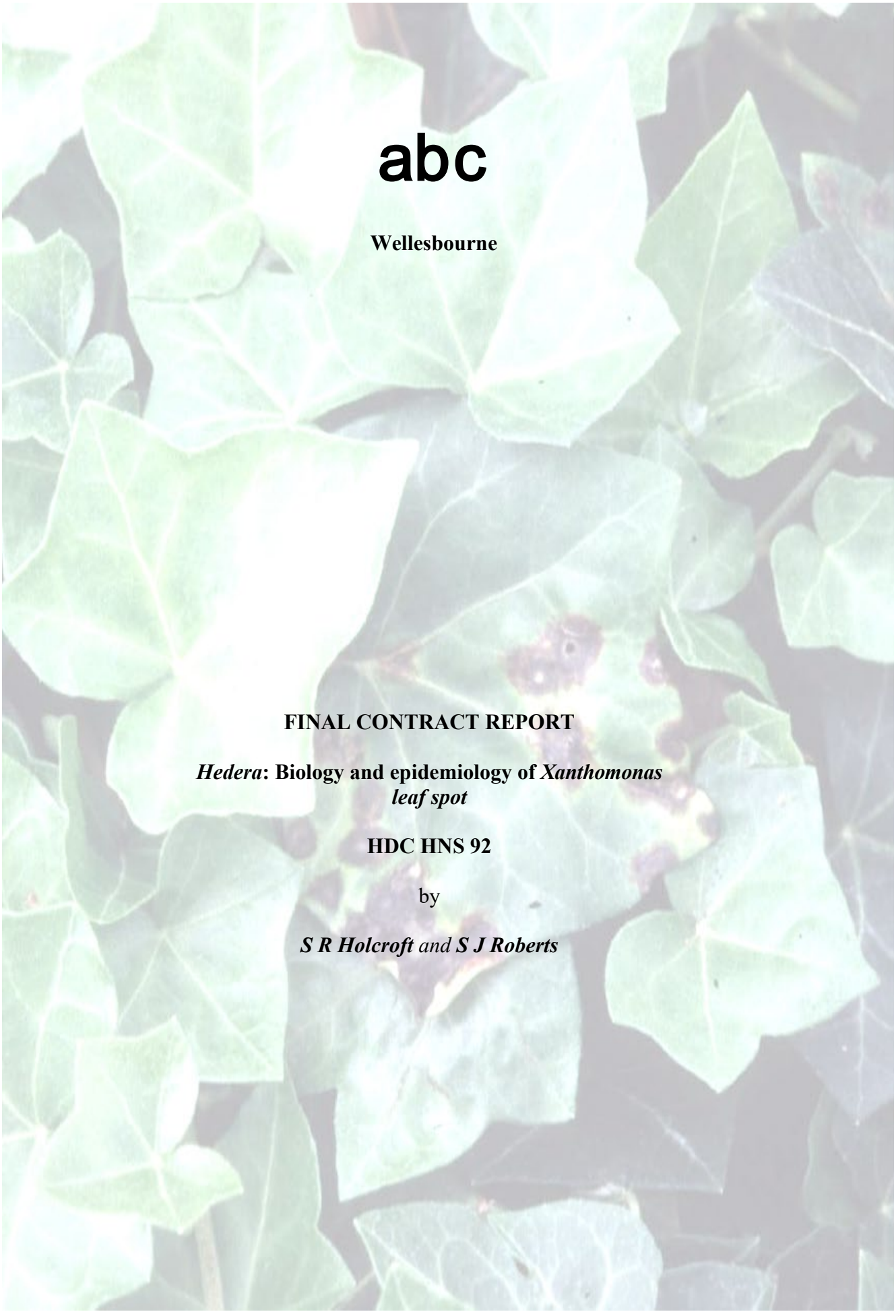

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Wellesbourne

FINAL CONTRACT REPORT

Hedera: Biology and epidemiology of *Xanthomonas leaf spot*

HDC HNS 92

by

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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: Roberts, 1997) 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, water-soaked 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 was almost no information on this disease available in the scientific literature, in particular, there was 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 was impossible to target control measures. There was 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 was 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 was 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 was 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 symptom production.

The main objectives of this project were to:

- Establish a collection of isolates of the pathogen
- Characterise isolates using physiological tests and DNA fingerprinting to assess phenotypic and genetic variability.

- Develop a routine method for pathogenicity testing
- Determine if there is any pathogenic variability amongst different strains of the pathogen
- Screen a collection of ivy cultivars for resistance to a range of isolates of the pathogen.
- Develop a routine detection method for use in epidemiological studies.
- Conduct epidemiological studies to identify the primary source(s) of the pathogen and conditions for spread and infection

Summary of results

- All bacterial isolates from diseased ivy, identified as *Xanthomonas,* were pathogenic on *Hedera helix* cv. Green Ripple and can therefore be considered as *Xanthomonas hortorum* pv. *hederae* (*Xhh*); none of the non-*Xanthomonas* isolates were pathogenic.
- Contrary to reports from the USA, isolates of *Xhh* did not infect *Brassaia* (syn. *Schefflera*) *actinophylla*.
- Carbon utilisation tests and DNA-fingerprinting indicated that all isolates of *Xhh* are very similar.
- Thirty-five ivy cultivars were tested for resistance to ten isolates of *Xhh*. Most were fully susceptible and none could be considered resistant, although some (*H. rhombea*, *H. rhombea* cv Variegata and *H. helix* cv. Tanja) were 'less susceptible'.
- There was no evidence for the existence of pathogen races, i.e. all isolates gave a similar responses on all cultivars.
- Two new semi-selective media, Brilliant Cresol Blue Cellobiose (BCBC) and modified Tween, were developed for use in epidemiology studies using data from carbon utilisation and antibiotic sensitivity tests.
- An antiserum to *Xhh* was produced for detection and rapid confirmation of the identity of isolates from selective media plates.
- Nursery-studies showed that *Xhh* could be present on stock plants with no visible symptoms of disease.
- The numbers of *Xhh* recovered from the cuttings decreased during rooting.
- Cross-infection between trays of cuttings was only detected after they had rooted, polythene covers had been removed and overhead watering started.
- Results from one nursery indicated that plants with visible symptoms provide a more important source of inoculum than plants without.

Action points for growers

- Bacterial leaf spot of ivy is caused by the pathogen *Xanthomonas hortorum* pv. *hederae* (*Xhh*) .
- Infection from 'wild' plants is unlikely; control therefore needs to be focused on the nursery.
- The disease is most likely disseminated with cuttings and plant material.
- Nursery studies indicated that the primary source of infection was the stock plants.
- Control measures need to be targeted at producing/cleaning-up/maintaining disease-free stock plants, and minimising the likelihood of cross-infection between batches of cuttings/plants.
- Keep stock plants separate from production.
- Ideally stock plants should be grown under protection with drip or capillary irrigation.
- Consider the value of an indexing scheme for stock plants.
- Do not take cuttings from plants with visible symptoms.
- Disinfect secateurs/knives between different stock plants (see HNS 91 for effectiveness of disinfectants)
- Do not mix cuttings/plants from different sources.
- Separate batches of ivy cuttings/plants from different sources by other genera.
- Inspect cuttings regularly: remove and destroy any showing symptoms.
- Remove and destroy plants showing symptoms. This is particularly important with batches which remain unsold due to presence of disease – GET RID OF THEM QUICKLY !
- Avoid or minimise overhead watering: use drip or capillary irrigation systems.
- Work is underway in HNS 91 to examine the effectiveness of copper sprays.
- Further work needs to be done on the rate of spread to set separation distances and tolerance standards for the disease indexing of stock plants.

Practical benefits

The project was developed to provide basic information which will be of use in developing a (non-chemical) control/management strategy for this disease based on disease avoidance and

resistance. The project has emphasised the need for disease-free stock plants combined with measures to reduce the likelihood of subsequent cross-contamination between batches of plants. Although no resistant cultivars have been identified, the project has identified some cultivars which are less susceptible; these may be worthy of further studies to determine the usefulness of this reduced susceptibility in practice or their potential as parents in a breeding programme. It is likely that the approach of a clean start using only certified healthy stock plants and subsequent minimisation of overhead watering and separation of batches of plants would make a significant contribution to the control of bacterial diseases of other species of vegetatively propagated HNS.

SCIENCE SECTION

Introduction

A survey of bacterial diseases of hardy nursery stock (HNS 71: Roberts, 1997) 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, water-soaked 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 have ceased 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 recorded in Germany by Lindau (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*. Pathogenicity was not confirmed until 1921 by Killian (1921). Burkholder and Gutterman (1932) re-named the pathogen *Phytomonas hederae.* In 1939, the genus *Xanthomonas* was proposed by Dowson (1939) and the ivy pathogen was re-named *Xanthomonas hederae*. Young *et al.* (1978) revised the nomenclature and classification of all plant pathogenic bacteria and re-named it *Xanthomonas campestris* pv. *hederae*. Vauterin *et al.* (1995) examined DNA homology and nutritional profiles of the species and pathovars within the genus *Xanthomonas* leading to a complete revision of the genus and re-named it *Xanthomonas hortorum* pv *hederae* (*Xhh*); this latest name will be used hereafter. The disease has also been observed in the United States (White and McCulloch 1934) and New Zealand (Dye 1967). Although isolates from Denmark and the UK have been deposited in the National Collection of Plant Pathogenic Bacteria (NCPPB), and Bradbury (1986) reports its occurrence in both these countries there appear to have been no formal reports of the disease in the UK prior to Roberts (1997).

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 was almost no information on this disease available in the scientific literature, in particular, there was 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 was impossible to target control measures. There was 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 was 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 on 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.

This final report summarises all of the work done during project. The main objectives of the project were to:

- Establish a collection of isolates of the pathogen
- Characterise isolates using physiological tests and DNA fingerprinting to assess phenotypic and genetic variability
- Develop a routine method for pathogenicity testing
- Determine if there is any pathogenic variability amongst different strains of the pathogen
- Screen a collection of ivy cultivars for resistance to a range of isolates of the pathogen.
- Develop a routine detection method for use in epidemiological studies.
- Conduct epidemiological studies to identify the primary source(s) of the pathogen and conditions for spread and infection

Isolation and characterisation of the pathogen

Introduction

A small number of isolates of *Xhh* have been included in larger studies looking at the taxonomy of *Xanthomonas*: SDS-PAGE (Vauterin *et al.* 1991), fatty acid profiling (Yang *et al.* 1993), carbon source oxidation (Vauterin *et al.* 1995b) and DNA-DNA homology (Vauterin *et al.* 1995b). However, there had been no systematic studies of a large number of isolates of the pathogen, and therefore the existence of and extent of phenotypic, genotypic and pathogenic variability was unknown. In this section we report work which was done to confirm the identity of the bacteria isolated from diseased ivy samples, and to establish the degree of phenotypic and genotypic variability amongst pathogenic strains.

Xhh has previously been reported to cause infection on other araliaceous host (*Dizgotheca*, *Schefflera*, *Brassaia*, *Polyscias*, *Fatsia* and *Fatshedera*) (Chase 1984) Following the start of this project Norman *et al*. (1999) reported that isolates from three genera (*H. helix*, *B. actinophylla* [syn. *Schefflera actinophylla*], *Polyscias fruticosa*) could cross-infect the others, but strains isolated from a particular host were more virulent on that host. Therefore in addition to testing the pathogenicity of isolates to *Hedera* we also examined the pathogenicity of isolates to *B. actinophylla.*

Materials and methods

Source of bacteria. A request for information and samples was published in HDC news (see Appendix I). Leaf samples were also collected during visits to nurseries

The origins of isolates used in this study are shown in Table 1. Isolations were attempted from leaves with typical symptoms by aseptically excising small (4 mm^2) pieces of tissue from the edges of lesions and comminuting on a sterile microscope slide in a drop of sterile water. Several isolations were attempted from each leaf sample. Slides were examined under a light microscope for bacterial streaming before being streaked onto Yeast Dextrose Chalk (YDC) agar plates (Lelliot and Stead 1987). Plates were incubated at 30°C for up to three days. Yellow fluidal/mucoid colonies appearing after two to three days incubation were presumed to be *Xhh* and single colonies were sub-cultured to further plates of YDC, to ensure purity, before further identification and characterisation tests. Isolates obtained as part of the previous HDC-funded project (HNS 71) were also included in the study and additional isolates were obtained from the NCPPB (National Collection of Plant Pathogenic Bacteria, CSL, York, UK). Long-term storage of isolates was by freezing in nutrient broth (Difco) containing 15% (w/v) glycerol on glass beads at -76°C (Feltham *et al.* 1978).

Preliminary Characterisation. Isolates were subjected to a number of bacteriological tests suggested by Lelliott & Stead (1987) as useful to establish the identity of *Xanthomonas* spp.. These tests were: Gram stain, cell morphology, Kovac's oxidase reaction, catalase reaction, inhibition by 0.1% and 0.02% TTC (tetrazolium chloride), production of yellow mucoid

growth on YDC agar, and oxidation/fermentation reaction. Additional tests for aesculin, gelatin and starch hydrolysis were also performed. All tests were performed according to the methods in Lelliott & Stead (1987).

Pathogenicity to ivy. Six-week-old rooted-cuttings of *H. helix* cv. Green Ripple growing in peat blocks were obtained from Fibrex Nurseries (Honeybourne, Warwickshire, UK) and maintained in a glasshouse with minimum temperatures of 16/13°C (day/night) and venting at 18/15°C (day/night). Plants were potted on after 7 weeks into 7 cm pots containing Levington M2 compost and grown on for a further month to provide new growth for inoculation.

A preliminary study was carried out with four isolates (7716, 7721B, 7723 and 7744) to determine the best inoculation method for routine testing. Isolates were grown on YDC agar plates for 48 h at 30°C and turbid suspensions prepared in sterile distilled water. Plants were inoculated by: (a) stabbing leaves with an insect pin charged with bacterial growth; (b) making small cuts in the edges of leaves with scissors dipped in bacterial suspension; (c) wounding the edges of leaves with rats-tooth tweezers wrapped in absorbent cotton wool and dipped in bacterial suspension; (d) spraying the underside of leaves with bacterial suspension using a DeVibliss atomiser connected to a compressor.

For each method the two youngest leaves on each of four plants were inoculated. Half of the plants were maintained in a mist chamber at 100% RH and a temperature of 18-20°C for 24 h before and after inoculation. Control plants were inoculated in a similar way with sterile distilled water. Following inoculation (and misting) plants were maintained in a glasshouse with minimum temperatures of 16/13°C (day/night) and venting at 18/15°C (day/night) . Plants were observed at regular intervals for the appearance of symptoms.

Subsequently all isolates were inoculated into rooted cuttings of *H. helix* cv. Green Ripple using the insect pin method (a) with pre- and post-inoculation misting. The two youngest leaves on each of two plants were inoculated with each isolate. Control plants were inoculated with a sterile insect pin. Plants were observed at regular intervals for the appearance of symptoms. Inoculations were repeated twice.

Pathogenicity to Brassaia. Plants of *B. actinophylla* growing in 15 cm pots were obtained from Sainsbury Homebase, Leamington Spa, UK. Four isolates from ivy (5691B, 7183, 7733B, 7744) and the two isolates from *Schefflera arboricola* (7789 and 7790) obtained from NCPPB were inoculated into *B. actinophylla* by stabbing the two youngest leaves on each plant with an insect pin, as for ivy. The plants were maintained in the mist chamber for 24 h before and after inoculation and then returned to the glasshouse as for ivy. Control leaves were inoculated with a sterile insect pin. Plants were observed at regular intervals for the appearance of symptoms. Inoculations were repeated twice.

Methionine requirement. Before carbon-utilisation tests could be performed it was first necessary to determine the need for growth factors, which are known to be required by some

strains of *Xanthomonas* (Starr 1945). Factorial combinations of glucose (0.1% w/v) and methionine (0.02% w/v) (i.e. no addition, glucose only, methionine only, glucose and methionine) were added to the mineral base agar medium of Palleroni and Doudoroff (1972). *Xhh* isolates 7219, 7730E, 7737C and 7744 were grown on YDC for 2 d at 30°C and suspended in distilled water to give a final concentration of approx. 10^5 cells/ml (not visibly turbid). Drops of the bacterial suspension (5 µl) were pipetted onto the surface of plates of each medium. Growth was recorded after incubation for 7 and 9 days at 30°C.

Carbon utilisation tests. Carbon sources were added to the basal agar medium of Palleroni and Doudoroff (1972), amended with 0.02% (w/v) methionine, as filter sterilised solutions in 0.033M Na-K buffer (KH_2P0_4, 2.27 g/l; Na_2HP0_4, 5.969 g/l, pH 6.8) after autoclaving to give final concentrations of 0.1%. Isolates were grown on the medium of Palleroni and Doudoroff (1972) amended with 0.02% (w/v) methionine and 1% glucose for 48 h at 30°C and suspended in sterile distilled water to give a concentration of approx. 10^5 cells/ml. Drops of the suspensions (1 µl) were inoculated onto the surface of the agar medium with a multi-point inoculator (Denley instruments, Sussex, UK). Two replicate plates of each carbon source were inoculated with up to 20 isolates per plate. The drops of inoculum were allowed to dry before plates were incubated at 30°C. After up to 9 d incubation, growth on plates containing carbon sources was compared with that on control plates containing no added carbon source. An isolate was considered to utilise the carbon source (i.e. positive) when growth on the plates containing the carbon source exceeded that on the control plates containing no carbon source. A negative result was recorded if growth was less than or equal to that on control plates. The tests were repeated three times.

DNA extraction. All isolates were grown on King's Medium B (King *et al.* 1954) (to obviate problems with excessive polysaccharide production on YDC) for 2 d 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 quantity of DNA was estimated using the mini-gel method of Sambrook *et al.* (1989). The extracts were diluted with RO water to give a working concentration of 2.5 ng DNA/µl.

DNA fingerprinting. Thirty-one isolates of *Xhh* selected as representative of the different geographic origins and range of *Hedera* spp/cultivars and the two isolates from *S. arboricola* together with *X. campestris* pv. *campestris*, *X. hortorum* pv. *pelargonii* and *Pseudomonas syringae* pv. *syringae* (included as controls)(Table 1) were subjected to DNA fingerprinting using RAPD-PCR (Random Amplified Polymorphic DNA-Polymerase Chain Reaction). A set of 20 high-GC random oligonucleotide primers was obtained from Operon, VH Bio, Newcastle-upon-Tyne, UK.

The PCR reaction mixture and conditions were optimised using a set of four isolates (5691B, 7193, 7732C and 7744) and a single oligonucleotide primer (OPG11, Operon).

Subsequently all PCR reactions were performed in a total volume of 25 µl containing PCR buffer (200 mM Tris-HCl, 500 mM KCl, pH 8.4), 0.75 mM $MgCl_2$, 400 µM deoxynucleotide triphosphate (100 µM of each dNTP) (Gibco, Life Technologies, Paisely, Yorkshire, UK), 15 pmoles RAPD primer (Operon), 5 ng of template DNA, and 1.5 Units of *Taq* polymerase (Gibco). DNA amplification was carried out in a Hybaid Omnigene thermal cycler (Hybaid Limited, Ashford, Middlesex, UK) programmed for 40 cycles of 30 sec at 94°C, 1 min at 37°C, 1 min at 72°C and a single final cycle of 5 min at 72°C. A negative control containing no template DNA was included in all reactions. To obtain 'fingerprints' the PCR products were separated by horizontal submerged gel electrophoresis in 1.4% agarose gels (12 x 14 cm) prepared with 0.5x TBE (Tris borate-EDTA, pH 8.0) buffer and low molecular grade agarose (Amersco, Solon, Ohio, USA) and incorporating ethidium bromide (0.5µg/ml). Size markers (1 Kb plus DNA ladder, Gibco) were included in the end lanes of each gel. Electrophoresis was performed in Hybaid gel tank (Hybaid) at 100 V for 1.5 hours in 0.5x TBE buffer. Gels were observed using a dual-intensity transilluminator (Ultra violet products, Cambridge, UK) and photographed using a Polaroid 4+ camera (Polaroid, Cambridge, UK).

Initially 20 primers from kit OPG were tested on a subset of ten isolates chosen as representative of the different sites of origin (5691B, 5863, 7053B, 7193, 7718, 7730E, 7732C, 7733B, 7738C and 7744). Primers which did not produce any bands with this subset were not used further. PCR was then done using all the chosen isolates with a reduced set of ten primers (Table 4). All reactions were performed three times

Data analysis. The migration distance of each band was measured and used to estimate the molecular weight by comparison with the size markers included in each gel. Bands which were not present in all three repetitions were excluded from the analysis. 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 Jaccard similarity coefficient (Payne *et al*., 1993). Hierarchical cluster analysis was used to generate a dendrogram from the similarity matrix by the average linkage method (Payne *et al.* 1993).

Results

Source of bacteria. Eight responses were received to the article placed in HDC news and four growers sent samples of diseased ivy for isolation.

A total of 92 bacterial isolates from ivy are now held in the culture collection at HRI-Wellesbourne. As some of these isolates were effectively duplicates from the same isolation or from the same cultivar at a particular site a reduced set of 74 was examined in detail in this study (Table 1). The isolates were obtained from 16 different cultivars/species of ivy and from 13 different geographical locations, including 3 different countries: UK, Denmark and the USA. Two isolates listed as *Xhh* from *S. arboricola* were obtained from the NCPPB and included in the studies.

Preliminary Characterisation. Fifty-four of the 74 isolates from ivy produced pale yellow mucoid growth on YDC agar and were Gram-negative, oxidase negative, catalase positive, had oxidative metabolism and were inhibited by 0.02% and 0.1% TTC and were therefore identified as belonging to the genus *Xanthomonas*. Further tests showed that these isolates hydrolysed aesculin and starch and 30% of them liquefied gelatin.

One isolate received from the NCPPB as *Xhh* from ivy (7745) produced orange mucoid growth on YDC agar was therefore not identified as *Xanthomona*s.

The two isolates from *S. arboricola* from the NCPPB produced dark yellow/orange mucoid growth on YDC, grew on media containing 0.1% TTC and produced acid from glucose under aerobic and anaerobic conditions and were therefore not considered to be *Xanthomonas*. These two isolates hydrolysed starch and aesculin and liquefied gelatin.

Pathogenicity to ivy. In the preliminary experiment, disease symptoms were obtained with all of the inoculation methods and all of the isolates. Inoculation with an insect pin or by spraying gave symptoms which were typical of those seen on naturally infected plants, but symptoms took longer to become clearly visible on the sprayed plants. Inoculation with scissors or claw tweezers gave symptoms which were more difficult to interpret: the symptoms were not typical of those that occurred through natural infection and the extent of wounding meant that they were difficult to quantify. Symptoms developed more rapidly with pre- and post-inoculation misting than without. Stabbing leaves with an insect pin charged with bacterial growth combined with pre- and post-inoculation misting was therefore selected for routine pathogenicity testing due to its simplicity, consistency and ease of interpretation.

All of the 54 isolates identified as *Xanthomonas* in the preliminary characterisation tests produced irregular dark-green water-soaked lesions within 10-12 d when inoculated into leaves of *H. helix* cv. Green Ripple. These isolates were therefore considered to be pathogenic and their identify confirmed as *Xhh.* Lesions were 2-5 mm in diameter 5 weeks after inoculation. Plants inoculated with the two isolates from *S. arboricola* and isolate 7745 from ivy, received as *Xhh* from the NCPPB, gave responses which were indistinguishable from the control. These isolates, which had not been identified as *Xanthomonas* in the preliminary tests, were considered to be non-pathogenic and therefore incorrectly identified in the NCPPB.

Pathogenicity to Brassaia. The four isolates from ivy characterised as *Xanthomonas* and pathogenic on ivy (5691B, 7183, 7733B and 7744) and the two isolates from *S. arboricola* (7789 and 7790) produced only limited necrosis immediately around the point of inoculation which was indistinguishable from the controls, and were therefore considered to be non-pathogenic on *Brassaia.*

Methionine requirement. In initial tests to establish the requirement for methionine as a growth factor, no growth was observed on basal medium without the inclusion of methionine and only very limited growth was observed on media containing only methionine. The basal

medium (Palleroni and Doudoroff 1972) was therefore amended with 0.02 % (w/v) methionine for carbon utilisation tests.

Carbon utilisation tests. The results of the carbon utilisation test for the 54 isolates which were pathogenic on ivy are given in Table 2. Thirty-six carbon sources gave identical results for all pairs of replicate plates and in all three repeat tests: all isolates utilised D-alanine, L-alanine, L-arabinose, L-asparagine, cellobiose, dextrin, D-fructose, fumarate, D-galactose, α-D-glucose, glycerol, glycogen, D-maltose, D-mannitol, D-mannose, D-melibiose, L-proline, pyruvate, serine, D-sorbitol, succinate, sucrose, D-trehalose, tween 40 and tween 80, and none of them utilised acetate, citrate, D-gluconate, L-glutamate, D,L-lactate, α-D-lactose, malonate, L-ornithine, starch, L-threonine. Four of the carbon sources (D-aspartate, D-raffinose, tween 20 and L-xylose) produced inconsistent results, with identical results for replicate plates but with variation between tests. Results for some isolates varied between repetitions from weak positive to positive on D-aspartate, D-raffinose, tween 20, and for other isolates varied from negative to weak positive. Isolates varied between tests from negative to weak positive on L-xylose.

DNA fingerprinting. All of the 31 isolates from ivy produced bands with the ten primers tested. The number of bands produced varied with the different primers. A total of 158 possible fragment lengths (bands) were produced by the ten primers. The dendrogram generated by cluster analysis of all pair-wise comparisons is shown in Fig. 1. Isolates of *Xhh* from ivy formed one major cluster (similarity >78%) which could be divided into two sub-groups at the 80% similarity level.

The 31 isolates of *Xhh* from ivy gave identical patterns of bands with five out of the ten primers (OPG2, OPG5, OPG11, OPG13 and OPG18), but gave varying patterns with the others (OPG3, OPG4, OPG10, OPG12 and OPG19). The majority of *Xhh* isolates (23 of 31) were contained within a single group (Group 1), 17 of these isolates gave identical patterns of bands with all of the primers tested. Isolates in Group 1 were differentiated from those in Group 2 by primers OPG3 and OPG12. Isolates in Group 1 produced four bands of approx. 1993, 850, 800 and 340 bp with OPG3 and one of 2250 bp with OPG12, whereas all isolates in Group 2 produced six bands of 1993, 1767, 1250, 1000 and 350 bp with OPG3 and eight bands of 1825, 1650, 1542, 1326, 1218, 850, 650 and 400 bp with OPG12. Isolate 7746 in Group 2 produced an additional band of 850 bp with primer OPG3.

Within each of the groups isolates produced different patterns with primers OPG4 and OPG10. Differences were also seen with primer OPG19 where the three *Xhh* isolates obtained from the NCPPB (7743, 7744 and 7746) produced identical fingerprints to each other but differed by one band (400 bp) from that produced by the other isolates of *Xhh*.

The two isolates from *S. arboricola* showed little similarity with each other (43%) and appeared un-related to isolates from ivy (similarity 14%). Isolates of *Xhh* from ivy showed

greater similarity with isolates of *X. hortorum* pv. *pelargonii* (49% similarity) and *X. campestris* pv. *campestris* (51% similarity), than with isolates received as *Xhh* from *S. arboricola*.

Discussion

All of the isolates identified as belonging to the genus *Xanthomonas* in the preliminary characterisation tests were also pathogenic on ivy and should therefore be correctly classified as *Xanthomonas hortorum* pv. *hederae* (*Xhh*)

Three isolates received from the NCPPB as *Xhh* (7745 from ivy, and 7789 and 7790 from *S. arboricola*) were not identified as belonging to the genus *Xanthomonas* in the preliminary tests and were not pathogenic on ivy. It would therefore appear that these isolates had been originally mis-identified, or had become contaminated during culture at NCPPB.

None of the six isolates (four from and pathogenic on ivy and two from *S. arboricola*) which we tested were pathogenic on *B. actinophylla*. As the two isolates from *S. arboricola* were not *Xanthomonas*, were not pathogenic on ivy and had clearly been mis-identified, or contaminated, we cannot draw any conclusions about the host range of *Xanthomonas* strains from *Schefflera.* However, the results obtained with the isolates from ivy, identified as *Xhh* on the basis of their pathogenicity to ivy, indicated that *Xhh* is not a pathogen of *B. actinophylla*.

Our conclusions do not agree with those of Chase (1984) who reported that *Xhh* is pathogenic on *B. actinophylla, Fatsia japonica*, *H. helix, Dizygotheca elegantissima*, *Polyscias fruticosa* and *S. arboricola* or those of Norman *et al.* (1999) who reported similar symptoms on *B. actinophylla, H. helix* and *P. fruticosa* when inoculated with isolates mainly from *Hedera* and *Polyscias.* It is difficult to account for the discrepancies between the work reported here and that of Chase (1984) and Norman *et al.* (1999). Chase (1984) inoculated plants with six isolates from *B. actinophylla* and *F. japonica* and three isolates presumed to come from ivy (he gives no details) by wounding leaves and then spraying on bacterial suspensions. Norman *et al.* (1999) did inoculations with six isolates from *Brassaia*, 28 from *Hedera,* 23 from *Polyscias* and two from *Schefflera* by spraying bacterial suspensions onto plants which had been misted for 12 h and were then covered by polythene bags for 24 h. Thus, although their inoculation methods differed slightly and were probably performed at higher temperatures than ours, it seems unlikely that this could account for the different conclusions. Despite several requests we were unable to obtain isolates directly from these authors, and they did not include any isolates from the UK in their work. However, the type strain of *Xhh* which we obtained from the NCPPB (7744 = NCPPB939 = ICMP453) was included in the work of Norman *et al.* (1999) (although incorrectly listed as originating in New Zealand rather than the USA). The two isolates from *Schefflera* which we obtained from the NCPPB apparently originated from Chase (1984) and were also used by Norman *et al.* (1999). Interestingly, Norman *et al.* (1999) report in a table of results that the two isolates

from *S. arboricola* caused no to slight symptoms on *Brassaia* and *Hedera* (mean scores of 1.6 and 1.5, where 1 equals no symptoms and two equals slight symptoms) and no symptoms on *Polyscias* (mean score 1.0). These two isolates were also quite distinct from the majority of other isolates in the phenotypic and genotypic tests they performed. Thus their results with these two isolates are not too dissimilar from ours and it is possible that Norman *et al* (1999) have misinterpreted their observations. Clearly further work, using a common set of isolates is needed to clarify the host range of *Xhh.*

The results of the carbon source utilisation tests indicate little phenotypic variability amongst the 54 isolates of *Xhh* examined in this study with results varying between isolates for only four of the 40 carbon sources. As results for these four carbon sources also varied between repetitions for a particular isolate, they were not considered sufficiently reliable to use as a basis for differentiation.

The results for carbon source utilisation generally agreed with carbon oxidation results obtained using the Biolog™ system by Norman *et al*. (1999) for *Xhh* from ivy and by Vauterin *et al*. (1995) for *X. hortorum*. Results differed for seven compounds but such differences are to be expected as the methods use different basal media and are quite different in interpretation: a positive result in the Biolog™ system requires electron transfer to produce a colour change in a tetrazolium dye and does not depend on growth of the organism; a positive result in the tests reported here requires that the organism can utilise the carbon source to produce substantial visible growth. Norman *et al.* (1999) also found that most isolates of *Xhh* from ivy had similar carbon oxidation (metabolic) profiles and were quite distinct from the profiles of isolates from other araliaceous hosts.

The results of the DNA fingerprinting using RAPD-PCR suggest that all isolates of *Xhh* from ivy are genetically very similar, although two distinct sub-groups can be distinguished. Norman *et al.* (1999) using a different DNA fingerprinting method (RFLP) also found that most *Xhh* isolates from ivy were genetically similar (27 out of 28 strains > 80% similarity) and in addition were genetically quite distinct (> 60% similarity) from isolates obtained from *Polyscias*.

Using three different methods of characterisation (Biolog™, fatty acid methyl esters - FAME, RFLP genetic fingerprinting), Norman *et al.* (1999) consistently separated *Xanthomonas* isolates from ivy (i.e. *Xhh*) and *Xanthomonas* isolates from other araliaceous hosts, especially *Polyscias.* The heterogeneity in *Xhh*, that they reported, resulted from the inclusion of isolates from araliaceous hosts other than ivy in the pathovar, and they suggested that this warranted its division into two pathovars. This would not be possible if isolates from these other araliaceous hosts are indeed pathogenic on ivy, as the definition of a pathovar is based solely on pathogenicity, this again emphasises the need for verification of the results obtained by Chase (1984) and Norman *et al.* (1999) on the host range of the two distinct groups of *Xanthomonas* isolates from members of the araliaceae. It is possible, however, that

the two groups of strains are sufficiently distinct to warrant separate *Xanthomonas* species; unfortunately Norman *et al.* (1999) did not include any control isolates belonging to other *Xanthomonas* species in their work.

Many bacterial plant pathogens occur as races which are distinguished on the basis of their pathogenicity to a range of host species/cultivars. RAPD-PCR has been shown to discriminate between races of other pathovars (Roberts *et al*. unpublished), it is possible that the two genetic sub-groups found here, although very similar, may represent races. However, there did not appear to be any relationship between the sub-groups within *Xhh* from ivy and their geographical or cultivar of origin.

Much more genetic variability has been observed in other pathovars of plant pathogenic bacteria using similar methods. The absence of variability observed here and the fact that the host (ivy) is propagated vegetatively suggests there is little genetic in-flow into the patho-system. Thus, infection of nursery-raised plants from wild plants growing locally seems unlikely and we have never observed the disease on wild ivy in the UK. It also seems more likely that the disease is primarily disseminated with the cuttings and spreads between cultivars during production.

Host-Pathogen interactions

Introduction

There is no information on pathogenic variability within *Xhh*. The DNA-fingerprinting studies indicated that UK isolates of *Xhh* can be divided into two closely related genetic groups, these groups could represent pathogenic races, which vary in their virulence on different ivy species/cultivars.

Arnaud (1920) reported that there was a difference in susceptibility between the two ivy cultivars used in his study: cv. Lierre des bois showed more severe symptoms than cv. Lierre d'Ecosse. White and McCulloch (1934) did varietal susceptibility tests but they did not observe any differences in the severity of symptoms, only that all cultivars tested were susceptible to the pathogen.

Osborne and Chase (1985) investigated the susceptibility of 12 ivy cultivars to two-spotted spider mite and *Xanthomonas* leaf spot. They reported that there was approximately a seven-fold difference in the number of lesions between the most susceptible and the least susceptible although none were completely resistant. However, Osborne and Chase (1985) used a single isolate from *Brassaia* in their tests.

The objective of this work was to investigate the susceptibility of a range of *Hedera* species/cultivars to *Xhh* isolates from ivy representing the different sites and cultivars of origin and to determine if there is any pathogenic variability amongst isolates of *Xhh*.

Materials and methods

Isolates.. Ten isolates of *Xhh* were selected (boldface type in Table 1) to represent the two DNA fingerprinting groups, and the range of different geographic sites and cultivars.

Plants. An initial batch of 20 ivy species/cultivars was selected on the basis of previous work by Osborne and Chase (1985) and to represent the most frequently grown cultivars in the UK. Stock plants of the selected species/cultivars were obtained from the UK National Collection of ivies which is maintained by 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 maintained at a minimum temperature of 18/12°C (day/night) and with venting at 20/14°C (day/night). After 6 weeks the cuttings were gradually hardened off by removing the polythene cover. At 8 weeks the cuttings were potted on into 7 cm pots of Levington M2 compost and then grown on for a further 2 months before inoculation. A second batch of 17 ivy species/cultivars was later obtained as rooted cuttings and grown on in a similar way.

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 by scraping growth from agar plates with an insect pin and stabbing the leaves in five places. The two youngest leaves on each of the other two plants were inoculated by spraying a turbid suspension containing approx. 10^8 cfu/ml 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 and recorded approximately 2, 3, 4 and 5 weeks after inoculation. 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.

Data analysis. Pin inoculation data (number of points showing infection and size of lesion) and spray inoculation data (number of lesions) recorded approx. five weeks after inoculation were analysed by generalised linear modelling methods or analysis of variance, as appropriate, using Genstat statistical analysis software (Payne *et al.*, 1993).

Results

Symptoms were produced by all of the isolates on all cultivars and first appeared after 12 d on pin inoculated plants and after 14 d on spray inoculated plants. Both methods gave symptoms that were typical of those seen on naturally infected plants. Lesions were smaller on spray inoculated (0.5-2 mm diameter after 37 d) than on pin-inoculated plants (0.5-10 mm diameter after 40 d).

All of the first set of twenty species/cultivars tested were considered to be fully susceptible to all of the isolates, therefore, a further set of 15 species/cultivars plus *H. helix* cv. Green Ripple and *H. maderiensis* subsp. *iberica* from the first set (included as controls) were tested.

Lesions were generally smaller in the second set of inoculations and results for each set of inoculations were analysed separately. The analyses of deviance indicated that isolate and cultivar were the most important terms in the model. There was some evidence of an isolate x cultivar interaction but this was much less important. Thus, although none of the cultivars could be considered to be completely resistant, they did vary in their susceptibility. The means of three measures of disease for each species/cultivar are shown in Tables 5 and 6, in ascending order of lesion size for pin inoculated leaves, i.e. from least to most susceptible. Ranking isolates on the basis of the mean number of lesions obtained for spray inoculated leaves would have produced a different ranking. However, as the number of lesions may be a function of leaf size this is probably not a reliable indicator of relative resistance (c.f. *H. helix* cv. Minima). In general, the non-*helix* species tended to be less susceptible than the *helix* species, with *H. maderiensis* subsp. *iberica* the least susceptible of the first batch of cultivars, and *H. rhombea* and *H. helix* cv. Tanja the least susceptible in the second batch.

Some cultivar/isolate combinations gave results which were inconsistent between pin and spray inoculated leaves: in the first batch *H. helix* cv. Chrysophylla and *H. canariensis* var. *algeriensis* cv. Ravensholst gave almost no lesions on leaves spray inoculated with isolate 7744 but 100% on pin inoculated leaves, and in the second batch *H. cypria* gave almost no lesions on leaves spray inoculated with isolates 7734C and 7744. Conversely, *H. rhombea,* gave no lesions on leaves pin inoculated with isolates 5863 and 7219, but high numbers on spray inoculated leaves, and similarly *H. helix* cv. Tanja with isolates 5993 and 7219.

The means for each isolate across all cultivars are shown in Table 7. Isolates 7731A, 7734C, 7738C consistently produced larger lesions than the other isolates. Isolate 7744 (the type strain from NCPPB) tended to produce smaller lesions than the other isolates.

Discussion

None of the 35 ivy species/cultivars tested could be considered resistant to *Xhh.* Although there was little difference in susceptibility between most of the species/cultivars, the non-*helix* species tended to be less susceptible than the *helix* species. A few species/cultivars were clearly less susceptible: *H. maderiensis* subsp. *iberica* in the first batch, and *H. rhombea* and *H. helix* cv. Tanja in the second batch.

Results differed for some cultivar/isolate combinations depending on the mode of inoculation. Some cultivars showed resistance to certain isolates when pin inoculated, however, the same cultivar/isolate combination resulted in infection when the plants were spray inoculated. This may indicate that different resistance mechanisms are operating in the case of pin and spray inoculation. It is likely that the difference in lesion size between pin and spray-inoculated plants is an artefact of the methods: as there were usually more lesions per unit area on the spray inoculated leaves, the amount of tissue available for expansion of each individual lesion was more limited than in pin-inoculated leaves.

Our results did not agree with those of Osborne & Chase (1985) for the seven *H. helix* cultivars included in both studies (cvs. Brokamp, California, Eva, Goldheart, Ivalace, Manda's Crested, Telecurl). We found no significant differences in the numbers of lesions on the spray inoculated leaves for these cultivars, whereas Osborne & Chase found significant differences, ranging from 5.7 for Eva to 22.1 for Brokamp. It should be emphasised, however, that whereas Osborne & Chase (1985) used only a single isolate obtained from a different genus (*Brassaia*) for inoculation, we used ten isolates all obtained from *Hedera*; it is probable therefore that our results are more robust and informative in practice.

The DNA fingerprinting results indicated a lack of genetic variability within the pathovar *Xhh*, with existence of only two closely-related genetic 'types'. We postulated that these two genetic types could represent pathogenic races. No relationship was observed between the pathogenicity of the isolates and their DNA fingerprinting group (Table 7). The absence of

any pathogenic specialisation (i.e. races) is consistent with the lack of resistance in any of the host species/cultivars.

Development of a routine detection method

Introduction

A pre-requisite for any epidemiological study is a method for routine detection, identification and quantification of the target pathogen. This section describes the development of a method based on dilution plating on semi-selective agar media followed by serological confirmation of identity.

Materials and methods

Isolates. A test array of 74 isolates (54 isolates of *Xhh* and 20 non-pathogenic bacterial isolates from ivy, see Table 1) was used throughout for the development and testing of media.

Multipoint inoculation. For many tests, isolates were inoculated onto test plates using a multipoint inoculator. Isolates were recovered from storage at –76˚C onto YDC medium and grown for 24-48 h at 30°C. Bacteria were suspended in 3 ml of sterile RO water to give a final concentration of approximately 10^5 cells/ml. The bacterial suspensions were inoculated onto the test media, under sterile conditions, using a multi-point inoculator (Denley Instruments) which simultaneously inoculates the surface of an agar plate with 1 µl of each of up to 20 bacterial suspensions. The drops of inoculum were allowed to dry, before plates were incubated at 30°C.

Antibiotic sensitivity. Isolates were tested for their sensitivity to a range of concentrations of sixteen antibiotics (Table 8) which have previously been used in selective media for other *Xanthomonas* spp. (Schaad 1988).

Antibiotics were incorporated into plates of nutrient agar (Difco) by adding an appropriate volume of a concentrated stock solution to cooled molten medium before pouring. Stock solutions were prepared in distilled water, 70% ethanol or 50% dimethyl formamide depending on their solubility. Stock solutions prepared in distilled water were filter sterilised before addition to the medium. Plates were inoculated with the multipoint inoculator and incubated at 30°C. Growth was recorded on test plates after 3 d by comparison with that on control plates containing no antibiotic. Each isolate was tested on duplicate plates of each antibiotic on three separate occasions.

Growth on media selective for other Xanthomonas *spp.*. The growth of isolates was examined on four media previously reported as selective for *Xanthomonas* spp.: NSCAA for *X. campestris* pv. *campestris* (Randhawa and Schaad 1984), D-5 for *X. hortorum* pv. *carotae* (Kuan *et al.* 1985), Tween for *X. vesicatoria* (McGuire *et al.* 1986) and CS for *X. campestris* pv. *diffenbachiae* (Norman and Alvarez 1989). In addition to the standard 74 isolates, positive control isolates known to grow on the medium were inoculated onto test plates using the multipoint inoculator. Plates were incubated at 30°C. Growth on test plates was compared after 3, 4 and 5 d with that of positive control isolates and control plates of non-selective

medium (YDC). Each isolate was tested on duplicate plates of each medium on two separate occasions.

Modification of Tween medium. Isolates were tested for their growth on Tween medium modified by varying the concentrations of the antibiotics in the original medium (McGuire *et al.*, 1986). Chlorothalonil was used as a replacement fungicide for cycloheximide as it was expected to be discontinued. Cephalexin, chlorothalonil, 5-fluorouracil and tobramycin were tested individually and then combined at varying concentrations to assess their interactions. Antibiotics were incorporated into the base of the original tween medium (calcium chloride, 0.25 g/l, peptone (Bacto Difco), 10 g/l, potassium bromide, 10 g/l, agar, 15 g/l) by adding an appropriate volume of a concentrated stock solution to cooled molten medium before pouring. Stock solutions were prepared in distilled water and filter sterilised apart from chlorothalonil which was suspended in 70% ethanol. The effect of a range of concentrations of tween 80 on the growth of *Xhh* was also examined. Test plates were inoculated with the multipoint inoculator and incubated at 30°C. Growth on test plates was compared after 3 and 4 d with 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 the antibiotic sensitivity and carbon utilisation tests. The basic basal medium for BCBC was contained: cellobiose, 10 g/l, K_2HPO_4, 0.8g/l, MgSO4, 0.1 g/l, yeast extract, 0.6 g/l, Bacto agar (Difco), 15 g/l) Isolates were tested for their growth on the basal medium containing varying concentrations of amoxicillin, brilliant cresol blue, cephalexin, chlorothalonil, 5-fluorouracil, pyridoxine and tobramycin. The antibiotics were first tested individually and then combined at varying concentrations to assess their interactions. Antibiotics were incorporated into the basal medium by adding an appropriate volume of a concentrated stock solution to cooled molten medium before pouring. Stock solutions were prepared in distilled water and filter sterilised apart from chlorothalonil which was suspended in 70% ethanol. Test plates were inoculated with the multipoint inoculator and incubated at 30°C. Growth on test plates was compared after 3 and 4 d with control plates containing no antibiotics.

The effect of pH on recovery of isolates on BCBC medium was examined by plating a ten-fold dilution series of three isolates of *Xhh* (7183, 7733C and 7744) onto plates of BCBC adjusted to pH 6.8, 7.0 and 7.2 using 1M NaOH and YDC. Isolates were grown on plates of YDC for 48 h at 30°C and suspended in sterile distilled water to give a concentration of approx. 10^8 cells/ml and a ten-fold dilution series prepared. 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 at appropriate dilutions was recorded after 3 and 4 d.

Recovery of Xhh on mTween and BCBC media. *Xhh* isolates 7183, 7733C and 7744 were suspended in sterile distilled water to give a concentration of approx. 10^8 cells/ml. A tenfold

dilution series was then prepared and 100 µl of each dilution pipetted onto the surface of plates of YDC, BCBC (Cellobiose, 10g/l, K_2HPO_4, 0.8 g/l, $MgSO_4$, 0.1 g/l, Yeast extract, 0.6 g/l, Difco Bacto agar, 15 g/l, Brilliant cresol blue, 5 mg/l, Cephalexin, 30 mg/l, Chlorothalonil, 20 mg/l, 5-fluorouracil, 6 mg/l, Tobramycin, 0.6 mg/l, pH 6.8) and mTween (modified Tween) (Difco Bacto peptone, 10 g/l, potassium bromide, 10 g/l, calcium chloride, 0.25 g/l, Difco Bacto agar, 15 g/l, cephalexin, 15 mg/l, chlorothalonil, 20 mg/l, 5-fluorouracil, 6 mg/l, Tobramycin, 0.6 mg/l, pH 6.8) and spread with a bent glass rod. Plates were incubated at 30°C and the number of colonies growing on each medium at appropriate dilutions was recorded after 2 and 3 d (YDC) or 3 and 4 d (BCBC and mTween)

Selectivity of mTween and BCBC media. Eighty ivy leaves from a range of ivy cultivars were collected from around the site at HRI-Wellesbourne. The leaves were mixed and twenty leaves were placed in each of four conical flasks containing 20 ml of sterile RO water and 0.02% tween 80. The flasks were then shaken vigorously on a wrist action shaker for 30 minutes. The liquid extracts from the four flasks were combined and series of four ten-fold dilutions in sterile RO water was prepared from the combined extract. Growth of three isolates of *Xhh* (7183, 7733C, 7744) from 48 h YDC plates was suspended in sterile RO water to make a suspension of approx. 10^8 cfu/ml and a ten fold dilution series prepared for each of them. One ml of each dilution of each isolate was then added to the equivalent dilution of the leaf washings and vortexed. An aliquot (100 µl) of each dilution of each isolate was pipetted onto the surface of duplicate pates of YDC, mTween and BCBC media and spread with a bent glass rod. As a control, 100 µl of each dilution of the leaf washings which had not been spiked with *Xhh* was plated onto YDC, BCBC and modified Tween to confirm that no *Xhh* were present on the leaves at the start of the test. Plates were allowed to dry and incubated at 30°C. The number of colonies growing on the plates was recorded after 2 and 3 d on YDC and after 3 and 4 d on the modified Tween and BCBC agar plates. Suspected colonies of *Xhh* were tested from each plate by agglutination with *Staph. aureus* conjugated antiserum (Lyons and Taylor 1990) to confirm their identity.

Agglutination with antisera raised to other Xanthomonas species. Isolates were tested for agglutination reactions with eight antisera, from the HRI antiserum collection, raised to different *Xanthomonas* spp. Each antiserum was conjugated to *Staphylococcus aureus* according to Lyons and Taylor (1990). 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. The homologous isolates for each antiserum were also tested as positive controls. 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. A rabbit antiserum was raised against whole cells of *Xhh* isolate 7183 using standard methods. Antigen consisted of cells harvested from a 48 h plate of King's Medium B (King *et al*., 1954) mixed with Freund's incomplete adjuvant. The new antiserum

was tested against the 74 isolates of the test array plus the eight controls from above in slide agglutination (antiserum diluted 1:30) as previously and in indirect ELISA.

ELISA. Antigens for ELISA were prepared from bacteria which had been grown on YDC plates 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 PBS + thiomersal (phosphate buffered saline containing thiomersal) using a Spectronic spectrophotometer (Milton Roy Company, Northampton, UK).

An initial ELISA was done, in which the antiserum was titrated against a single concentration of the homologous antigen, to determine the optimum antiserum concentration. Subsequently duplicate wells of ELISA plates were coated overnight at 4°C with 100 µl of each antigen, 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 antiserum diluted 1:102,000. A blank control was also included in the plate using PBSA instead of antiserum. Plates were then washed and incubated for 45 min with goat anti-rabbit conjugated antiserum, then washed again before finally adding substrate and incubating in the dark. Absorbance at a wavelength of 405 nm was measured with a plate reader at 30 min intervals until the absorbance of the homologous (positive control) isolate exceeded a value of one.

Results

Antibiotic sensitivity tests. The results of the antibiotic sensitivity tests are shown in Table 8 as the percentages of isolates resistant to each antibiotic. All fifty-four isolates of *Xhh* gave identical results with nine of the antibiotics tested and were resistant to brilliant cresol blue, chlorothalonil, cycloheximide, 5-fluorouracil, gentamycin, pyridoxine, trimethoprim and vancomycin and sensitive to kanamycin; results for the other seven antibiotics (ampicillin, bacitracin, boric acid, methyl green, nitrofurantoin, penicillin G) varied between isolates. All of the twenty non-pathogenic isolates from ivy gave identical results for seven of the antibiotics tested and were resistant to boric acid, chlorothalonil, 5-fluorouracil, gentamycin, pyridoxine, trimethoprim and were sensitive to kanamycin; results for the other nine antibiotics (ampicillin, bacitracin, brilliant cresol blue, cephalexin, cycloheximide, methyl green, nitrofurantoin, penicillin and vancomycin) varied between isolates.

Evaluation of selective media for other Xanthomonas species. The growth of *Xhh* and the non-pathogenic isolates from ivy on the four media originally developed for other *Xanthomonas* spp./pvs is shown in Table 9. All of the 54 *Xhh* isolates grew on D-5 and produced circular, yellow mucoid colonies, but the selectivity of the medium was poor and 18 of the non-pathogenic isolates also grew. None of the fifty-four isolates of *Xhh* grew on either NSCAA or CS media. Growth of *Xhh* and inhibition of non-pathogenic isolates was most successful on the Tween medium: 53 of the 54 *Xhh* isolates and only seven of the 20 non-pathogenic isolates grew; *Xhh* isolates produced circular, yellow mucoid colonies on the

medium, although, the colonies were not surrounded by zones of crystals which the medium was originally designed to show.

Modification of Tween medium. All isolates of *Xhh* grew on tween base medium which contained either cephalexin up to 50 mg/l, chlorothalonil up to 20 mg/l, 5-fluorouracil up to 6 mg/l, tobramycin up to 0.6 mg/l or tween up to 5 g/l. The antibiotics were then combined at various concentrations to assess whether inhibition occurred due to their interactions.

All isolates of *Xhh* grew on a modified Tween medium (mTween) containing cephalexin (15 mg/l), chlorothalonil (20 mg/l), 5-fluorouracil (6 mg/l) and tobramycin (0.6 mg/l) (see Appendix II). Only one non-pathogenic isolate (7735C) grew on this medium. It produced yellow non-mucoid growth and was easily differentiated from colonies of *Xhh* which were small (2 mm diameter), cream, circular and raised after 4 d at 30 °C.

Brilliant cresol blue cellobiose (BCBC) medium. All isolates of *Xhh* grew on BCBC medium containing cephalexin (30 mg/l), chlorothalonil (20 mg/l), 5-fluorouracil (6 mg/l) and tobramycin (0.6 mg/l) (see Appendix III). Isolate 7735C was again the only non-pathogenic isolate that grew on the medium. It produced yellow non-mucoid growth and was easily differentiated from colonies of *Xhh* which were large (4 mm diameter), blue, smooth, domed and mucoid after 4 d at 30°C. Recovery of each of the three isolates of *Xhh* was greatest at pH 6.8 (Table 10).

Recovery of Xhh on mTween and BCBC media. There was no difference in recovery of *Xhh* on BCBC and mTween media. Greater numbers of *Xhh* were apparently recovered from the selective media than on the non-selective YDC medium (Table 11)

Selectivity of media. All suspect colonies of *Xhh* were positive in slide agglutination. *Xhh* was not detected in control samples which had not been spiked with a suspension of *Xhh.* Although both media showed high levels of selectivity and recovery compared to the non-selective medium YDC, BCBC medium appeared to be slightly better than the Tween 80 medium.

Reactions with antisera raised to other Xanthomonas species. Most (46) of the 54 isolates of *Xhh* tested did not react with any of the antisera raised to other *Xanthomonas* spp. Twelve isolates reacted with one or more antisera (Table 12). The antisera prepared to *Xanthomonas fragariae* and *Xanthomonas vesicatoria* reacted with the greatest number of isolates (seven and four) and six *Xhh* isolates reacted with more than one antiserum, 5863 (4), 7053B (3), 7730E (3), 5998 (2), 7219 (2) and 7735B (2).

Antiserum to Xhh. All 54 *Xhh* isolates and *X. campestris* pv. *campestris*, *X. hortorum* pv. *carotae* and *X. vesicatoria* gave positive slide agglutination results with the new antiserum raised to *Xhh*. All of the non-*Xhh* isolates from ivy gave a negative reaction.

ELISA. All isolates of *Xhh* gave an absorbance value of ≥0.8 after 90 min compared to the reagent blank control values of 0.1 and were considered as positive. Cross-reactions occurred with isolates of *X. campestris*, *X. vesicatoria* and *X. hortorum* pv. *pelargonii* which gave mean absorbance values after 90 min of 0.7, 0.8 and 0.7 respectively. All non-*Xhh* isolates from ivy gave absorbance values of <0.33 after 90 min and were considered as negative results.

Discussion

Prior to this work there were no selective media for the isolation of *Xhh.* Two selective media were developed for the isolation of *Xhh*. Modified Tween was based an original recipe developed by McGuire *et al*. (1986) for isolation of *Xanthomonas vesicatoria*. The medium was adapted to be more specific to *Xhh* by varying the concentration of antibiotics. The antifungal compound cycloheximide was replaced with chlorothalonil as its manufacture was expected to be discontinued.

Brilliant cresol blue cellobiose medium was developed on the basis of the carbon source utilisation and antibiotic sensitivity tests and from the recipes of media for other *Xanthomonas* spp.

Some isolates agglutinated with antisera prepared to other *Xanthomonas* spp. The greatest cross reactivity was observed with an antiserum produced to *Xanthomonas fragariae*. It had been expected that cross-reactions would be more likely to occur with antisera produced to *X. hortorum* pv. *pelargonii* and/or *X. hortorum* pv. *carotae* as these pathovars belong to the same species as *Xhh*. *X. fragariae* is closely related to *Xanthomonas hortorum* based on DNA homology (Vauterin *et al*. 1995) and may therefore possess some common antigens. However, *X. fragariae* did not react in either agglutination or ELISA with the antiserum prepared to *Xhh.*

Although the new antiserum to *Xhh*, produced as part of this project, cross-reacted with other *Xanthomonas* spp. from other hosts in agglutination tests, it did not react with any of the saprophytic isolates from ivy. As the *Xanthomonas* strains from other hosts are unlikely to be present on ivy, these cross-reactions will be of no consequence in practice.

The development of two semi-selective media and antiserum to *Xhh* will allow the detection and confirmation of the pathogen from leaf material. Although other leaf-inhabiting bacteria are not completely eliminated on these media, the distinctive colony characteristics of *Xhh* means it can be easily distinguished from these other bacteria. Further confirmation that individual colonies growing on the media are *Xhh* can then be obtained by using *Staphylococcus aureus* slide agglutination test.

Epidemiology studies

Introduction

There is no information in the scientific literature on the epidemiology of bacterial leaf spot of ivy, specifically the primary source and dissemination of the pathogen. In order to develop a disease management strategy such information is essential.

The aims of this study were to determine if the pathogen was present on stock plants and could be transmitted with cuttings in the absence of symptoms.

Materials and methods

Design. Studies were done at two nurseries that practised different methods of propagation. At *Site 1* internodal cuttings of *H. helix* cv. Glacier were taken in early September from four-year-old stock plants grown as hanging baskets in a polytunnel and inserted into peat-based compost in '104' module trays (two cuttings per cell). Trays were placed on the gravel floor of a glasshouse (with sub-heating via hot-water pipes at 18°C and minimum air temp of 10°C) and covered with a low tunnel of clear polythene. Cuttings were watered once when first set out, then left to root for six weeks during which time they were not watered. After rooting the polythene was gradually removed over a period of two weeks and the rooted cuttings were grown on *in situ* for a further six months. Following the removal of polythene the rooted cuttings were watered daily by hand using a hose-pipe fitted with a hand lance.

At *Site 2* internodal cuttings of *H. helix* cv. Jesters Gold were taken in November from the previous years liners growing in 14 cm pots and inserted into peat-based compost in '104' module trays (one per cell). Trays were then placed on upturned crates in a glasshouse with a minimum temperature of 10°C and covered with white polythene (in contact with the foliage). Cuttings were watered once when first set out then left to root for three months and watered when the compost appeared dry (approx. every two weeks). After rooting the polythene was gradually removed and the rooted cuttings were grow on *in situ* for a further six months. Following the removal of the polythene the rooted cuttings were watered daily by hand using a hose-pipe fitted with a hand lance

At both sites the stock plants were numbered. Material for cuttings was removed from each stock plant using secateurs which were disinfected between each stock plant. Material from each stock plant was then kept in separate black polythene bags until preparation of cuttings. Cuttings from different stock plants were inserted into separate trays. Thus each tray of cuttings could be traced to a particular stock plant; some stock plants were represented by a single tray of cuttings others by two.

At *Site 1* an additional three trays of cuttings of the same cultivar (*H. helix* cv. Glacier), obtained from Fibrex nurseries (Warwickshire, UK), were set out in random positions amongst the other trays to act as 'bait' plants. These cuttings had been taken from stock

plants which had previously been tested (by leaf washing as described below) and found free from *Xhh.* At *Site 1* a batch of cuttings of another *H. helix* cultivar (Green Ripple) was rooted immediately adjacent to the cuttings in the trial under the same low polythene tunnel. At *Site 2* there were no 'bait' plants and the cuttings in the trial were kept separate from any other batches of cuttings.

Leaf washings. Leaf washings were done to estimate the proportion of contaminated symptomless leaves throughout propagation (approx. every 2-4 weeks). One sample of 20 leaves and one of two leaves was taken from each tray by cutting at the base of the petiole using scissors, which were sterilised with 70% ethanol between trays, on nine occasions at *Site 1* and seven occasions at *Site 2*. Leaves were selected at random using a different series of random numbers for each tray/occasion (generated using Genstat, Payne *et al.* 1993). Samples were kept in separate new clean polythene bags overnight at 4°C until processing.

Leaves were put into conical flasks containing sterile RO water and 0.02% tween 80. The volume of water was adjusted according to the size of the sample (1 ml per leaf). Flasks were shaken using a wrist-action shaker for 30 minutes. The sample extracts were then diluted (10^{-1}, 10^{-2}, 10^{-3}) and 100 µl of each dilution and the undiluted extract spread onto plates of mTween and BCBC media using a bent glass rod. Plates were incubated for 3-4 days at 30°C and the numbers of typical *Xhh* colonies were counted. The identity of a selection of typical colonies was confirmed by *Staph. aureus* slide agglutination.

Data analysis. Bacterial counts were used to estimate the mean numbers of *Xhh* per leaf for each site and sampling date using a generalised linear model with Poisson error and logarithmic link function. An arbitrary value of 0.1 (equivalent to $1/10^{th}$ of the theoretical detection threshold of the assay) was added to all counts before analysis, in order to obtain meaningful values for samples in which *Xhh* was not detected. The proportions of leaves contaminated at each site and sampling date were estimated using a generalised linear model with binomial errors and a complementary log-log link function. Models were fitted using Genstat (Payne *et al.* 1993). The data were used to produce maps of the distribution of *Xhh* at each stage of propagation.

Results

Results are summarised as maps showing the locations of individual trays of cuttings in which *Xhh* was detected at each site and sampling date in Figs. 2 and 3 and as overall summaries of the proportion of contaminated leaves and numbers of *Xhh* per leaf in Figs. 4 and 5.

At *Site 1 Xhh* was detected on 5 of the 15 stock plants with a mean across all stock plants of 16 cfu *Xhh* per leaf and 3.5% of leaves contaminated. *Xhh* was not then detected on the cuttings whilst they were rooting until after the polythene was removed and overhead watering resumed. The numbers peaked shortly afterwards then declined until the pathogen

was not detected at the final two sampling dates. Disease symptoms were never observed on any of the cuttings of cv. Glacier during the trial, but were visible on the adjacent plants of cv. Green Ripple throughout the trial period. Following rooting, contamination was found more frequently in the trays nearest the Green Ripple, even when *Xhh* had not been detected on the stock plants from which these cuttings had been derived.

Xhh was not detected in the 'bait' trays of cuttings until after rooting and overhead watering had been resumed, and was only detected in the two trays which were adjacent to the trays of cv. Green Ripple with symptoms.

At Site 2 *Xhh* was detected on 4 of the 20 liners used as mother plants with a mean across all stock plants of 6 cfu *Xhh* per leaf and 1.5% of leaves contaminated. *Xhh* was detected on the cuttings shortly after insertion but was not then detected again over the following two months during rooting until the polythene was removed and overhead watering was resumed. Following a peak after covers were removed, numbers showed a gradual decline

Discussion

Xhh was present on the leaves of stock plants at both sites without any visible symptoms of disease. However, it was not detected on all stock plants; it is possible that *Xhh* was present on some of these stock plants but in numbers which were below the detection threshold of the test. At both sites the numbers of *Xhh* on the cuttings decreased to undetectable levels during rooting, although it is likely that the bacterium was still present on some these trays of cuttings but in numbers which were below the threshold level of detection of the test. After rooting, removal of polythene covers, and resumption of frequent overhead watering, the numbers of *Xhh* detected on the leaves of cuttings increased to a peak. *Xhh* was also detected in trays of cuttings in which it had not been detected before and in the 'bait' trays of cuttings, indicating lateral spread of the pathogen between trays of cuttings. At both sites the numbers of *Xhh* then declined towards the end of the experiment.

The greater frequency of detection in trays adjacent to plants with symptoms at *Site 1*, indicates that infected symptomatic plants provide a much stronger source of inoculum than asymptomatic contaminated plants. Conversely, the very low frequency of detection in the trays furthest away from the symptomatic plants suggests that under the conditions of the trial, the rate of spread from tray to tray was relatively low.

Throughout the experimental period, symptoms were not seen on any of the cuttings which were part of the trial, suggesting that conditions for infection had not occurred. Thus it is likely that the *Xhh* detected on the cuttings was present as an a epiphyte on the surface of the leaves, and given the gradual decline in numbers toward the end of the experiment, that little multiplication was occurring.

It is clear from these results that symptomless stock plants can act as a primary source of the pathogen, highlighting the critical importance of producing and maintaining clean stock

plants for the control of this disease. In addition, at *Site 1* a neighbouring batch of cuttings with visible symptoms provided a stronger source of inoculum than the symptomless stock plants. This not only highlights the importance of separating batches of cuttings to prevent cross-infection but also demonstrates the critical importance of removing and destroying plant/leaves with visible symptoms. Clearly, growers should not take cuttings from plants showing visible symptoms, should inspect cuttings regularly and remove any with symptoms.

Spread of the pathogen between trays of cuttings was not detected until frequent overhead watering had been resumed after rooting. This is consistent with many other bacterial diseases in which spread occurs via water-splash. Overhead watering should therefore be avoided if possible by the use of drip or sub-irrigation systems (capillary matting, sand beds) or kept to the absolute minimum. This would also have other benefits in terms of reducing occurrence of weeds, mosses and liverworts and water conservation.

In practical terms, these results have highlighted some aspects of ivy propagation that will have an influence on subsequent disease development. However, further work is necessary to examine methods to produce and maintain clean stock plants on the nursery either using cultural approaches or chemicals; and to prevent re-infection of cuttings taken from clean stock plants. There is also the need to develop a disease indexing system for stock plants to ensure their health status; this could be based on the leaf wash system combined with selective media and antisera used here. Quantitative data on rates of spread and infection are also required in order to set minimum separation distances between batches of plants in different environmental conditions. There is no information on whether and for what length of time *Xhh* can survive as an epiphyte on ivy stock plants and other HNS.

Conclusions

These results confirm that *Xanthomonas hortorum* pv. *hederae* is the cause of a leaf spot disease of a range of ivy cultivars in the UK. *Xhh* has characteristics typical of a *Xanthomonas* sp. and requires the amino acid methionine as a growth factor in defined agar media.

A number of non-pathogenic isolates were obtained from diseased tissues, they probably represent secondary invaders of already moribund tissues or may be part of the normal leaf surface micro-flora. These isolates were used in the development of selective media and diagnostic reagents. Some isolates obtained as *Xhh* from the NCPPB appear to have been mis-identified.

Isolates of *Xhh* were not pathogenic on *Brassaia actinophylla* as reported by researchers in the USA.

The carbon utilisation results indicated little phenotypic variability amongst isolates of *Xhh* from ivy in the UK.

The results of the DNA fingerprinting using RAPD-PCR and ten primers indicated 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 seemed likely that the disease is primarily disseminated with the cuttings and spreads between cultivars during production.

The carbon utilisation and antibiotic sensitivity data were used to develop two semi-selective media, modified Tween and Brilliant cresol blue cellobiose (BCBC), for the detection and isolation of Xhh during epidemiological studies. Both media apparently give a high level of recovery of and selectivity for the pathogen *Xhh*.

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.

Host resistance studies were done using thirty-five cultivars of ivy and ten isolates of *Xhh* representative of the different geographical/cultivars of origin and the two genotypic groups. None of the cultivars tested were resistant to *Xhh*, but some showed reduced susceptibility to all isolates. The least susceptible species/cultivars to all the isolates tested were *Hedera rhombea*, *Hedera rhombea* cv. Variegata and *Hedera helix* cv. Tanja. In general, non-*helix* species tended to be the least susceptible.

There were no indications of the existence of pathogenic races within *Xhh.*

Nursery studies showed that *Xhh* could be present on stock plants with no visible symptoms of disease. Spread of the pathogen between batches of cuttings/plants occurs via water-splash resulting from overhead watering. Overhead watering therefore should be kept to a minimum, and batches of plants should if possible be separated by other genera.

Recommendations for further work

- The nursery based studies showed that the primary source of the pathogen was the stock plants. Further work should be directed at the development of methods produce and maintain disease-free stock plants.
- Development of an indexing system to assure that stock plants are disease-free.
- The resistance studies indicated that some species/cultivars are less susceptible. Further resistance screening should be done targeted at species most closely related to those identified as less susceptible.
- Direct comparisons of the benefits of using 'less susceptible' varieties compared to the more common varieties
- Obtain quantitative data on the rate of spread in both time and space. This would then be used to set separation distances and set tolerance standards for the health of stock plants/cuttings

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Table 1. Sources of isolates of used in this study. Continued on next page

Isolate No.	Species	Cultivar	Country	County	Site	DNA[1]
Xanthomonas hortorum *pv*. hederae *isolates*						
5690	*Hedera helix*	Green Ripple	UK	Suffolk	1	
5691B	*Hedera helix*	Green Ripple	UK	Suffolk	1	*
5691C	*Hedera helix*	Green Ripple	UK	Suffolk	1	
5863[2]	***Hedera helix***	**Green Ripple**	**UK**	**Yorkshire**	**2**	*
5867	*Hedera hibernica*	-	UK	Yorkshire	2	*
5887	*Hedera helix*	Green Ripple	UK	Yorkshire	2	
5889	*Hedera helix*	Glacier	UK	Yorkshire	2	*
5993	***Hedera helix***	**Goldheart**	**UK**	**Yorkshire**	**2**	*
5998	*Hedera hibernica*	-	UK	Yorkshire	2	
7053B	***Hedera hibernica***	**-**	**UK**	**Hampshire**	**3**	*
7063	*Hedera helix*	Green Ripple	UK	Hampshire	3	*
7169	*Hedera hibernica*	-	UK	Hampshire	4	
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**	*
7220	*Hedera helix*	-	UK	Hampshire	5	
7714	***Hedera colchica***	**Dentata**	**UK**	**Hampshire**	**6**	*
7715	*Hedera colchica*	Dentata	UK	Hampshire	6	
7716	*Hedera colchica*	Dentata	UK	Hampshire	6	
7717B	*Hedera algeriensis*	Ravensholst	UK	Hampshire	6	*
7718	*Hedera helix*	Angularis Aurea	UK	Hampshire	6	*
7719B	*Hedera helix*	Angularis Aurea	UK	Hampshire	6	
7720	*Hedera helix*	Cristata	UK	Hampshire	6	*
7721B	*Hedera helix*	Chrysophylla	UK	Hampshire	6	
7722	*Hedera hibernica*	-	UK	Hampshire	6	*
7723	*Hedera hibernica*	-	UK	Hampshire	6	
7724B	*Hedera maderiensis* subsp. *iberica*		UK	Hampshire	6	
7725B	*Hedera maderiensis* subsp. *iberica*		UK	Hampshire	6	*
7726B	*Hedera hibernica*	Deltoidea	UK	Hampshire	6	*
7730E	*Hedera helix*	Green Ripple	UK	Worcestershire	7	*
7731A	***Hedera helix***	**Buttercup**	**UK**	**Oxfordshire**	**8**	*
7731B	*Hedera helix*	Buttercup	UK	Oxfordshire	8	
7732A	*Hedera helix*	Goldheart	UK	Oxfordshire	8	*
7732C	*Hedera helix*	Goldheart	UK	Oxfordshire	8	
7733A	*Hedera colchica*	Dentata Variegata	UK	Herefordshire	9	
7733B	*Hedera colchica*	Dentata Variegata	UK	Herefordshire	9	*
7733C	*Hedera colchica*	Dentata Variegata	UK	Herefordshire	9	
7734A	*Hedera algeriensis*	-	UK	Herefordshire	9	*
7734B	*Hedera algeriensis*	-	UK	Herefordshire	9	
7734C	***Hedera algeriensis***	**-**	**UK**	**Herefordshire**	**9**	
7735A	*Hedera helix*	Green Ripple	UK	Herefordshire	9	*
7735B	*Hedera helix*	Green Ripple	UK	Herefordshire	9	
7736B	*Hedera helix*	Jesters Gold	UK	Herefordshire	9	*
7737C	*Hedera helix*	Goldchild	UK	Herefordshire	9	*
7738B	*Hedera helix*	Cristata	UK	Hampshire	10	
7738C	***Hedera helix***	**Cristata**	**UK**	**Hampshire**	**10**	*
7738D	*Hedera helix*	Cristata	UK	Hampshire	10	
7738E	*Hedera helix*	Cristata	UK	Hampshire	10	

Table 1 continued. Sources of isolates of used in this study.

Isolate No.	Species	Cultivar	Country	County	Site	DNA[1]
Xanthomonas hortorum *pv*. hederae *obtained from NCPPB*						
7743 (NCPPB 2011)	*Hedera helix*	-	UK	-	-	*
7744 (NCPPB 939)	***Hedera helix***	**-**	**USA**	**-**	**-**	*
7746 (NCPPB 642)	***Hedera helix***	**-**	**Denmark**	**-**	**-**	*
7747 (NCPPB 2336)	*Hedera helix*	-	UK	-	-	
Obtained from NCPPB as Xanthomonas hortorum *pv.* hederae *but non-pathogenic*						
7745 (NCPPB 987)	*Hedera helix*	-	USA	-	-	
7789 (NCPPB 3588)	*Schefflera arboricola*	-	USA	Florida (ex Chase)	-	*
7790 (NCPPB 3589)	*Schefflera arboricola* (*Brassaia actinophylla* in on-line ATCC database)		USA	Florida (ex Chase)	-	*
Non-pathogenic non-Xanthomonas *isolates*						
7043	*Hedera helix*	Green Ripple	UK	Yorkshire	2	
7717A	*Hedera algeriensis*	Ravensholst	UK	Hampshire	6	
7719A	*Hedera helix*	Angularis Aurea	UK	Hampshire	6	
7721A	*Hedera helix*	Chrysophylla	UK	Hampshire	6	
7724A	*Hedera maderiensis*	Iberica	UK	Hampshire	6	
7725A	*Hedera maderiensis*	Iberica	UK	Hampshire	6	
7726A	*Hedera hibernica*	Deltoidea	UK	Hampshire	6	
7730AA	*Hedera helix*	Green Ripple	UK	Worcestershire	7	
7730AB	*Hedera helix*	Green Ripple	UK	Worcestershire	7	
7730B	*Hedera helix*	Green Ripple	UK	Worcestershire	7	
7730C	*Hedera helix*	Green Ripple	UK	Worcestershire	7	
7730D	*Hedera helix*	Green Ripple	UK	Worcestershire	7	
7731C	*Hedera helix*	Buttercup	UK	Oxfordshire	8	
7732B	*Hedera helix*	Goldheart	UK	Oxfordshire	8	
7735C	*Hedera helix*	Green Ripple	UK	Herefordshire	9	
7736C	*Hedera helix*	Jesters Gold	UK	Herefordshire	9	
7737A	*Hedera helix*	Goldchild	UK	Herefordshire	9	
7737B	*Hedera helix*	Goldchild	UK	Herefordshire	9	
7738A	*Hedera helix*	Cristata	UK	Hampshire	10	
Xanthomonas hortorum *pv*. pelargonii						
5616	*Pelargonium* sp.	-	UK	-	-	
6012	*Pelargonium* sp.	-	Italy	-	-	
Xanthomonas campestris *pv*. campestris						
5421 (NCPPB 528)	*Brassica oleracea*	-	UK	-	-	
Pseudomonas syringae *pv*. syringae						
5411 (NCPPB 281)	*Syringa vulgaris*	-	UK	-	-	
Xanthomonas axonopodis *pv*. malvacearum						
5232 (NCPPB 633)	*Gossypium* sp.	-	Sudan	-	-	
Xanthomonas arboricola *pv*. pruni						
6014	*Prunus* sp.	-	Italy	-	-	
Xanthomonas fragariae						
6013	*Fragaria* sp.	-	Italy	-	-	
Xanthomonas hortorum *pv*. carotae						
6301 (NCPPB 3440)	*Daucus carota*	-	Brazil	-	-	
Xanthomonas oryzae *pv*. oryzae						
5233 (NCPPB 3002)	*Oryzum* sativa	-	India	-	-	
Xanthomonas vesicatoria						
5235 (NCPPB 422)	*Lycopersicon esculentum*		N. Zealand	-		

[1]Isolates used for DNA fingerprinting indicated with *.
[2]Isolates in **bold typeface** were used for resistance screening.

Table 2. Phenotypic characteristics of 54 isolates of *Xanthomonas hortorum* pv. *hederae* from *Hedera* spp. Values in the table are the percentage of isolates which were positive for a particular character.

Yellow mucoid growth on YDC	100	Oxidative metabolism	100
Gram reaction	100	Growth on 0.1% TTC	0
Catalase	100	Growth on 0.02% TTC	0
Oxidase	100	Pathogenic to *H. helix* cv. Green Ripple	100
Hydrolysis of:			
Aesculin	100	Starch	100
Gelatin	30		
Carbon utilisation:			
Acetate	0	Malonate	0
D-Alanine	100	D-Maltose	100
L-Alanine	100	D-Mannitol	100
L-Arabinose	100	D-Mannose	100
L-Asparagine	100	D-Melibiose	100
L-aspartate	81[1]	L-Ornithine	0
Cellobiose	100	L-Proline	100
Citrate	0	Pyruvate	100
Dextrin	100	D-Raffinose	11[1]
D-Fructose	100	L-Serine	100
Fumarate	100	D-Sorbitol	100
D-Galactose	100	Starch	0
Gluconate	0	Succinate	100
α-D-Glucose	100	Sucrose	100
L-Glutamate	0	L-Threonine	0
Glycerol	100	D-Trehalose	100
Glycogen	100	Tween 20	31[1]
D,L-Lactate	0	Tween 40	100
α-D-Lactose	0	Tween 80	100
D,L-Lysine	0	L-Xylose	0[2]

[1]Variable results: values represent the percentage of isolates giving weak positive to positive results.
[2]Variable results: isolates either negative or weak positive

Table 3. Optimised composition of RAPD-PCR reaction mix for a 25 µl reaction volume.

Component	Quantity (µl)	Stock concentration
RO water	16.0	
PCR buffer (Gibco)	2.5	10x
$MgCl_2$ (Gibco)	0.37	0.75 mM
DNTPs (Gibco)	0.025 each	100 µM
Taq (Gibco)	0.3	5 Units/µl
Template DNA	2.0	2.5 ng/µl
Primer	3.75	0.6 µM

Table 4. PCR primer sequences used for genetic fingerprinting of *Xanthomonas hortorum* pv. *hederae*

Primer	Sequence 5'-3'
OPG2	GGCACTGAGG
OPG3	GAGCCCTACA
OPG4	AGCGTGTGTG
OPG5	CTGAGACGGA
OPG10	AGGGCCGTCT
OPG11	TGCCCGTCGT
OPG12	CAGCTCACGA
OPG13	CTCTCCGCCA
OPG18	GGCTCATGTG
OPG19	GTCAGGGCAA

Table 5. Proportion of infected wounds (p) and their mean diameter (diam.) for pin inoculated leaves, and mean no. of lesions per leaf for spray inoculated leaves for the first batch of ivy species and cultivars inoculated with ten isolates of *Xanthomonas hortorum* pv. *hortorum.* Species/cultivars are listed in ascending order of mean lesion diameter (i.e. from least to most susceptible).

Hedera species	Cultivar	Isolate																	
		5863			5993			7053			7219			7714			7731		
		p	diam	no.	p	diam	no.	p	diam	no.	p	diam	no.	p	diam	no.	p	diam	no.
maderiensis subsp. *iberica*		1.0	1.0	38.3	1.0	2.0	47.8	1.0	1.8	13.5	1.0	1.6	31.3	1.0	1.9	32.5	1.0	1.7	31.3
helix	Chrysophylla	1.0	2.5	29.3	1.0	2.3	60.0	1.0	2.2	33.8	1.0	2.0	60.0	1.0	3.5	12.8	1.0	2.4	6.3
hibernica	Deltoidea	1.0	2.6	31.3	1.0	2.3	25.8	1.0	2.1	30.5	1.0	3.0	33.5	1.0	2.5	15.0	1.0	2.6	20.0
canariensis var. *algeriensis*	Ravensholst	1.0	2.2	60.0	1.0	2.7	49.5	1.0	2.1	42.5	1.0	2.3	60.0	1.0	2.8	47.5	1.0	2.5	50.0
canariensis var. *algeriensis*	un-named	1.0	3.2	48.0	1.0	2.9	52.0	1.0	2.9	35.8	1.0	2.9	53.0	1.0	3.7	47.3	1.0	2.4	49.0
colchica	Dentata	1.0	2.7	60.0	1.0	3.2	41.8	1.0	2.4	26.8	1.0	2.8	49.0	1.0	2.6	47.5	1.0	3.4	35.5
helix	Angularis Aurea	1.0	2.9	33.5	1.0	2.9	44.0	1.0	3.1	44.3	1.0	2.5	43.3	1.0	3.0	60.0	1.0	4.0	47.0
helix	Brokamp	1.0	2.6	41.3	1.0	2.6	49.8	1.0	2.5	26.0	1.0	3.6	57.0	1.0	3.5	54.5	1.0	4.2	29.3
helix	un-named	1.0	2.3	47.5	1.0	2.9	47.5	1.0	2.3	60.0	1.0	3.2	38.3	1.0	3.1	36.8	1.0	2.9	34.3
helix	California	1.0	3.1	30.5	1.0	3.3	42.0	1.0	2.6	47.8	1.0	3.2	41.5	1.0	3.4	28.0	1.0	3.4	52.5
colchica	Dentata Variegata	1.0	3.5	26.8	1.0	2.6	36.3	1.0	3.0	37.5	1.0	2.8	44.8	1.0	2.6	39.0	1.0	3.7	50.5
helix	Green Ripple	1.0	3.2	45.5	1.0	4.1	60.0	1.0	3.0	39.0	1.0	3.3	60.0	1.0	3.4	50.3	1.0	3.2	49.0
helix	Eva	1.0	3.7	40.8	1.0	3.9	38.5	1.0	3.3	50.0	1.0	3.5	27.0	1.0	3.1	60.0	1.0	3.1	47.8
helix	Glacier	1.0	3.8	35.5	1.0	3.6	44.0	1.0	3.5	54.5	1.0	3.5	46.0	1.0	3.3	42.8	1.0	3.9	24.5
helix	Goldheart	1.0	3.0	40.0	1.0	2.9	53.8	1.0	2.7	39.0	1.0	4.3	32.3	1.0	3.3	48.0	1.0	5.2	46.8
helix	Ivalace	1.0	3.8	39.8	1.0	3.9	44.0	1.0	3.4	32.5	1.0	3.3	42.5	1.0	3.9	52.5	1.0	4.0	37.8
helix	Telecurl	1.0	3.7	49.8	1.0	3.7	54.0	1.0	3.6	44.3	1.0	3.7	34.8	1.0	3.4	38.3	1.0	4.3	38.0
helix	Goldchild	1.0	3.7	24.3	1.0	3.0	51.3	1.0	3.7	23.3	1.0	3.1	21.0	1.0	4.2	43.8	1.0	4.1	50.5
helix	Buttercup	1.0	3.6	46.0	1.0	3.5	45.0	1.0	4.1	39.8	1.0	4.0	48.5	1.0	4.1	42.3	1.0	4.4	42.5
helix	Manda's Crested	1.0	4.1	46.5	1.0	4.7	50.0	1.0	4.3	47.3	1.0	5.5	53.8	1.0	5.2	44.0	1.0	4.1	55.0

Table 5 continued. Proportion of infected wounds (p) and their mean diameter (diam.) for pin inoculated leaves, and mean no. of lesions per leaf for spray inoculated leaves for the first batch ivy species and cultivars inoculated with ten isolates of *Xanthomonas hortorum* pv. *hortorum.* Species/cultivars are listed in ascending order of mean lesion diameter (i.e. from least to most susceptible).

Hedera species	Cultivar	Isolate																	
		7734			7738			7744			7746			Mean					
		p	diam	no.	p	diam	no.	p	diam	no.	p	diam	no.	p	s.e.	diam	s.e.	no.	s.e.
maderiensis subsp. *iberica*		1.0	3.7	44.8	1.0	1.6	32.8	1.0	1.6	41.5	1.0	1.8	52.5	1.00	0.00	1.9	0.05	36.6	2.53
helix	Chrysophylla	1.0	3.0	60.0	1.0	2.9	32.5	1.0	1.8	0.0	1.0	2.0	44.3	1.00	0.00	2.4	0.05	33.9	2.43
hibernica	Deltoidea	1.0	2.6	14.0	1.0	2.8	31.0	1.0	1.8	7.8	1.0	2.4	8.0	1.00	0.00	2.5	0.05	21.7	1.95
canariensis var. *algeriensis*	Ravensholst	1.0	4.0	41.3	1.0	3.6	45.3	1.0	1.9	0.0	1.0	2.2	16.3	1.00	0.00	2.6	0.05	41.2	2.69
canariensis var. *algeriensis*	un-named	1.0	3.1	42.5	1.0	2.2	29.0	1.0	1.4	60.0	1.0	2.2	43.3	1.00	0.00	2.7	0.05	46.0	2.84
colchica	Dentata	1.0	2.8	60.0	1.0	3.9	21.3	1.0	2.1	30.8	1.0	2.4	21.0	1.00	0.00	2.8	0.05	39.4	2.62
helix	Angularis Aurea	1.0	3.3	60.0	1.0	3.3	49.8	1.0	1.4	15.8	1.0	2.1	60.0	1.00	0.00	2.8	0.05	45.8	2.83
helix	Brokamp	1.0	2.5	46.3	1.0	3.2	18.0	1.0	1.6	17.3	1.0	2.5	45.5	1.00	0.00	2.9	0.05	38.5	2.59
helix	un-named	1.0	4.4	60.0	1.0	4.1	43.3	1.0	1.8	43.5	1.0	2.3	60.0	1.00	0.00	2.9	0.05	47.1	2.87
helix	California	1.0	3.2	46.3	1.0	3.5	41.5	1.0	2.0	17.5	1.0	2.6	34.5	1.00	0.00	3.0	0.05	38.2	2.59
colchica	Dentata Variegata	1.0	3.9	39.5	1.0	3.4	41.3	1.0	2.0	21.8	1.0	3.4	41.0	1.00	0.00	3.1	0.05	37.8	2.57
helix	Green Ripple	1.0	4.0	53.8	1.0	3.4	54.8	1.0	1.2	46.3	1.0	2.5	52.5	1.00	0.00	3.1	0.05	51.1	2.99
helix	Eva	1.0	3.7	60.0	1.0	3.3	42.5	1.0	1.8	24.3	1.0	2.9	21.5	1.00	0.00	3.2	0.05	41.2	2.69
helix	Glacier	1.0	3.4	60.0	1.0	3.8	43.3	1.0	1.5	34.0	1.0	2.8	51.0	1.00	0.00	3.3	0.05	43.6	2.76
helix	Goldheart	1.0	4.2	60.0	1.0	3.2	55.0	1.0	2.1	44.0	1.0	3.4	50.0	1.00	0.00	3.4	0.05	46.9	2.86
helix	Ivalace	1.0	3.2	60.0	1.0	3.6	31.3	1.0	2.9	17.5	1.0	2.6	13.8	1.00	0.00	3.4	0.05	37.2	2.55
helix	Telecurl	1.0	2.5	60.0	1.0	4.1	30.5	1.0	2.7	24.5	1.0	3.3	60.0	1.00	0.00	3.5	0.05	43.4	2.76
helix	Goldchild	1.0	4.8	60.0	1.0	4.0	60.0	1.0	2.8	12.8	1.0	3.6	49.3	1.00	0.00	3.7	0.05	39.6	2.63
helix	Buttercup	1.0	5.0	60.0	1.0	3.7	49.0	1.0	2.6	19.3	1.0	3.3	42.5	1.00	0.00	3.8	0.05	43.5	2.76
helix	Manda's Crested	1.0	5.2	60.0	1.0	3.7	60.0	1.0	2.3	17.8	1.0	4.0	37.8	1.00	0.00	4.3	0.05	47.2	2.87

Table 6. Proportion of infected wounds (p) and their mean diameter (diam.) for pin inoculated leaves, and mean no. of lesions per leaf for spray inoculated leaves for the second batch of ivy species and cultivars inoculated with ten isolates of *Xanthomonas hortorum* pv. *hortorum.* Species/cultivars are listed in ascending order of mean lesion diameter (i.e. from least to most susceptible).

Hedera species	Cultivar	Isolate																
		5863			5993			7053			7219			7714			7731	
		p	diam	no.	p	diam	no.	p	diam	no.	p	diam	no.	p	diam	no.	p	diam	no.
rhombea	Variegata	1.0	1.0	44.0	0.6	0.6	56.3	0.8	0.4	41.0	0.5	0.4	54.5	1.0	0.9	50.3	0.4	0.5	42.3
rhombea	un-named	0.0	0.0	47.0	0.8	0.5	38.3	0.8	1.2	44.3	0.0	0.0	60.0	0.5	0.4	46.0	1.0	2.2	32.5
helix	Tanja	1.0	1.3	19.5	0.1	0.0	24.5	0.5	0.4	38.3	0.3	0.1	48.0	0.8	0.9	43.8	1.0	1.5	7.3
nepalensis var. *sinensis*	un-named	0.9	1.2	39.5	0.5	0.4	60.0	1.0	1.3	52.5	1.0	0.8	60.0	1.0	1.0	60.0	1.0	1.6	37.5
nepalensis	un-named	1.0	1.5	35.5	1.0	1.5	60.0	1.0	1.4	41.3	1.0	1.0	42.5	1.0	1.0	43.0	1.0	1.7	43.5
maroccana	Spanish Canary	1.0	1.0	56.3	1.0	1.9	60.0	1.0	0.8	45.3	1.0	0.9	44.3	1.0	1.0	60.0	1.0	1.8	37.3
cypria	un-named	0.5	0.5	12.3	1.0	1.4	42.3	1.0	0.9	35.3	0.5	0.3	55.0	1.0	1.1	29.3	1.0	1.9	50.0
azorica	Pico	1.0	1.2	60.0	0.5	0.6	60.0	1.0	1.0	56.0	1.0	0.6	60.0	1.0	1.4	56.3	1.0	1.8	56.8
helix	Cuspidata Major	1.0	1.9	24.0	1.0	1.2	24.3	1.0	1.1	29.3	0.9	0.9	15.5	1.0	1.6	24.5	1.0	2.4	19.5
maderiensis subsp. *iberica*	un-named	1.0	1.0	45.5	1.0	1.4	40.3	1.0	1.3	7.8	1.0	1.2	39.8	1.0	1.5	19.8	1.0	1.9	31.3
maroccana	Marocco	1.0	1.1	54.3	1.0	1.3	60.0	1.0	1.5	40.3	1.0	0.9	53.8	1.0	0.5	60.0	1.0	2.4	52.0
helix	Pittsburgh	1.0	1.4	60.0	1.0	1.5	55.5	1.0	1.4	36.3	1.0	1.0	45.0	1.0	1.3	47.0	1.0	2.4	55.0
helix	Minima	1.0	1.5	7.5	1.0	1.1	7.3	1.0	1.7	8.8	1.0	1.4	31.5	1.0	1.7	4.8	1.0	1.7	11.3
canariensis var. *algeriensis*	Gloire de Marengo	1.0	1.1	60.0	1.0	2.3	56.3	1.0	1.0	38.0	1.0	1.2	60.0	1.0	1.2	60.0	1.0	2.1	60.0
colchica	Sulphur Heart	1.0	1.5	38.8	1.0	1.6	51.8	1.0	2.3	55.0	1.0	1.3	60.0	1.0	1.5	35.5	1.0	1.7	46.5
helix	Golden Ingot	1.0	1.8	60.0	1.0	1.9	60.0	1.0	1.5	60.0	1.0	1.5	60.0	1.0	1.9	60.0	1.0	3.2	52.0
helix	Green Ripple	1.0	2.2	60.0	1.0	2.6	48.0	1.0	1.9	35.0	1.0	2.0	42.3	1.0	2.1	60.0	1.0	2.4	24.3

Table 6 continued. Proportion of infected wounds (p) and their mean diameter (diam.) for pin inoculated leaves, and mean no. of lesions per leaf for spray inoculated leaves for the second batch ivy species and cultivars inoculated with ten isolates of *Xanthomonas hortorum* pv. *hortorum.* Species/cultivars are listed in ascending order of mean lesion diameter (i.e. from least to most susceptible).

Hedera species	Cultivar	Isolate												Mean					
		7734			7738			7744			7746								
		p	diam	no.	p	diam	no.	p	diam	no.	p	diam	no.	p	s.e.	diam	s.e.	no.	s.e.
rhombea	Variegata	0.3	0.1	27.8	0.9	0.5	25.0	0.1	0.1	60.0	0.3	0.3	41.3	0.57	0.04	0.5	0.13	44.2	2.78
rhombea	un-named	0.5	0.3	22.3	1.0	1.4	32.3	0.3	0.1	60.0	1.0	1.1	42.5	0.58	0.03	0.7	0.13	42.5	2.73
helix	Tanja	1.0	1.6	37.0	1.0	1.7	28.0	0.8	0.5	39.8	1.0	0.9	25.8	0.72	0.03	0.9	0.13	31.2	2.34
nepalensis var. *sinensis*	un-named	1.0	1.3	47.8	1.0	1.4	50.5	1.0	0.9	49.3	1.0	0.5	42.5	0.93	0.02	1.0	0.13	50.0	2.96
nepalensis	un-named	1.0	1.1	40.5	1.0	1.5	39.3	1.0	0.9	54.0	1.0	1.2	21.0	1.00	0.00	1.3	0.13	42.1	2.71
maroccana	Spanish Canary	1.0	2.6	54.3	1.0	2.0	60.0	1.0	0.9	47.0	0.8	0.5	32.8	0.98	0.01	1.3	0.13	49.7	2.95
cypria	un-named	1.0	2.6	0.0	1.0	2.7	45.5	1.0	1.3	0.0	1.0	0.9	8.3	0.89	0.02	1.4	0.13	27.8	2.20
azorica	Pico	1.0	2.2	60.0	1.0	2.1	53.0	1.0	1.4	60.0	1.0	1.6	55.8	0.95	0.02	1.4	0.13	57.8	3.18
helix	Cuspidata Major	1.0	1.6	16.3	1.0	2.1	37.0	1.0	1.0	45.0	1.0	0.6	40.3	0.98	0.01	1.4	0.13	27.6	2.20
maderiensis subsp. *iberica*	un-named	1.0	2.5	32.0	1.0	1.8	26.8	1.0	1.1	32.5	1.0	1.2	34.0	1.00	0.00	1.5	0.13	31.0	2.33
maroccana	Marocco	1.0	2.2	60.0	1.0	3.3	60.0	1.0	1.0	60.0	1.0	1.1	48.0	1.00	0.00	1.5	0.13	54.8	3.10
helix	Pittsburgh	1.0	2.2	34.8	1.0	2.1	45.5	1.0	1.1	43.8	1.0	1.0	60.0	1.00	0.00	1.5	0.13	48.3	2.91
helix	Minima	1.0	2.0	6.3	1.0	1.8	21.8	1.0	1.1	4.5	1.0	1.4	11.0	1.00	0.00	1.5	0.13	11.5	1.42
canariensis var. *algeriensis*	Gloire de Marengo	1.0	2.7	60.0	1.0	1.9	60.0	1.0	1.3	55.0	1.0	0.9	60.0	0.99	0.01	1.6	0.13	56.9	3.16
colchica	Sulphur Heart	1.0	2.3	60.0	1.0	1.9	53.8	1.0	1.2	47.5	1.0	1.0	42.5	1.00	0.00	1.6	0.13	49.1	2.93
helix	Golden Ingot	1.0	2.4	60.0	1.0	2.1	49.8	1.0	1.6	60.0	1.0	1.3	44.0	1.00	0.00	1.9	0.13	56.6	3.15
helix	Green Ripple	1.0	2.7	42.8	1.0	2.6	42.3	1.0	1.3	46.3	1.0	2.3	52.0	1.00	0.00	2.2	0.13	45.3	2.81

Table 7 Proportion of infected wounds (p) and their mean diameter (diam.) for pin inoculated leaves, and mean no. of lesions per leaf for spray inoculated leaves for ten isolates of *Xanthomonas hortorum* pv. *hortorum* inoculated into two batches of ivy cultivars.

Isolate	DNA Group	p	s.e.[1]	diam	s.e.	no.	s.e.
First batch (20 ivy species/cvs.)							
5863	2	1.00	0.00	3.0	0.03	40.7	1.9
5993	1	1.00	0.00	3.1	0.03	46.8	2.0
7053	2	1.00	0.00	2.9	0.03	38.4	1.8
7219	1	1.00	0.00	3.2	0.03	43.9	2.0
7714	1	1.00	0.00	3.3	0.03	42.1	1.9
7731	1	1.00	0.00	3.5	0.03	39.9	1.9
7734	2	1.00	0.00	3.6	0.03	52.4	2.1
7738	1	1.00	0.00	3.3	0.03	40.6	1.9
7744	1	1.00	0.00	1.9	0.03	24.8	1.5
7746	2	1.00	0.00	2.7	0.03	40.2	1.9
Second Batch (17 ivy species/cvs.)							
5863	2	0.91	0.01	1.2	0.1	42.6	2.1
5993	1	0.84	0.02	1.3	0.1	47.3	2.2
7053	2	0.94	0.02	1.2	0.1	39.1	2.0
7219	1	0.83	0.02	0.9	0.1	48.9	2.2
7714	1	0.96	0.01	1.2	0.1	44.7	2.2
7731	1	0.96	0.01	1.9	0.1	38.8	2.0
7734	2	0.92	0.01	1.9	0.1	38.9	2.0
7738	1	0.99	0.01	1.9	0.1	43.0	2.1
7744	1	0.89	0.01	1.0	0.1	45.0	2.2
7746	2	0.94	0.01	1.0	0.1	38.9	2.0

[1] s.e.: standard error

Table 8. Antibiotic sensitivity of *Xanthomonas hortorum* pv. *hederae* (*Xhh*) and saprophytes from ivy (non-Xhh). Values in the table are the percentage of isolates which grew on Nutrient agar medium containing the antibiotic.

Antibiotic	Dissolved in:	Conc. (µg/ml)	*Xhh* (54 isolates)	Non-*Xhh* (20 isolates)
Ampicillin	RO water[1]	20	81	60
Bacitracin	RO water	100	81	90
Boric acid	RO water	1500	91	100
Brilliant cresol blue	RO water	40	100	85
Cephalexin	RO water	80	74	80
Chlorothalonil	70% EtOH[2]	200	100	100
Cycloheximide	70% EtOH	200	100	90
5-fluorouracil	RO water	20	100	100
Gentamycin	RO water	0.4	100	100
Kanamycin	RO water	50	0	0
Methyl green	RO water	40	91	90
nitrofurantoin	50% DMF[3]	10	83	95
Penicillin G	RO water	100	26	50
Pyridoxine	RO water	40	100	100
trimethoprim	RO water	30	100	100
Vancomycin	RO water	10	100	90

[1] RO: reverse osmosis
[2] EtOH: ethanol
[3] DMF: dimethyl formamide

Table 9. Percentage of *Xanthomonas hortorum* pv. *hederae* (*Xhh*) and saprophytes (non-*Xhh*) from ivy able to grow on four selective media developed for *Xanthomonas* spp.

	Medium			
	NSCAA	D-5	Tween	CS
Xhh (54 isolates)	0	100	98	0
Non-*Xhh* (20 isolates)	55	90	35	40

NSCAA: Nutrient starch cellobiose agar for isolation of *Xanthomonas campestris* pv. *campestris* (Randhawa and Schaad, 1984)
D-5: for isolation of *Xanthomonas hortorum* pv. *carotae* (Kuan *et al*., 1985).
Tween: for isolation of *Xanthomonas vesicatoria* (McGuire, 1986).
CS: Cellobiose starch medium for isolation of *Xanthomonas campestris* pv. *diffenbachiae* (Norman and Alvarez, 1989)

Table 10. Effect of pH on the recovery of *Xanthomonas hortorum* pv. *hederae* isolates on Brilliant Cresol Blue Cellobiose medium (BCBC). Values are the mean counts (± s.e.) of two replicate plates at the 10^{-6} dilution.

Medium	Isolate		
	7183	7733C	7744
BCBC pH 6.8	65 ± 4.0	55 ± 3.7	42 ± 3.2
BCBC pH 7.0	40 ± 3.2	42 ± 3.2	33 ± 2.9
BCBC pH 7.2	28 ± 2.6	30 ± 2.7	20 ± 2.2

Table 11. Recovery of *Xanthomonas hortorum* pv. *hederae* on non-selective (YDC) and semi-selective (BCBC and modified Tween) media. Values are the mean counts (± s.e.) on two replicate plates at the dilution.

Medium	Isolate (dilution)		
	7183 (x 10^{-6})	7733C (x 10^{-6})	7744 (x 10^{-5})
YDC	11 ± 2.4	11 ± 2.3	10 ± 2.2
BCBC	69 ± 5.9	39 ± 4.4	49 ± 5.0
modified Tween	70 ± 5.9	31 ± 3.9	40 ± 4.5

Table 12. *Xanthomonas hortorum* pv. *hederae* isolates from ivy agglutinating with *Staphylococcus aureus*-conjugated antisera raised to different *Xanthomonas* spp.

Antiserum	Isolates giving positive agglutination
Xanthomonas axonopodis pv. *malvacearum*	5863
Xanthomonas arboricola pv. *pruni*	5889, 7735B, 7737C
Xanthomonas campestris pv. *campestris*	5863, 7053B
Xanthomonas fragariae	5863, 5993, 5998, 7053B, 7220, 7716, 7730E
Xanthomonas hortorum pv. *carotae*	7730E
Xanthomonas hortorum pv. *pelargonii*	7730E
Xanthomonas oryzae pv. *oryzae*	5998, 7219, 7735B
Xanthomonas vesicatoria	5863, 7053B, 7204, 7219,

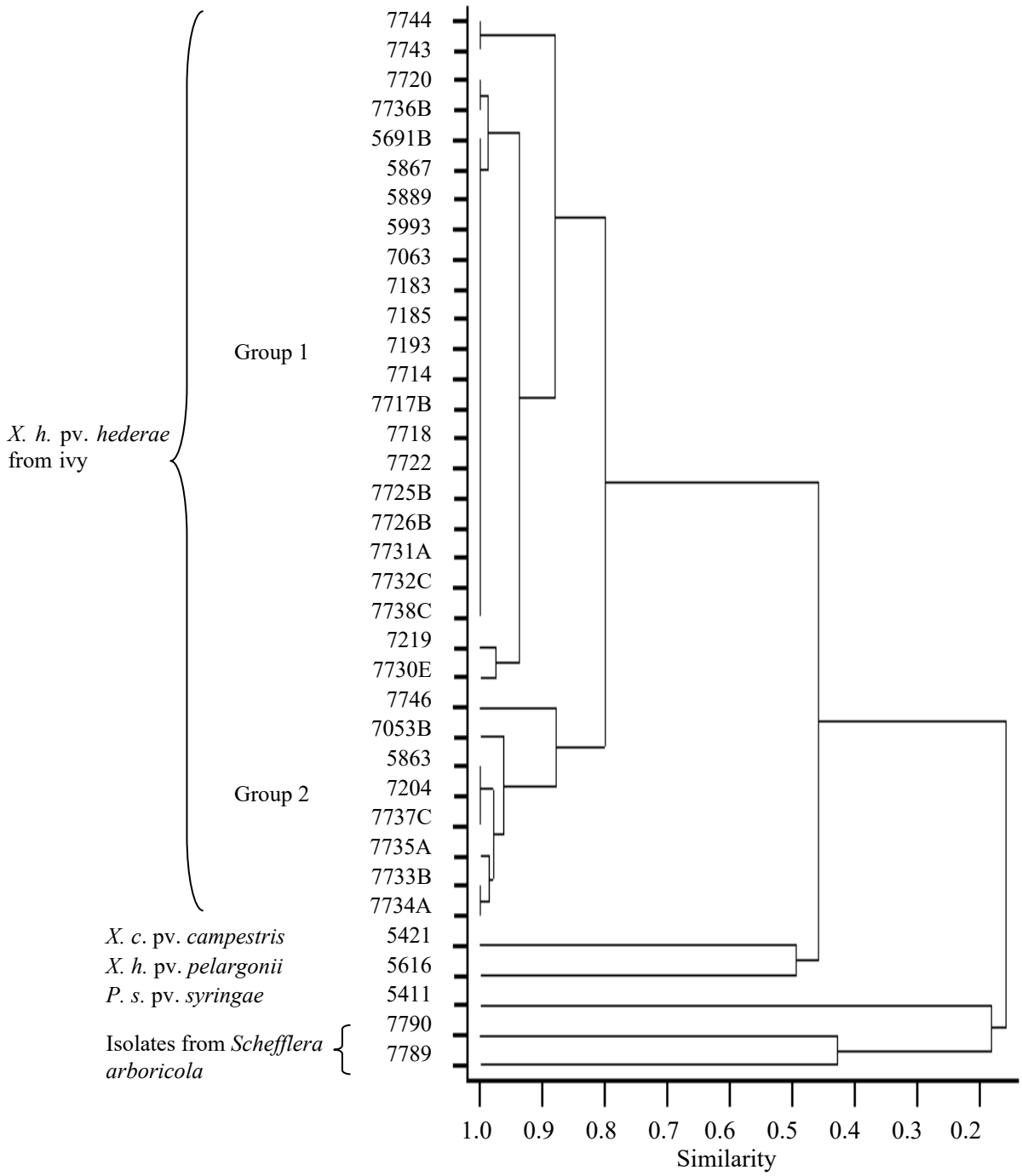

Fig 1. Dendrogram obtained by the average linkage method showing the similarity of DNA fingerprints amongst isolates of *Xanthomonas hortorum* pv. *hederae* from ivy, based on RAPD-PCR with ten 10-mer oligonucleotide primers.

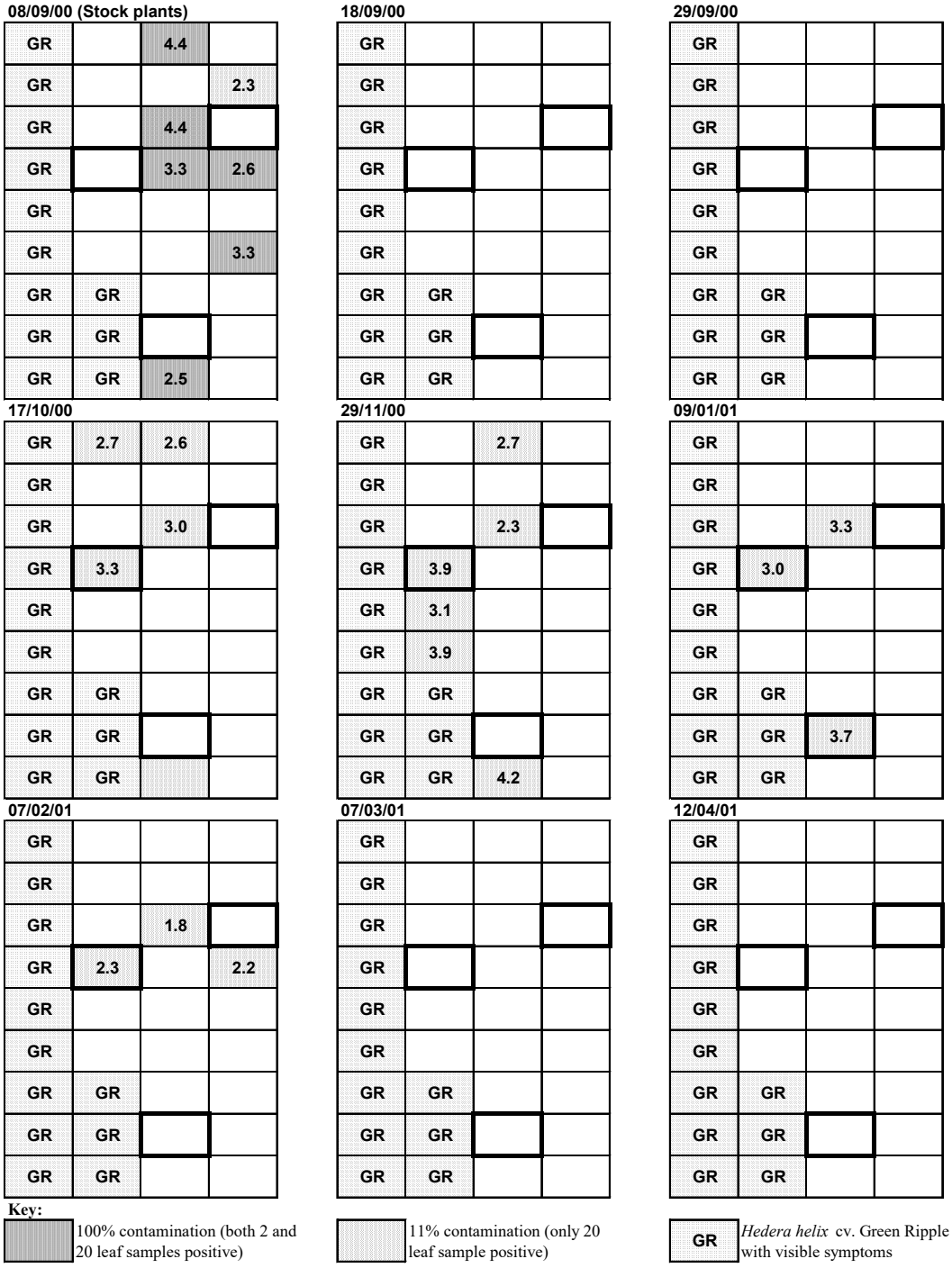

Fig. 2. Maps for Site 1 showing layout of trays of cuttings of *Hedera helix* cv. Glacier and those in which *Xanthomonas hortorum* pv. *hederae* (*Xhh*) was detected at each sampling date. Values in cells are the mean $\log_{10}$ cfu *Xhh* per leaf; 'bait' trays of clean cuttings are shown with a thick border.

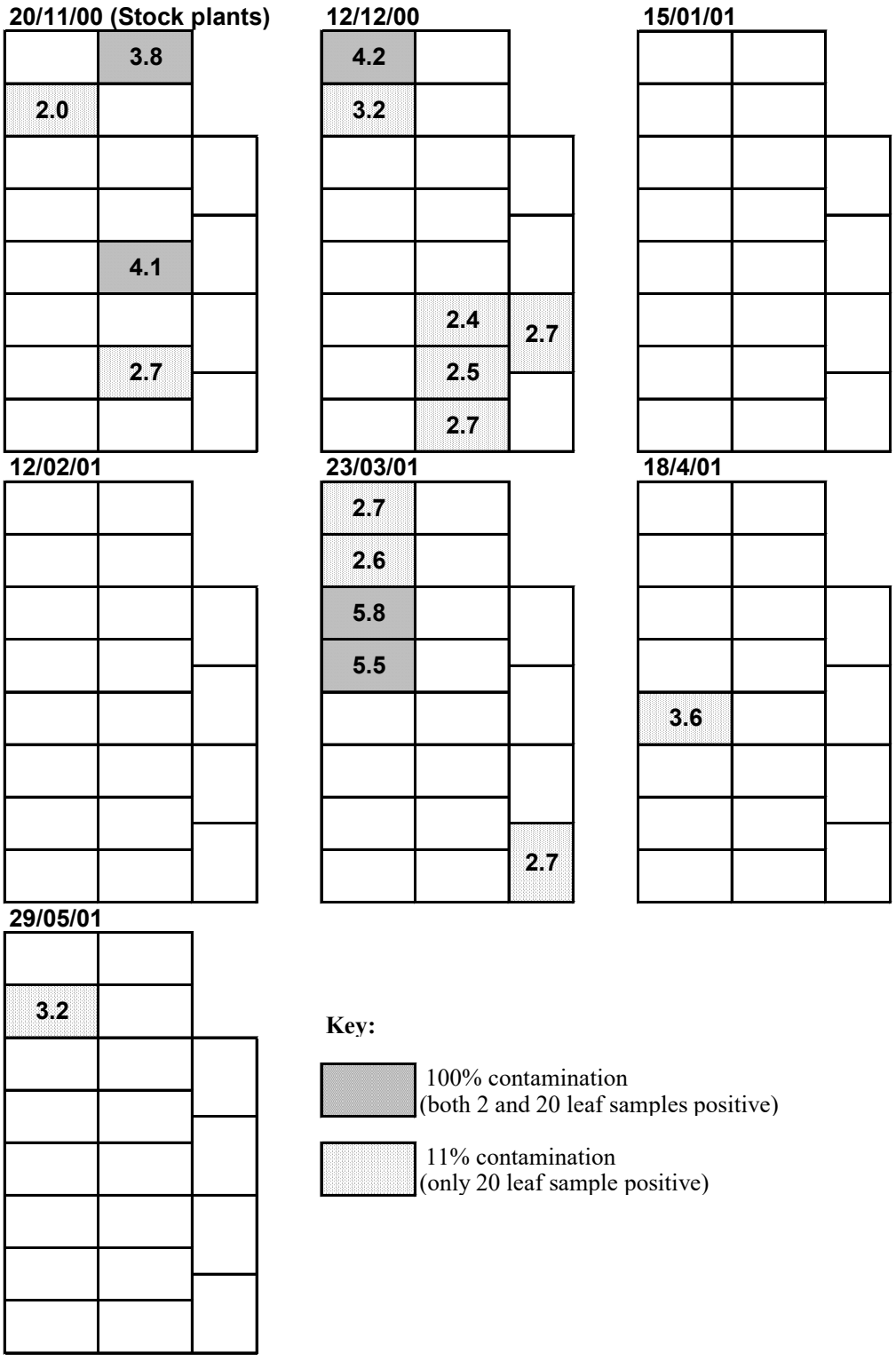

Fig. 3. Maps for Site 2 showing layout of trays of cuttings of *Hedera helix* cv. Jester's Gold and those in which *Xanthomonas hortorum* pv. *hederae* (*Xhh*) was detected at each sampling date. Values in cells are the mean $\log_{10}$ cfu *Xhh* per leaf.

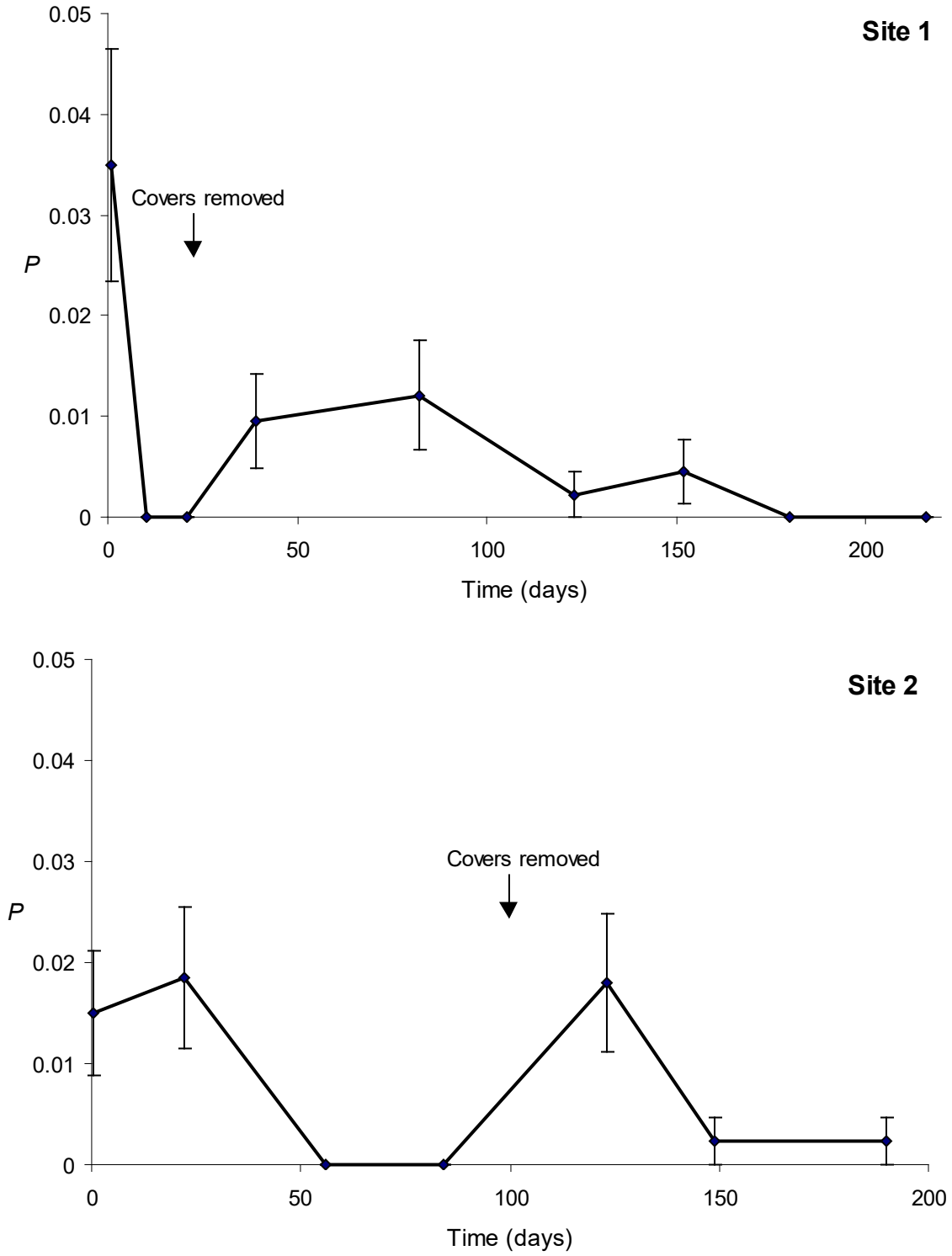

Fig. 4. Proportion of leaves (*P*) contaminated with *Xanthomonas hortorum* pv. *hedera* in trays of *Hedera helix* cuttings (mean across all trays) during rooting and after polythene covers were removed at two commercial nurseries. Means and standard errors (bars) were estimated by fitting a generalised linear model.

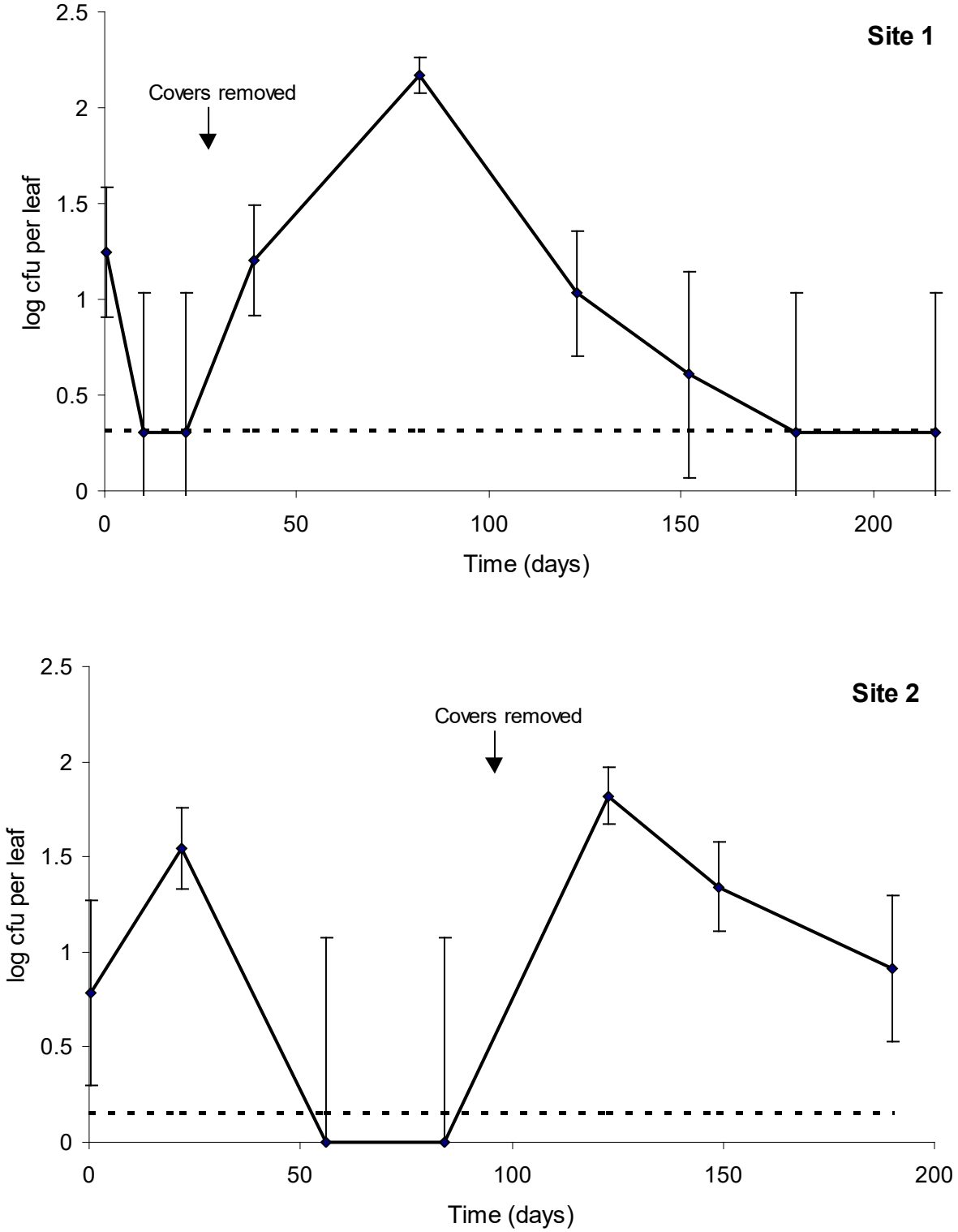

Fig. 5. Mean numbers (log_{10}) of *Xanthomonas hortorum* pv. *hederae* in trays of *Hedera helix* cuttings (mean across all trays) during rooting and after polythene covers were removed at two commercial nurseries. Means and standard errors (bars) were estimated by fitting a generalised linear model, with zero counts assigned an arbitrary value of 0.1. The dashed horizontal lines represent the detection threshold of the assay.

APPENDIX I - ARTICLE IN HDC NEWS

HAVE YOU SPOTTED IT ?

(BACTERIAL LEAF SPOT ON IVY)

A new project has just been started at HRI Wellesbourne investigating Bacterial leaf spot on ivy. The HDC-funded PhD studentship will study the biology and epidemiology of the causal pathogen *Xanthomonas hortorum* pv *hederae.*

Symptoms of the disease are brown water soaked lesions on the leaves. At the very least this can make the plants unmarketable or even kill them outright.

As a vital step in the research, the student Sarah Holcroft is keen to hear from ivy growers, to establish the range of different propagation and cultural practices in use. If you are an ivy grower with 5 minutes to spare to fill in a simple questionnaire and/or you suspect you may have a problem with the disease please contact Sarah by phone, fax or E-mail.

Sarah Holcroft, Horticultural Research International, Plant Pathology and Microbiology Department, Wellesbourne, Warwick, CV35 9EF, Tel 01789 470382
Fax 01789 470552 or E-mail Sarah.Holcroft@HRI.ac.uk

APPENDIX II - MODIFIED TWEEN 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)	15 mg (2 ml)	7.5 mg (1 ml)
Chlorothalonil [3] (20mg/ml 70% 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) Steam to dissolve
4) Adjust pH to 6.8 with 1 M NaOH, and add agar.
5) Autoclave at 121°C, 115 psi for 15 minutes.
6) Autoclave tween 80 in a separate container.
7) Prepare antibiotic solutions and filter sterilise as appropriate.
8) Cool medium to approx. 50°C in a water bath and add tween 80 and antibiotics.
9) 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 2 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 distilled water. 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 III - BRILLIANT CRESOL BLUE CELLOBIOSE MEDIUM

Compound	g/l	g/500 ml
KH_2PO_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[1] (5 mg/ml H_2O)	5 mg (1 ml)	2.5 mg (0.5 ml)
Cellobiose[2] (0.25 g/ml H_2O)	10 (40 ml)	5 (20 ml)
Cephalexin[3] (7.5 mg/ml H_2O)	30 mg (4 ml)	15 mg (2 ml)
Chlorothalonil[4] (20 mg/ml 70%v 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 5-fluorouracil, cellobiose and agar into a suitable container
2) Add 960 ml (or 480 ml) of distilled water.
3) Add brilliant cresol blue stock solution.
4) Steam to dissolve.
5) Adjust pH to 6.8 with 1M NaOH (use dye as an indicator: the medium should be bright blue in colour) and add agar.
6) Autoclave at 121°C, 115 psi for 15 minutes.
7) Prepare antibiotics and cellobiose and filter sterilise as appropriate.
8) Cool medium to approx. 50°C in a water bath and add antibiotics and cellobiose.
9) Mix gently to avoid air bubbles and pour plates (22 ml per 9.0 cm plates).
10) Leave plates to dry in the flow bench or similar before use.

Additions

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

Storage

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

