MINISTRY OF AGRICULTURE, FISHERIES AND FOOD

Research and Development

Final Project Report

(Not to be used for LINK projects)

Date project completed:

31/03/1998

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Section 1 : Identification sheet

1. (a)	MAFF Project Code	**HH1731SHN**	
(b)	Project Title	Aetiology and control of bacterial leaf spot of cherry laurel	
(c)	MAFF Project Officer	Dr S C Popple	
(d)	Name and address of contractor	Horticulture Research International Wellesbourne Warwick Postcode CV35 9EF	
(e)	Contractor's Project Officer	Dr S J Roberts	
(f)	Project start date	01/04/1995	Project end date 31/03/1998
(g)	Final year costs:	**approved** expenditure	£0
		actual expenditure	£0
(h)	Total project costs / total staff input:	**approved** project expenditure	£0
		actual project expenditure	£0
		***approved** staff input	£0
		***actual** staff input	£0
(i)	Date report sent to MAFF		
(j)	Is there any Intellectual Property arising from this project ?		

***staff years of direct science effort**

Section 2 : Scientific objectives / Milestones

2. Please list the scientific objectives as set out in CSG 7 (ROAME B). If necessary these can be expressed in an abbreviated form. Indicate where amendments have been agreed with the MAFF Project Officer, giving the date of amendment.

To characterise the causal agent of shot-hole of Cherry laurel and determine its host range and variability, thereby allowing the development of routine methods for detection of the pathogen. These methods will then be applied in epidemiological studies to identify the major sources of the pathogen.

3. List the primary milestones for the final year.

It is the responsibility of the contractor to check fully that ALL primary milestones have been met and to provide a detailed explanation if this has not proved possible

Milestones		Target date	Milestones met?	
Number	Title		in full	on time
vi	Recovery of pathogenic isolates on selective media	30/04/1997	YES	YES
viia	Identify nurseries for epidemiological studies	30/04/1997	YES	YES
viib	Occurrence of pathogen on stock plants, cuttings, liners, marketable plants throughout season determined	31/03/1997	YES	YES
ii	Complete and refine analysis of characterisation data	31/03/1997	YES	YES

If any milestones have not been met in the final year, an explanation should be included in Section 5.

Section 3 : Declaration

4. I declare that the information I have given in this report is correct to the best of my knowledge and belief. I understand that the information contained in this form may be held on a computer system.

Signature Date

Name

Position in Organistation

Section 4 : Executive summary

The UK nursery stock industry is worth over £200M. Cherry laurel (*Prunus laurocerasus*) is one of the most important single species in production; large numbers are produced for and used in landscaping and amenity plantings. Nurserymen consider that bacterial shot-hole/leaf spot is the major cause of losses in Cherry laurels and can result in up to 30% of stock becoming unmarketable. Bacterial leaf spot of Cherry laurel, particularly on ground cover varieties such as cv. Otto Luyken and Zabeliana, has been known in the UK, Europe and N. America at least since the early 1980s. The disease also occurs on cv. Rotundifolia and *P. lusitanica*. The disease is characterised by brown necrotic lesions which frequently drop out of the leaves to give a shot-hole appearance. These shot hole symptoms have often in the past been mistaken for insect damage. Shoot tips may also be killed by stem cankers. Recently, particularly severe problems have been experienced with cv. Otto Luyken and *P. lusitanica* which seem to be exacerbated by herbicide treatment. There are currently no recommended control measures for this disease.

The aims of this project were therefore to characterise the causal agent of shot-hole of Cherry laurel and determine its host range and variability, thereby allowing the development of routine methods for detection of the pathogen. These methods would then be applied in epidemiological studies to identify the major sources of the pathogen.

Isolations of bacteria were attempted from leaves with leaf spot and shot-hole symptoms, with marginal leaf necrosis, and from stem dieback symptoms on *Prunus laurocerasus* and *Prunus lusitanica*. Strains of the bacterium *Pseudomonas syringae* were isolated with relatively low frequency from leaf spot and shot-hole symptoms and from dieback symptoms but never from marginal leaf necrosis symptoms. Although there have been reports that shot-hole of cherry laurels can be caused by fungi or by the bacterium *Xanthomonas arboricola* pv. *pruni*, these were never isolated. A small number of samples were also tested for viral infection and found to be negative. Thus it would appear that shot-hole, leaf spot and dieback of cherry laurel in the UK is caused by the bacterium *P. syringae* and that the marginal leaf necrosis which is frequently seen in cherry laurels has a physiological origin.

In addition to the isolates obtained as part of this study, isolates of *P. syringae* from cherry laurel were also obtained from France and from the National Collection of Plant Pathogenic Bacteria. Fifty-eight isolates were characterised on the basis of over sixty characters - antibiotic sensitivity, nutritional and physiological tests, toxin production, serological reactions. The results of these tests were subject to hierarchical cluster analysis to produce a dendrogram. The groups obtained from the cluster analysis were then used to select a subset of isolates for pathogenicity testing.

Cherry laurel cv Otto Luyken was successfully micropropagated at HRI-Efford. Explants were multiplied to provide plantlets for pathogenicity testing/inoculation studies and to provide a nucleus of material of potential as pathogen-free mother plants. Micropropagated lilac plantlets of known susceptibility to *P. syringae* were obtained from the USA and multiplied for use in inoculation studies.

In pathogenicity studies with selected isolates on *Prunus laurocerasus* (plants and micropropagated explants), *Syringa vulgaris* (explants of two cultivars), *Pisum sativum*, *Forsythia* and *Weigela,* not all of the isolates of *P. syringae* from cherry laurel were pathogenic. Some isolates which were pathogenic on cherry laurel also induced symptoms on lilac and *Forsythia*. Isolates from lilac (*Syringa vulgaris*) and *Prunus avium* also induced symptoms on cherry laurel. Thus it would appear that pathogenic isolates from cherry laurel should correctly be called *Pseudomonas syringae* pv *syringae.* There is therefore potential for cross infection between different host species.

The recovery of isolates on two selective media (P3 and S4) was examined, as counts did not differ significantly between selective and non-selective media, these two selective media were therefore chosen for routine use in epidemiological studies. Epidemiological studies were done at two commercial nurseries. Pathogenic isolates of *P. syringae* were detected and quantified at all stages of crop production (stock plants, cuttings, liners, finals) on both nurseries. The contamination of stock plants with the pathogen suggests that control strategy based on the production and maintenance of pathogen-free stock plants (from which cuttings are taken) together with the development of an indexing system to monitor their health status may have considerable potential for the control of this disease. Further work will therefore be needed to produce a nucleus of pathogen-free stock plants and a cost-effective indexing system to ensure their health status. In order to assess the potential effectiveness of such a clean-stock-plant control strategy there is also a need for quantitative data on the rate of re-infection of plants during the production cycle.

Section 5 : Scientific report

Background and Objectives

Leaf-spotting pathogens of containerised hardy ornamental nursery stock cause major losses to an industry with an annual value of approximately £200M. The precise extent of losses caused by bacterial or any other diseases is unknown. These losses result from rejection of poor quality plants and the expense of applying prophylactic or curative chemical control. A better knowledge of disease epidemiology and, in particular, sources of inoculum is likely to result in more effective control especially for those diseases caused by pathogens thought to reside in or on mother plants.

Cherry laurel (*Prunus laurocerasus*) is the most important single species in production; large numbers are produced for and used in landscaping and amenity plantings. Nurserymen consider that bacterial shot-hole/leaf spot is the major cause of losses in Cherry laurels and can result in up to 30% of stock becoming unmarketable. Bacterial leaf spot of Cherry laurel, particularly on ground cover varieties such as cv. Otto Luyken and Zabeliana, has been known in the UK, Europe and N. America at least since the early 1980s. The disease also occurs on cv. Rotundifolia and *P. lusitanica*. It is characterised by brown necrotic lesions which frequently drop out of the leaves to give a shot-hole appearance. These shot hole symptoms have often in the past been mistaken for insect damage. Shoot tips may also be killed by stem cankers. Recently, particularly severe problems have been experienced with cv. Otto Luyken and *P. lusitanica* which seem to be exacerbated by herbicide treatment. Although the disease is generally considered to be caused by a bacterium, a strain of *Pseudomonas syringae,* there is also a record in the scientific literature of *Xanthomonas* causing the disease. In addition there have also been reports that the disease may have a fungal origin, or result from compost problems. There are currently no recommendations for the control of this disease.

Assuming that the disease is caused by a strain of *P. syringae,* it would then be vital to understand the taxonomic position of the pathogen in order to develop a control strategy. *P. syringae* is a widely occurring bacterial species which is divided into pathovars on the basis of distinctive pathogenicity to one or more hosts. Based on experience with a range of other bacterial pathosystems, strains characterised as belonging to a particular pathovar tend to have a more limited host range, a close association with host propagating material and cause disease over a range of environmental conditions. It is often the case that strains which are not assigned to a particular pathovar or which are often incorrectly assigned to pv. *syringae* tend to have more ubiquitous occurrence and cause disease in a more narrowly defined set of environmental conditions, usually involving some form of host stress and frost damage. Thus defining the taxonomic position of the causal agent may suggest whether a control strategy aimed at producing clean planting material (specific pathovar) or at avoiding a particular set of environmental conditions (non-specific pathovar) is the more appropriate. An understanding of the taxonomy of the pathogen is also a pre-requisite the development of diagnostic tools and procedures which are vital for progress in studies of epidemiology.

The major aims of this project were therefore to isolate and characterise the causal agent of shot-hole of Cherry laurel and determine its host range and variability. This would then enable the development of routine methods for detection of the pathogen. These methods would then be applied in epidemiological studies to identify the major sources of the pathogen, which would in turn indicate possible control strategies. All of these objectives have been achieved.

Isolation and characterisation of the pathogen

Samples of plant material with symptoms were requested from consultants and growers and were also collected during visits to nurseries and garden centres. Samples were obtained from throughout the UK and from the range of different *Prunus* spp./cvs. Isolations were attempted from symptomatic tissue by comminuting small pieces from the leading edge of lesions in a drop of sterile tap water on a sterile microscope slide and streaking out the resulting suspension on King's B and 5% sucrose agar media. Following incubation, isolates were sub-cultured and then subjected to a number of preliminary identification tests. More than 180 isolations were attempted from over 80 samples. Isolates initially identified as *Pseudomonas syringae* were isolated from 52% of samples, but only from

shot-hole, leaf spot and shoot dieback/blight symptoms, and never from general marginal leaf necrosis symptoms. On a few occasions bacteria suspected of being potentially *Xanthomonas* were also isolated but these later proved to be non-pathogenic. No evidence of fungal or viral infections was ever found.

In addition to the isolates of *P. syringae* obtained as part of this study, isolates of *P. syringae* from cherry laurel were also obtained from France (Dr L. Gardan, INRA, Angers) and from the National Collection of Plant Pathogenic Bacteria (NCPPB). Fifty-eight isolates were selected for further characterisation on the basis of over sixty characters including antibiotic sensitivity, nutritional and physiological tests, toxin production, ice nucleation activity, serological in agglutination and ELISA and bacteriophage reactions.

The results of these tests were used to generate a similarity matrix and then subject to hierarchical cluster analysis to produce a dendrogram. The isolates could be divided into 21 groups at the 90% similarity level. These groups were then used as a basis for the selection of a subset of isolates for pathogenicity testing.

Pathogenicity testing

A consistent and reliable source of healthy plants at an appropriate growth stage is a necessary pre-requisite for any pathogenicity testing. This is particularly problematical in the case of vegetatively propagated perennial crops and even more so in the case of *Prunus laurocerasus* where most commercial stock appears to be infected. Micropropagation of cherry laurel was therefore attempted at HRI-Efford, although unsuccessful in the first year of the project it was successfully achieved in the second year. Explants were then multiplied to provide material for pathogenicity testing/inoculation studies and to provide a nucleus of material of potential as pathogen-free mother plants. Micropropagated lilac plantlets of known susceptibility to *P. syringae* were obtained from the USA and multiplied for use in inoculation studies.

Methods for inoculation of both micropropagated and conventionally propagated (normal) plants were developed following tests of several techniques. For micropropagated plants, explants were removed from agar medium, either wounded or not, and immersed in a bacterial suspension before replacement on agar medium and incubation. The same method was used for inoculation of micropropagated plants of both *Prunus* and Lilac. For normal plants inoculating leaves and stems with an insect needle charged with bacterial growth from an agar plate proved to give the most consistent results.

Pathogenicity of 30 isolates was tested on explants of *Prunus* cv Otto Luyken and plants of cv Rotundifolia, on explants of lilac cv Sensation. A more limited number of isolates were also tested for pathogenicity on *Pisum sativum, Phaseolus vulgaris*, *Weigela* and *Forsythia* . Not all isolates of *P. syringae* from cherry laurel were pathogenic. Isolates which were pathogenic on *Prunus* belonged to 7 of the 21 groups from the cluster analysis. All were also pathogenic on lilac explants, were ice-nucleation positive and belonged to one of two sero-groups. Some isolates which were pathogenic on lilac did not give symptoms on *Prunus.* Some isolates which were pathogenic on *Prunus* also induced symptoms on *Forsythia* but not on *Weigela.*

Detection methods

As the pathogenic isolates from cherry laurel showed similar antibiotic sensitivity to *P. syringae* pvs *pisi* and *syringae*, the recovery of isolates on the selective media (P3 and S4) previously developed for *pisi* and *syringae* was examined. Counts did not differ significantly between selective and non-selective media. These two selective media were therefore chosen for routine use in epidemiological studies. Comparisons were also made shaking and stomaching for washing/detection of the pathogen on leaves. No differences were found in spiked samples and as stomaching is more convenient in terms of laboratory consumables, it was selected as the method for use in epidemiological studies.

Epidemiological studies

Two nurseries were selected for epidemiological studies with the aim of providing quantitative estimates of

contamination of the crop with the pathogen at different stages of production, in order to identify the major source of the pathogen. One of the nurseries selected for this study has suffered considerable problems with the disease for several years. This has led him to reduce production on the nursery and import final plants from France to fulfil contracts. The other nursery considered that it was able to limit the severity of damage by the use of disinfectants. In addition the two nurseries were located in different parts of the country (South coast and North West) and followed differing crop production practices.

Six replicate samples of thirty healthy leaves were collected from plants at each stage of production (stock plants, cuttings, rooted cuttings, liners, finals) at each nursery on three occasions. Samples were then stomached and the extracts diluted and plated on the selective media. Suspect isolates were then sub-cultured to sectored plates, tested for levan and oxidase production, tobacco hypersensitivity, syringomycin production and agglutination reactions. These tests were then used as a basis for selecting a sub-set of isolates for pathogenicity testing on explants of *Prunus* and lilac.

Pathogenic isolates of *P. syringae* were detected on healthy leaves at all stages of production on both nurseries. Results were interpreted using maximum likelihood methods to estimate the proportion of contaminated leaves from the numbers of positive and negative sub-samples. Contamination varied from less than 0.6% to over 6% and varied with production stage, site and sampling date. In general, the site with the most severe disease problems also had the highest levels of contamination and plants grown in protected environments (cuttings and liners) had lower levels of contamination than plants grown in the open (stock plants, finals). Although initial characterisation work had indicated that syringomycin production and serological reaction were useful indicators of pathogenicity, this proved not to be the case with many of the isolates from the epidemiological studies, where isolates which did not produce syringomycin in bioassay produced disease symptoms in pathogenicity tests and vice versa.

Conclusions and general discussion

The results of the isolation and characterisation work clearly indicate that shot-hole, leaf spot and dieback symptoms in *Prunus laurocerasus* and *Prunus lusitanica* are caused by strains of *Pseudomonas*. The evidence to date suggests that strains which cause disease in cherry laurels represent a subset of the pathovar *syringae*, in that not all strains of pv. *syringae* which cause symptoms on lilac cause symptoms on *Prunus.* However, some taxonomic problems remain to be resolved e.g. by definition strains belonging to pv. *syringae* should produce syringomycin. It is possible that a molecular approach could go someway to resolving these problems.

Tissues showing marginal necrosis symptoms which were frequently seen in cherry laurels were consistently free from any pathogenic organisms. These symptoms also tended to develop on plants which could be considered to be under some sort of stress, e.g. due to over/under watering, poor root development, lifting/transplanting, high temperatures, inappropriate compost mixes/nutrition. Thus it would seem that generalised marginal necrosis symptoms represent a generalised stress response.

The successful micropropagation of cv Otto Luyken proved invaluable as a means of producing a consistent supply of healthy plants for inoculation and pathogenicity testing on a routine basis. Pathogenicity results obtained with explants were identical to those obtained for normal plants indicating the reliability of this approach. The pathogenicity results on lilac explants and *Forsythia* and taxonomic results indicate that there may be considerable potential for cross-infection between these species in the nursery situation, however as there are some indications of specialised adaptation to *Prunus* further work is necessary to clarify this potential and quantify the risks.

Although the selective media worked satisfactorily, considerable effort was still required to reliably identify isolates obtained in the epidemiological studies as it was not possible to identify pathogenic isolates based solely on serological and reactions and toxin production. Thus it was always necessary to confirm pathogenicity of isolates in planta.

The presence of pathogenic strains was demonstrated on symptomless leaves at all stages of crop production: stock plants, cuttings, rooted cuttings, liners, finals. In particular the contamination of stock plants with the pathogen

suggests that a strategy based on the production and maintenance of pathogen-free stock plants (from which cuttings are taken) together with the development of an indexing system to monitor their health status may have considerable potential in the control of this disease. The success of such a strategy will also depend on the rate of re-infection of plants in production. Further work will therefore be needed to produce a nucleus of stock plants from micropropagated material, and to develop a cost-effective indexing system to ensure their health status. In addition there is a need for basic quantitative data on the rate of re-infection of plants in production in order to assess the potential effectiveness of a clean-stock-plant control strategy.

During this work, non-pathogenic strains of *Pseudomonas syringae* were frequently isolated from *Prunus* leaves. The ecological role of these strains is unclear but it may be possible to select strains from amongst them which are effective and aggressive leaf surface colonisers for use as biocontrol agents

Future work

The taxonomic position of the cherry laurel pathogen needs to be conclusively established. This could be achieved by means of a molecular taxnomic study of cherry laurel and related strains of *P. syringae*.

The contamination of stock plants with the pathogen suggests that a strategy based on the production and maintenance of pathogen-free stock plants (from which cuttings are taken) together with the development of an indexing system to monitor their health status may have considerable potential in the control of this disease. The success of such a strategy will also depend on the rate of re-infection of plants in production. This rate will be determined by a number of factors such as: proximity to other sources of inoculum, rate of removal of other sources of inoculum (i.e. general nursery hygiene), environmental and climatic factors, relative infectivity of pathogenic strains from different hosts, frequency of use of chemical control treatments and disinfectants. Further work will therefore be needed to produce a nucleus of stock plants from micropropagated material, and to develop a cost-effective indexing system to ensure their health status. In addition there is a need for basic quantitative data on the rate of re-infection of plants in production in order to assess the potential effectiveness of a clean stock plant control strategy. This might best be assessed empirically using bait/trap plants which were known to be pathogen-free prior to placement on the nursery and which could then be monitored for disease/pathogen contamination and/or replaced at regular intervals to identify critical factors determining re-infection

One potential source for re-infection is infected/contaminated plants of other (unrelated) plant species. Although pathogenic in artificial laboratory/glasshouse host tests it is not known whether strains from a particular host show any host preference. As this has implications for the rate of re-infection, we need to determine the relative host specificity and infectivity for strains originating on different host species. Isolates of *P. syringae* from a range of different HNS are already held in the culture collection at HRI. Although it would be impractical to test all of these on all potential hosts, molecular typing of these isolates might give indications of accociations with particular hosts and allow selection of a limited representative set of strains which could then be tested for pathogenicity on a number of key host species.

As part of this project, a considerable number of isolations were attempted from symptomatic cherry laurel leaves. As well as the pathogenic isolates of *P. syringae*, a number of isolates of *P. syringae* were obtained but which do not appear to be pathogenic. This phenomenon is not unique and non-pathogenic strains of *P. syringae* have been found on a range of different plant species and have been assumed to be part of the general epiphytic (leaf surface) microflora. If, as circumstantial evidence suggests, pathogenic strains are present as epiphytes prior to infection it may be possible to use non-pathogenic strains which are effective and aggressive colonisers as natural biocontrol agents. In addition, as they would be naturally occurring members of the epiphytic microflora the perceived risks associated with their use should be minimal. In the USA naturally occuring epiphytic strains of *P. syringae* have already been used as biocontrol agents for microbially-mediated frost damage. Further work could examine the potential of non-pathogenic strains already collected as biocontrol agents.

Publications/outputs

Roberts, S.J (1997) Bacterial diseases: a blight on the landscape. *HDC Hardy Nursery Stock Conference: New Directions, Stoke-on-Trent 22-23 July 1997,* pp. 43-45.

Informal discussions with growers during visits to nurseries.

Roberts, S.J. & Brough, J. (1998) Phenotypic charaterisation and pathogencity of isolates of *P. syringae* from cherry laurel (*Prunus laurocerasus*). *In preparation.*