiated from colonies of *Xhh* that develop as small (2 mm diameter), circular, raised, cream colonies after 4 days at 30 °C.

Brilliant cresol blue cellobiose (BCBC) medium. All isolates of *Xhh* were able to grow in BCBC media containing cephalexin (30 mg/l), chlorthalonil (20 mg/l), 5-fluorouracil

(6 mg/l) and tobramycin (0.6 mg/l) (Table 7) (see Appendix II). Isolate 7735C was again the only non-*Xhh* isolate from ivy that was able to grow on the media. However, as the isolate produces yellow non-mucoid growth on BCBC, it can be differentiated from colonies of *Xhh* which develop as large (4 mm diameter), smooth, domed, mucoid, blue colonies after 4 days at 30°C. Recovery of each of the three isolates of *Xhh* was greatest on BCBC media at pH 6.8 (see Table 8).

Recovery of Xhh on Tween and BCBC media. Greater numbers of *Xhh* were apparently recovered from a suspension of a pure culture of *Xhh* on both the BCBC and Tween media than on the non-selective medium YDC (Table 9)

Serology

Reaction with antisera raised to other Xanthomonas species. Very few of the 54 *Xhh* isolates from ivy (8 or less) gave any reactions with antisera previously prepared to other *Xanthomonas* spp. (Table 10). A new antiserum to *Xhh* was therefore produced.

Antiserum to Xhh. All 54 *Xhh* isolates from ivy gave positive slide agglutination results with the new antiserum raised to *Xhh*. None of the non-*Xhh* isolates from ivy gave a positive result. Cross reactions were observed with isolates of *Xanthomonas campestris* pv. *campestris*, *Xanthomonas hortorum* pv. *carotae* and *Xanthomonas vesicatoria*. However, it is unlikely that these organisms will be isolated from ivy leaves and produce a false positive result.

ELISA. All isolates of *Xhh* tested gave positive results. All non-*Xhh* isolates from ivy gave negative results. All isolates gave a reading of over 1 after 30 minutes, except for isolate 7304 (0.8). Some cross-reactivity of the antiserum was observed with isolates of *Xanthomonas campestris* pv. *campestris*, *X. vesicatoria* and *Xanthomonas hortorum* pv. *pelargonii* which gave mean absorbance readings of 0.806, 0.734 and 0.71 respectively after 30 minutes, however it is unlikely that these pathogens would be present in association with ivies.

Resistance and pathogenic variability

Symptoms first appeared on plants that were inoculated using the pin inoculation method after 12 days. Symptoms on plants that were spray inoculated began to appear after 14 days. Both methods gave symptoms that were typical of those seen on naturally infected plants.

Pin inoculation data (number of points showing infection and size of lesion) and spray inoculation (number of lesions and average lesion diameter) were analysed by Generalised linear modelling methods or Analysis of variance, as appropriate, using Genstat statistical analysis software (Payne *et al.*, 1993).

There were significant differences between cultivars and between isolates, but no interaction, suggesting that each cultivar responded similarly to all isolates and that each isolate gave a similar reaction on all cultivars. None of the cultivars tested showed complete resistance to all the isolates, but they did vary in their susceptibility. The least susceptible cultivar to all the isolates tested using both the pin and spray inoculation was *Hedera maderensis* subsp. *iberica.* One isolate (7744) was significantly less pathogenic than other isolates of *Xhh*. However, this isolate was obtained from the NCPPB and originally isolated in 1961 and could therefore have lost pathogenicity during storage.

Conclusions

The results of the DNA fingerprinting indicate that all isolates of *Xhh* from ivy are genetically very similar. There does not appear to be any relationship between the sub-groups within *Xhh* from ivy and their geographical or cultivar of origin. Given this absence of variability and as ivy is propagated vegetatively it seems likely that the disease is primarily disseminated with the cuttings and spreads between cultivars during production.

Two selective media, Tween and Brilliant cresol blue (BCBC) were developed for the detection and isolation of *Xhh* during epidemiological studies. Both media show an apparently high level of recovery, but this will require further monitoring when used in epidemiology studies

An antiserum to *Xhh* was produced to provide a rapid method for the confirmation of suspected *Xhh* colonies on selective media plates and so far appears to be sufficiently specific in both agglutination and ELISA for routine use, although this will require further monitoring when used in the epidemiology studies.

Host resistance studies were done using twenty cultivars of ivy and ten isolates of *Xhh* representative of the different geographical/cultivars of origin and the three genotypic groups. None of the cultivars tested were resistant to *Xhh*, but some showed reduced susceptibility to all isolates. The least susceptible species/cultivar of ivy to all the isolates tested was *Hedera maderensis* subsp. *iberica*. Further testing using a wider range of isolates will be needed to confirm this.

The lack of a cultivar x isolate interaction in the inoculation studies indicates that *Xhh* is not differentiated into pathogenic races. This is further supported by the lack of genetic diversity indicated by the DNA-fingerprinting.

Future work

The main emphasis during the final year of the project will be on epidemiology studies on commercial nurseries. These studies will attempt to provide quantitative data on the spread and numbers of the pathogen present at different stages of production. Additional targets will be a thorough analysis of the data already collected and completion of the student's PhD thesis.

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Table 1. Sources of isolates used for DNA fingerprinting.

Isolate No.	Species	Cultivar	Country	County	Site
5691	*Hedera helix*	Green Ripple	UK	Suffolk	1
5863	*Hedera helix*	Green Ripple	UK	Yorkshire	2
5867	*Hedera hibernica*	-	UK	Yorkshire	2
5889	*Hedera helix*	Glacier	UK	Yorkshire	2
5993	*Hedera helix*	Goldheart	UK	Yorkshire	2
7053	*Hedera hibernica*	-	UK	Hampshire	3
7063	*Hedera helix*	Green Ripple	UK	Hampshire	3
7183	*Hedera helix*	Glacier	UK	Oxfordshire	4
7185	*Hedera helix*	Green Ripple	UK	Oxfordshire	4
7193	*Hedera helix*	Glacier	UK	Oxfordshire	4
7204	*Hedera hibernica*	-	UK	Hampshire	5
7219	*Hedera colchica*	Dentata variegata	UK	Hampshire	5
7714	*Hedera colchica*	Dentata	UK	Hampshire	6
7717	*Hedera algeriensis*	Ravensholst	UK	Hampshire	6
7718	*Hedera helix*	Angularis Aurea	UK	Hampshire	6
7720	*Hedera helix*	Cristata	UK	Hampshire	6
7722	*Hedera helix*	-	UK	Hampshire	6
7725	*Hedera maderiensis subsp. iberica*		UK	Hampshire	6
7726	*Hedera colchica*	Dendroides	UK	Hampshire	6
7730	*Hedera helix*	Green Ripple	UK	Worcestershire	7
7731	*Hedera helix*	Buttercup	UK	Oxfordshire	8
7732	*Hedera helix*	Goldheart	UK	Oxfordshire	8
7733	*Hedera colchica*	Dentata Variegata	UK	Herefordshire	9
7734	*Hedera algeriensis*	-	UK	Herefordshire	9
7735	*Hedera helix*	Green Ripple	UK	Herefordshire	9
7736	*Hedera helix*	Jesters Gold	UK	Herefordshire	9
7737	*Hedera helix*	Goldchild	UK	Herefordshire	9
7738	*Hedera helix*	Cristata	UK	Hampshire	10
7743 (NCPPB 2011)	*Hedera helix*	-	UK		-
7744 (NCPPB 939)	*Hedera helix*	-	USA		-
7746 (NCPPB 642)	*Hedera helix*	-	Denmark		-
7789 (NCPPB 3588)	*Schefflera arboricola*	-	USA		-
7790 (NCPPB 3589)	*Schefflera arboricola*	-	USA		
Xanthomonas hortorum pv. *pelargonii*					
5616	*Pelargonium sp.*	-	UK		-
Xanthomonas campestris pv. *campestris*					
5421 (NCPPB 528)	*Brassica oleracea*	-	UK		-
Pseudomonas syringae pv. *syringae*					
5411 (NCPPB 2815)	*Syringa vulgaris*	-	UK		-

Table 2. Optimised composition of RAPD-PCR reaction mix for a 25 µl reaction volume.

Component	Quantity (µl)	Stock concentration
RO water	15.98	-
PCR buffer (Gibco)	2.5	10x reaction
$MgCl_2$ (Gibco)	0.37	0.75 mM
DNTPs (Gibco)	0.025 of each	100 µM
Taq (Gibco)	0.3	1.5 Units
Genomic DNA	2	5 ng
Primer	3.75	0.6 µM

Table 3. Optimised thermal cycling conditions for RAPD-PCR

Temperature (°C)	Time (minutes)	Number of cycles	Step No.
-	-	-	1
94	30 sec	40	2
37	1	40	2
72	1	40	2
72	5	1	3

Table 4. PCR primer sequences used to examine genetic diversity amongst isolates of *Xanthomonas hortorum* pv. *hederae*

Primer	Sequence 5'-3'
OGP2	GGCACTGAGG
OPG3	GAGCCCTACA
OPG4	AGCGTGTGTG
OPG5	CTGAGACGGA
OPG10	AGGGCCGTCT
OPG11	TGCCCGTCGT
OPG12	CAGCTCACGA
OPG13	CTCTCCGCCA
OPG18	GGCTCATGTG
OPG19	GTCAGGGCAA

Table 5. The percentage of *Xanthomonas hortorum* pv. *hederae* and non-pathogenic isolates from ivy able to grow on four selective media.

	Selective medium			
Isolates	NSCAA	D-5	Tween	CS
Xhh	0	100	98	0
Non *Xhh*	55	90	35	40

Table 6. Effect of varying antibiotic concentrations on the growth in Tween medium of 54 *Xanthomonas hortorum* pv. *hederae (Xhh)* and 20 non-pathogenic isolates from ivy. Values in the table are the percentage of isolates growing.

Antibiotic (concentration)	*Xhh*	Non-*Xhh*
Cephalexin (30 mg/l)	100	20
Cephalexin (40 mg/l)	100	20
Cephalexin (50 mg/l)	100	20
Chlorthalonil (20 mg/l)	100	15
Chlorthalonil (40 mg/l)	98	5
Chlorthalonil (80 mg/l)	80	0
Chlorthalonil (120 mg/l)	33	0
Chlorthalonil (160 mg/l)	24	0
Chlorthalonil (200 mg/l)	24	0
5-fluorouracil (4.0 mg/l)	100	20
5-fluorouracil (5.0 mg/l)	100	10
5-fluorouracil (6.0 mg/l)	100	5
5-fluorouracil (10 mg/l)	74	5
Tobramycin (0.25 mg/l)	100	10
Tobramycin (0.3 mg/l)	100	5
Tobramycin (0.35 mg/l)	100	5
Tobramycin (0.4 mg/l)	100	5
Tobramycin (0.6 mg/l)	100	5
Tobramycin (1 mg/l)	44	5
Tobramycin (3 mg/l)	0	5
Tobramycin (5 mg/l)	0	5
Tobramycin (7 mg/l)	0	5
Tobramycin (10 mg/l)	0	0
Tween (1 g/l)	100	30
Tween (2 g/l)	100	30
Tween (3 g/l)	100	30
Tween (4 g/l)	100	30
Tween (5 g/l)	100	30
Cephalexin (20 mg/l), Chlorthalonil (20 mg/l), 5-fluorouracil (6 mg/l),Tobramycin (0.6 mg/l)	74	5
Cephalexin (15 mg/l), Chlorthalonil (20 mg/l), 5-fluorouracil (3 mg/l),Tobramycin (0.3 mg/l)	100	30
Cephalexin (15 mg/l), Chlorthalonil (20 mg/l), 5-fluorouracil (6 mg/l), Tobramycin (0.6 mg/l)	100	5

Table 7. Effect of varying antibiotic concentrations on the growth in BCBC medium of 54 *Xanthomonas hortorum* pv. *hederae (Xhh)* and 20 non-pathogenic isolates from ivy. Values in the table are the percentage of isolates growing.

Antibiotic (concentration)	*Xhh*	Non-*Xhh*
Cephalexin (30 mg/l)	100	5
Cephalexin (40 mg/l)	100	5
Chlorthalonil (20 mg/l)	100	5
Chlorthalonil (40 mg/l)	98	5
5-fluorouracil (6 mg/l)	100	5
5-fluorouracil (10mg/l)	74	5
Tobramycin (0.6 mg/l)	100	5
Tobramycin (0.8 mg/l)	74	5
Cephalexin (30 mg/l), Chlorthalonil (20 mg/l), 5-fluorouracil (6 mg/l), Tobramycin (0.6 mg/l)	100	5

Table 8. Effect of pH on recovery of *Xanthomonas hortorum* pv. *hederae* on BCBC medium.

Medium	Number recovered (cfu/ml)		
	7183	7733C	7744
YDC	1×10^8	2×10^8	1×10^8
BCBC pH 6.8	7×10^8	6×10^8	4×10^8
BCBC pH 7.0	4×10^8	5×10^8	2×10^8
BCBC pH 7.2	3×10^8	3×10^8	1×10^8

Table 9. Recovery of *Xanthomonas hortorum* pv. *hederae* on Tween and BCBC media.

Medium	Number recovered (cfu/ml)		
	7183	7733C	7744
YDC	2×10^9	1×10^8	1×10^8
Tween	1.1×10^{10}	1.7×10^9	4×10^8
BCBC	6.0×10^9	3×10^9	1×10^9

Table 10. The percentage of 54 *Xanthomonas hortorum* pv. *hederae* isolates and 20 non-pathogenic isolates from ivy agglutinating with *Staphylococcus aureus* conjugated antisera raised to different *Xanthomonas* spp.

	% reacting with antiserum raised to							
	Xcc	*Xhc*	*Xf*	*Xoo*	*Xv*	*Xap*	*Xam*	*Xhp*
Xhh	3.6	1.	14.3	5.4	4	5.4	1.8	1.8
Non *Xhh*	0	0	0	0	0	0	0	0

Key: *Xcc* - *Xanthomonas campestris* pv. *campestris*, *Xhc* - *Xanthomonas hortorum* pv. *carotae*, *Xf* - *Xanthomonas fragariae*, *Xoo – Xanthomonas oryzae* pv. *oryzae*, *Xv – Xanthomonas vesicatoria*, *Xap – Xanthomonas arboricola* pv. *pruni*, *Xam – Xanthomonas axonopodis* pv. *malvacearum*, *Xhp – Xanthomonas hortorum* pv. *pelargonii*, *Xhh – Xanthomonas hortorum* pv. *hederae*

Table 11. Isolates used for host resistance and pathogen variability study

Isolate No.	Species	Cultivar	County	Site	Genetic group
5863	*Hedera helix*	Green Ripple	Yorkshire	2	1
5993	*Hedera helix*	Goldheart	Yorkshire	2	1
7053	*Hedera hibernica*	-	Hampshire	3	2
7219	*Hedera colchica*	Dentata variegata	Hampshire	5	1
7714	*Hedera colchica*	Dentata	Hampshire	6	1
7731	*Hedera helix*	Buttercup	Oxfordshire	8	1
7734	*Hedera algeriensis*	-	Herefordshire	9	2
7738	*Hedera helix*	Cristata	Hampshire	10	1
7744 (NCPPB 939)	*Hedera helix*	-	-	-	3
7746 (NCPPB 642)	*Hedera helix*	-	-	-	3

Table 12. Susceptibility of 20 ivy species/cultivars to ten isolates of *Xanthomonas hortorum* pv. *hederae.* Values in the table are the means of twenty plants.

Species/cultivar	Pin % infected	Spray No. of lesions	± s.e.
Hedera canariensis var. *algeriensis*			
Ravensholst	93	7.3	1.3
un-named	98	26.4	2.5
Hedera colchica			
Dentata	94	12.3	1.7
Dentata variegata	97	21.6	2.3
Hedera helix			
Angularis aurea	100	21.4	2.3
Brokamp	97	14.5	1.8
Buttercup	100	33.6	2.8
California	100	32.0	2.8
Chrysophylla	91	14.0	1.8
Eva	100	32.4	2.8
Goldchild	100	34.2	2.8
Goldheart	100	35.5	2.9
Glacier	100	30.1	2.7
Green Ripple	99	25.1	2.4
Ivalace	100	32.9	2.8
Manda's Crested	100	40.4	3.1
Telecurl	100	30.4	2.7
un-named	100	27.9	2.6
Hedera hibernica			
Deltoidea	94	12.0	1.7
Hedera maderensis subsp. *iberica*	86	2.1	0.7

Table 13. Pathogenicity of ten isolates of *Xanthomonas hortorum* pv. *hederae*. Values in the table represent means of 2 plants x 20 cultivars

	Pin	Spray	
Isolate	% infected	No. of lesions	± s.e.
5863	98.6	21.2	1.6
5993	94.2	21.0	1.6
7053	97.2	13.3	1.3
7219	96.7	22.8	1.6
7714	96.5	37.2	2.1
7731	100.0	31.6	1.9
7734	100.0	39.5	2.2
7738	99.7	25.1	1.7
7744	92.0	10.2	1.1
7746	99.0	21.0	1.6

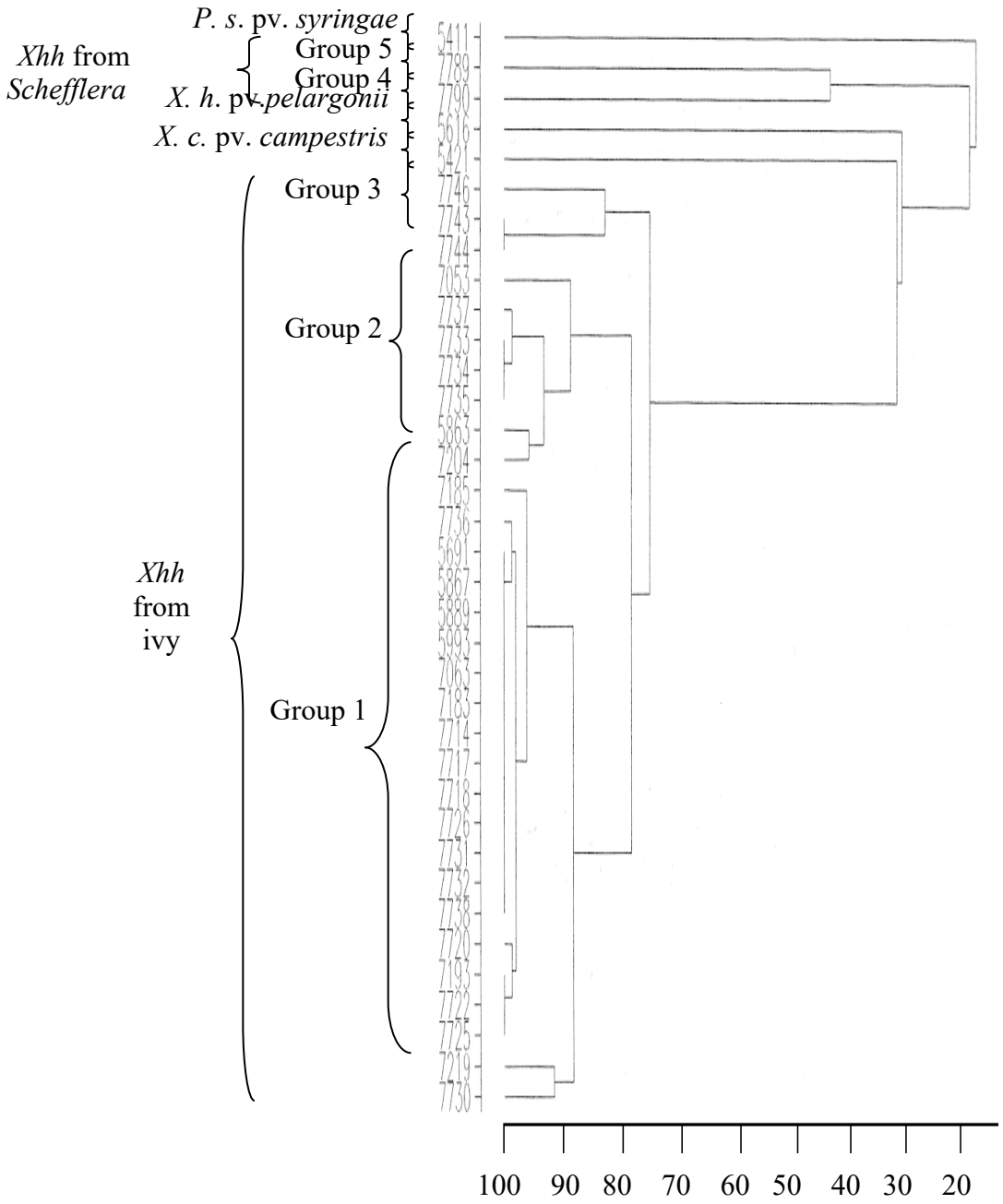

Fig.1. Dendrogram showing similarity between isolates of *X. h.* pv. *hederae* (*Xhh*) from different geographical/species of origin. Similarity values were obtained using UPGMA analysis of bands produced from RAPD-PCR using ten 10-mer oligonucleotide primers.

APPENDIX I

Tween 80 medium

Compound	g/l	g/500 ml
Peptone (Bacto Difco)	10	5
Potassium Bromide	10	5
Calcium Chloride	0.25	0.125
Agar	15	7.5
Tween 80 [1]	5	2.5
Cephalexin [2] (7.5mg/ml H_2O)	30 mg (4 ml)	15 mg (2 ml)
Chlorothalonil [3] (20mg/ml EtOH)	20 mg (1 ml)	10 mg (0.5 ml)
5-fluorouracil [4] (6 mg/ml H_20)	6 mg (1 ml)	3 mg (0.5 ml)
Tobramycin [5] (0.6 mg/ml H_2O)	0.6 mg (1 ml)	0.3 mg (0.5 ml)

[1,2,3,4,5] Added after autoclaving

Preparation

1) Weigh out ingredients except antibiotics, tween and agar into a suitable container
2) Add 1000 ml (or 500 ml) of distilled water.
3) Adjust media to pH 6.8 with 1M NaOH.
4) Autoclave at 121°C, 115 psi for 15 minutes.
5) Autoclave tween 80 separately and add to hot media.
6) Prepare antibiotic solutions and filter sterilise as appropriate.
7) Allow medium to cool to approx. 50°C and add antibiotic solutions.
8) Mix gently to avoid air bubbles and pour plates (22 ml per 9.0 cm plate).

Antibiotics

[2] Dissolve 150 mg cephalexin in 20 ml of distilled water, filter sterilise. Add 4 ml/l (or 2 ml/500 ml).
[3] Dissolve 200 mg chlorothalonil in 10 ml 70% ethanol. Add 1 ml/l (or 0.5 ml/500 ml).
[4] Dissolve 60 mg 5-fluorouracil in 10 ml 70% ethanol. Add 1 ml/l (or 0.5 ml/500 ml).
[5] Dissolve 6 mg tobramycin in 10 ml of distilled water, filter sterilise. Add 1 ml/l (or 0.5 ml/500 ml).

Storage

Stored prepared plates inverted in polythene bags in fridge or coldroom.
Use within 2 weeks of preparation to ensure activity of antibiotics.

APPENDIX II

Brilliant cresol blue cellobiose medium

Compound	g/l	g/500 ml
Cellobiose [1]	10	5
KH_2PO_4	0.8	0.4
K_2HPO_4	0.8	0.4
$MgSO_4$	0.1	0.05
Yeast extract	0.6	0.3
Difco Bacto Agar	15	7.5
Brilliant cresol blue [2] (5 mg/ml H_2O)	5 mg (1 ml)	2.5 mg (0.5 ml)
Cephalexin [3] (7.5 mg/ml H_2O)	30 mg (4 ml)	15 mg (2 ml)
Chlorothalonil [4] (20 mg/ml EtOH)	20 mg (1 ml)	10 mg (0.5 ml)
5-fluorouracil [5] (6 mg/ml H_20)	6 mg (1 ml)	3 mg (0.5 ml)
Tobramycin [6] (0. 6 mg/ml H_2O)	0.6 mg (1 ml)	0.3 mg (0.5 ml)

[1, 3, 4, 5 , 6] Added after autoclaving

Preparation

1) Weigh out ingredients except antiobitics, cellobiose and agar into a suitable container
2) Add 960 ml (or 480 ml) of distilled water
3) Adjust media to pH 6.8 with 1M NaOH (use dye as an indicator the media should be bright blue in colour) and add agar.
4) Autoclave at 121°C, 115 psi for 15 minutes.
5) Prepare 5-fluorouracil and cellobiose and filter sterilise as appropriate
6) Allow media to cool to approx. 50°C and add antibiotic solutions.
7) Mix gently to avoid air bubbles and pour plates (22 ml per 9.0 cm plates).
8) Leave plates to dry in the flow bench or similar before use.

Antibiotics

[1] Dissolve 10 g (or 5 g/500 ml) cellobiose in 40 ml (or 20 ml/ 500 ml) of distilled water. Filter sterilise and add to media when cool.
[2] Dissolve 50 mg Brilliant cresol blue in 10 ml of distilled water and filter sterilise. Add 1 ml/l (or 0.5 ml/500 ml)
[3] Dissolve 150 mg cephalexin in 20 ml of distilled water, filter sterilise. Add 4 ml/l (or 2 ml/500 ml).
[4] Dissolve 200 mg chlorothalonil in 10 ml 70% ethanol. Add 1 ml/l (or 0.5 ml/500 ml).
[5] Dissolve 60 mg 5-fluorouracil in 10 ml 70% ethanol. Add 1 ml/l (or 0.5 ml/500 ml).
[6] Dissolve 6 mg tobramycin in 10 ml of distilled water, filter sterilise. Add 1 ml/l (or 0.5 ml/500 ml).

Storage

Store prepared plates inverted in polythene bags in a fridge or coldroom.
Use within two weeks of preparation to ensure activity of antibiotics.