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SID 5 Research Project Final Report

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Project identification

1. Defra Project code: PH0309

2. Project title: Improved detection of Xanthomonas fragariae and X. arboricola pv fragariae in UK strawberry imports

3. Contractor organisation(s): Central Science Laboratory
Sand Hutton
York
YO41 1LZ

4. Total Defra project costs: £ 40,000

5. Project: start date .... 01 April 2004

   end date .... 31 March 2006

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(b) If you have answered NO, please explain why the Final report should not be released into public domain

Executive Summary

7. The executive summary must not exceed 2 sides in total of A4 and should be understandable to the intelligent non-scientist. It should cover the main objectives, methods and findings of the research, together with any other significant events and options for new work.

Xanthomonas fragariae (*Xf*) causes bacterial angular leaf spot of strawberry and is listed as a quarantine organism in the EPPO region (A2) and in EU plant health legislation (Council Directive 2000/29/EC Annex IIAII). It was first reported in the USA and spread to Australia, New Zealand and Brazil. By the late 1980's, it had spread to several European countries including Greece, Italy and Portugal. There have been few reports of serious losses but further findings of the disease, or interceptions of the pathogen in planting material, in other EU member states have raised awareness of the pathogen in recent years. The UK is considered free although there have been several potential interceptions. In some cases these were not confirmed because of the difficulties of detecting this insidious pathogen.
Xanthomonas arboricola pv. *fragariae* (*Xaf*) causes bacterial leaf blight of strawberry. It is a newly described bacterial pathogen of strawberries of which little is known, especially concerning risks to the UK industry. It has been recently identified in Italy and there is also some evidence that this pathogen may have been confused with *X. fragariae* elsewhere in Southern Europe.

Another concurrent project on these strawberry pathogens was also funded by the Horticultural Development Council. Although some project objectives were shared between these projects (e.g. real-time PCR assay development), other objectives were different, e.g. chemical control studies against *Xf* and *Xaf* required as part of the HDC funded project.

Objective 1. Develop real-time PCR assays for both pathogens
Objective 2. Validate these against existing technologies
Objective 3. Establish standard operating procedures for an improved testing service.

Four real-time PCR assays have been developed during the course of the project. These have been named as *Xf* 16S PCR; *Xf hrpB* PCR; *Xf gyraseB* PCR; and *Xaf pep* PCR. The properties of these PCRs can be summarised:

- *Xf* 16S PCR - designed within the DNA sequence of the *Xf* ribosomal 16S gene, associated with protein synthesis. Detects both *Xf* and *Xaf* as well as other *Xanthomonas* species. Sensitivity of 10^3 cells ml^{-1} in water and 10^3 cells per strawberry leaf disc.
- *Xf hrpB* PCR – designed within DNA sequence of *Xf hrpB* gene, associated with pathogenicity. Detects only *Xf* strains. Sensitivity of 10^5 cells ml^{-1} in water and 10^5 cells per leaf disc.
- *Xf gyraseB* PCR – designed within DNA sequence of *Xf gyraseB*, associated with DNA replication. This assay was developed during another DEFRA funded project. It detects *Xf* strains and not *Xaf* strains (of those tested) and has a sensitivity of 10^3 cells ml^{-1} in water and 10^3 cells per strawberry leaf disc.

Further validation of this assay is required.
- *Xaf pep* PCR – designed within *Xaf pep* protease gene, thought to be associated with pathogenicity. Detects *Xaf* strains and two other *Xanthomonas* strains. Does not detect *Xf*. Sensitivity of 10^3 cells ml^{-1} in water and 10^3 cells per leaf disc.

Although the *Xf* 16S assay has poor selectivity it was retained for use as a screening assay in view of the poor sensitivity of the highly specific *Xf hrpB* PCR. If used exclusively for routine detection it was felt that the *Xf hrpB* assay would lead to many false negative results being returned, especially from latently infected plant material. Pathogen levels of 10^3 cells per ml^{-1} (or disc) were found to be associated with asymptomatic material during work in the HDC funded project. The development of the *Xf gyraseB* assay late in the project will hopefully lead to the discontinuation in use of *Xf* 16S and *Xf hrpB* assays in routine screening as there will be two assays, *Xf gyraseB* and *Xaf pep,* both specific and sensitive enough to allow reliable detection and differentiation of the two pathogens.

In order to avoid complex and time consuming isolation protocols, for the establishment of pure bacterial cultures of *Xf* and *Xaf*, a DNA extraction protocol direct from leaf tissues is also necessary. This extract can then be used as the template in the real-time PCR assays mentioned previously. A technique, using a proprietary kit (Extract-N-Amp Plant PCR Kit, Sigma Aldrich), was adapted to provide a suitable DNA template of *Xf* and *Xaf* for the real-time PCR assays described above. The protocol extracts DNA from 0.7cm leaf discs, produced using a hole punch. The staff time required to process one leaf disc has been estimated at 3 minutes per disc, with a total consumables cost of £2 per sample. Protocols have been written to allow staff in the CSL Molecular Technology Unit to test large numbers of samples using these assays and extraction technique. These assays are now being used for routine diagnosis of both pathogens at CSL.

Objective 4. Initial pathogenesis studies on *Xanthomonas arboricola* pv. *fragariae.*

Three artificial inoculation methods were tested to determine the most suitable protocol for producing strawberry material infected with *Xaf*. These methods involved trying to inoculate suspensions of *Xaf* into plant tissues using either syringe infiltration, puncture inoculation, or leaf abrasion. None resulted in the development of definite *Xaf* symptoms despite the syringe infiltration and puncture inoculation methods being able to induce angular leaf spot symptoms with *Xf* suspensions.
A hydroponic experiment was set up to determine if *Xaf* could be taken up via the rhizosphere of strawberry plants. Unlike *Xf* inoculated plants, *Xaf* was found to be taken up into strawberry leaves via the rhizosphere, though no bacterial leaf blight symptoms developed in infected plants.

Project Report to Defra

8. As a guide this report should be no longer than 20 sides of A4. This report is to provide Defra with details of the outputs of the research project for internal purposes; to meet the terms of the contract; and to allow Defra to publish details of the outputs to meet Environmental Information Regulation or Freedom of Information obligations. This short report to Defra does not preclude contractors from also seeking to publish a full, formal scientific report/paper in an appropriate scientific or other journal/publication. Indeed, Defra actively encourages such publications as part of the contract terms. The report to Defra should include:
   - the scientific objectives as set out in the contract;
   - the extent to which the objectives set out in the contract have been met;
   - details of methods used and the results obtained, including statistical analysis (if appropriate);
   - a discussion of the results and their reliability;
   - the main implications of the findings;
   - possible future work; and
   - any action resulting from the research (e.g. IP, Knowledge Transfer).

BACKROUND

Xanthomonas fragariae causes bacterial angular leaf spot of strawberry and is listed as a quarantine organism in the EPPO region (A2) and in EU plant health legislation (Council Directive 2000/29/EC Annex IIAII). It is a potentially serious and insidious disease, first reported in the USA. From the USA it probably spread, in infected planting material, to Australia, New Zealand and Brazil. By the late 1980's, it had spread to several European countries including Greece, Italy and Portugal. There have been few reports of serious losses but further findings of the disease, or interceptions of the pathogen in planting material, in other EU member states (including Belgium, France, Germany, The Netherlands, Spain and Switzerland) have raised awareness of the pathogen in recent years. The UK is considered free although there have been several potential interceptions. In some cases these were not fully confirmed because of the difficulties of detecting this insidious pathogen.

Xanthomonas arboricola pv. *fragariae* causes bacterial leaf blight of strawberry. It is a newly described bacterial pathogen of strawberries of which little is known, especially concerning risks to the UK industry. It has been recently identified in Italy and there is also some evidence that this pathogen may have been confused with *X. fragariae* elsewhere in Southern Europe.

OVERALL AIM of PROJECT

To increase the reliability of excluding *Xanthomonas fragariae* and *Xanthomonas arboricola* pv. *fragariae* from the UK production system by developing and validating robust, cost-effective diagnostic methods and improving general knowledge on the current geographic distribution and ease of spread of the pathogen.

OBJECTIVE 1. Develop real-time PCR assays for both pathogens
OBJECTIVE 2. Validate these against existing technologies
OBJECTIVE 3. Establish standard operating procedures for an improved testing service.

INTRODUCTION

Prior to the commencement of this project testing methods for *Xanthomonas fragariae* were unreliable, slow, difficult to interpret and costly. In addition, apart from isolation of the pathogen directly from infected plant material there existed no rapid diagnostic protocols for the detection of *Xanthomonas arboricola* pv. *fragariae*. CSL has pioneered the use of real-time PCR for the detection of many plant pests and pathogens and thus this was selected as the method of choice for developing assays to detect both *Xf* and *Xaf*.

PCR utilises short DNA sequences (primers) to amplify a specific target DNA sequence. The target sequence (between the two primers) is amplified to a point where there are enough copies of the sequence for it to be detected. In conventional PCR these copies are observed by running the reaction mix through an electrophoretic gel and staining the DNA with ethidium bromide. Real-time (or TaqMan) PCR exploits the 5′ nuclease activity of *Taq* DNA polymerase in conjunction with fluorogenic DNA probes. Each probe, designed to hybridise specifically to the target PCR product, is labelled with a fluorescent reporter dye and a quencher dye. During PCR amplification the probe is digested by *Taq* DNA polymerase, separating the dyes, and resulting in an increase in reporter fluorescence. Repeated PCR cycles result in exponential amplification of the PCR product and corresponding increase in fluorescence intensity. Real-time analysis also facilitates quantification of the amount of sample DNA present in the reaction by ascertaining when (i.e. during which PCR reaction) fluorescence in a given reaction tube exceeds that of a threshold (Threshold Cycle (C_T)). Lower C_T values indicate higher amounts of target DNA. Comparison between reaction tubes and / or known standards can quantify the amount of DNA template present in a given tube.

In order to avoid complex and time consuming isolation protocols, for the establishment of pure bacterial cultures of *Xf* and *Xaf*, a DNA extraction protocol direct from leaf tissues is also necessary. This extract can then be used as the template in the real-time PCR assays mentioned previously. Strawberry tissues are known to contain many compounds which can inhibit the PCR reaction so any extraction techniques must either produce a suspension of pure DNA or contain steps which negate the effect of any PCR inhibitors.

In this project summary we report the development of four real-time PCR assays for the detection of both *Xf* and *Xaf*. We have also adapted an existing technique to extract bacterial DNA from strawberry leaf tissue which facilitates rapid and reliable detection of both pathogens by the relevant real-time PCRs.

MATERIALS and METHODS

Bacterial strains and DNA extraction from pure bacterial culture

Bacterial strains tested in this study are listed in Table 1. *Xf* strains were maintained on sucrose peptone agar (SPF) and *Xaf* strains were maintained on nutrient agar (NA). All strains were grown at 28°C. Genomic DNA was obtained from pure cultures of each strain using the Wizard Genomic DNA Purification Kit (Promega). These extracts were then used as template in real-time PCR reactions.

Real-time PCR primer and probe design

Sequences of primers and probes were designed following sequencing and comparison of specific genes harboured by *Xf, Xaf* and certain other closely related bacteria for which gene sequences were already available. The specifics of each assay are as follows:

1. *Xanthomonas* 16S screening assay (*Xf* 16S)
   An alignment of sequences from the ribosomal 16S gene was prepared following sequencing of the 16S gene from *Xf*, *Xaf* and other closely related bacteria. This alignment was used to design real-time PCR primers and probes for specific detection of *X. fragariae*. The 16S gene encodes for the small ribosomal RNA sub unit.

2. *Xanthomonas fragariae hrpB* assay (*Xf hrp*)
   An alignment of sequences from the *hrpB* operon of various *Xanthomonas* taxa (the *X. axonopodis* pathovars *glycinea, citri, dieffenbachia* and *syngonii*, the *X. campestris* pathovars *campestris and vesicatoria*, *X. oryzae* and *X. fragariae*) was used to design primers and probes for specific detection of *X. fragariae*. This gene is known to be essential for pathogenesis in *Xanthomonas* spp.

3. *Xanthomonas arboricola* pv. *fragariae* assay (*Xaf pep*)
   Primers were designed to amplify a *c*500 bp region of the *Xanthomonas fragariae* and *X. arboricola* pv. *fragariae* Pep protease gene. Amplified products were sequenced and an alignment used to design a primer/probe combination for specific detection of *X. arboricola* pv. *fragariae.* This gene is thought to be associated with pathogenesis.

4. *Xanthomonas fragariae gyrB* assay (*Xf gyrB*)

   At the end of this project an alignment of sequences from the *gyraseB* gene was produced during a CSL horizon scanning and future proofing project (funded by DEFRA Plant Health Division). This alignment was obtained from sequences also obtained from *Xf*, *Xaf* and other closely related bacteria and was used in the main project to design a primer / probe combination for specific detection of *X. fragariae.* The *gyraseB* gene encodes for a protein involved in DNA replication and like the 16S gene is present in all bacteria.

Extraction of bacterial DNA from strawberry leaf tissue

A technique, using conventional PCR and a proprietary kit, was published during the course of this project with a reported sensitivity of detection (of *Xf*) to 10^3 cells per sample (Stoger and Ruppitsch, 2004). This was modified to facilitate reliable and robust detection of both *Xf* and *Xaf* direct from leaf tissue using the real-time PCR assays developed in this project.

The extraction technique uses Extraction and Dilution Buffers from an Extract-N-Amp Plant PCR Kit (Sigma Aldrich). The protocol extracts DNA from 0.7cm leaf discs, produced using a hole punch, and is briefly as follows:

1. 0.7cm leaf disc placed in to a small stomacher bag.
2. Add 100µl of Extraction Buffer to bag. Homogenise disc in this buffer using roller. Transfer leaf suspension to sterile 1.5 mL microtube. Vortex.
3. Incubate the tube at 95˚C for 15 minutes. Add 100µl of Dilution Buffer. Vortex
4. Sample is further diluted 1:50 in sterile ultra pure water by removing 10 µl of the leaf suspension and transferring this to a fresh microtube containing 490 µl of sterile ultra pure water. This is used as template for the real-time PCR assays and can be stored at –30°C.

Sensitivity of real-time PCR assays

Strains of *X. fragariae* and *X. arboricola* pv. *fragariae* were grown on SPF or NA and used to prepare a decimal dilution series from 1 x 10^8 cells ml^{-1} to 1 x 10^1 cells ml^{-1}. These dilution series were then tested against all real-time PCR assays and also used to prepare decimal dilution series in leaf disc samples of 10^6 cells per disc to 10^1 cells per disc. This was achieved by adding 0.01 mL of each suspension (from 10^8 to 10^3 cells ml^{-1} suspensions) to individual leaf discs and then adding 0.09 mL of extraction buffer to each disc. From then on DNA was extracted from these discs using the extraction technique described previously. Each dilution series was used as template in respective real-time PCR assays.

RESULTS

Specificity of real-time PCR assays

PCR results from pure cultures of 35 *Xf*, *Xaf* and other closely related strains are summarised in Table 1.1. The *Xf* 16S PCR assay was shown to detect all *Xf*, *Xaf* and 15 other *Xanthomonas* strains. The *Xf hrpB* assay was shown to detect all 10 *Xf* strains analysed but no other bacterial strains in the panel. The *Xaf pep* PCR was shown to detect all 7 *Xaf* strains. The *Xf gyraseB* PCR developed in the last month of the project was shown to detect all 6 *Xf* strains tested and none of the 2 *Xaf* strains tested (data not shown). Further development work on this assay is ongoing.

Sensitivity of real-time PCR assays

The sensitivities of each individual TaqMan PCR were measured by testing 10-fold dilution series (in water and in leaf tissue extracts) of each pathogen. Mean C_T values for each assay against each dilution series are listed in Tables 1.2 and 1.3. The *Xf* 16S PCR assay detected target DNA down to a rate of 10^3 cells in both water and leaf tissue dilution series. The *Xf hrpB* assay was less sensitive, only being able to detect down to a rate of 10^5 cells. The new *Xf gryaseB* PCR was also shown to detect as little as 10^3 cells per disc as did the *Xaf pep* PCR in both water and leaf disc extracts.

Table 1.1. Panel of *Xanthomonas fragariae* and *Xanthomonas arboricola* pv. *fragariae* and other closely related strains tested by three real-time PCR assays

Strain	ID Code[a]	*Xf* 16S PCR	*Xf hrpB* PCR	*Xf gyraseB PCR*	*Xaf pep* PCR
Erwinia herbicola	NCPPB 2971	-	-	nt	-
Pseudomonas syringae pv. *syringae*	NCPPB 281	-	-	nt	-
Xanthomonas campestris pv. *campestris*	NCPPB 528	+	-	nt	-
Xanthomonas hortorum pv. *pelargonii*	NCPPB 2985	+	-	nt	-
Xanthomonas axonopodis pv. *phaseoli*	NCPPB 3035	+	-	nt	-
Xanthomonas campestris pv. *vesicatoria*	NCPPB 422	+	-	nt	-
Xanthomonas campestris pv. *campestris*	NCPPB 3207	+	-	nt	-
Xanthomonas arboricola pv. *corylina*	NCPPB 3037	+	-	nt	+
Xanthomonas axonopodis pv. *begoniae*	NCPPB 1925	+	-	nt	-
Xanthomonas arboricola pv. *juglandis*	NCPPB 362	+	-	nt	-
Xanthomonas hortorum pv. *hederae*	NCPPB 2011	+	-	nt	-
Xanthomonas arboricola pv. *pruni*	NCPPB 417	+	-	nt	+
Xanthomonas vesicatoria	NCPPB 422	+	-	nt	-
Xanthomonas albilineans	NCPPB 2969	-	-	nt	-
Xanthomonas axonopodis	NCPPB 457	+	-	nt	-
Xanthomonas cucurbitae	NCPPB 2597	+	-	nt	-
Xanthomonas melonis	NCPPB 3434	-	-	nt	-
Xanthomonas oryzae pv. oryzae	NCPPB 3002	+	-	nt	-
Xanthomonas pisi	NCPPB 762	+	-	nt	-
Xanthomonas fragariae	NCPPB 1469	+	+	nt	-
Xanthomonas fragariae	NCPPB 2949	+	+	+	-
Xanthomonas fragariae	NCPPB 3028	+	+	nt	-
Xanthomonas fragariae	NCPPB 4055	+	+	+	-
Xanthomonas fragariae	NCPPB 4177	+	+	+	-
Xanthomonas fragariae	NCPPB 4181	+	+	nt	-
Xanthomonas fragariae	PD 2802	+	+	+	-
Xanthomonas fragariae	PD 3145	+	+	nt	-
Xanthomonas fragariae	PD 3163	+	+	+	-
Xanthomonas fragariae	PD 3168	+	+	nt	-
Xanthomonas arboricola subsp. *fragariae*	NCPPB 4182	+	-	-	+
Xanthomonas arboricola subsp. *fragariae*	NCPPB 4183	+	-	-	+
Xanthomonas arboricola subsp. *fragariae*	PD 2694	+	-	nt	+
Xanthomonas arboricola subsp. *fragariae*	PD 2698	+	-	nt	+
Xanthomonas arboricola subsp. *fragariae*	PD 2780	+	-	nt	+
Xanthomonas arboricola subsp. *fragariae*	PD 3160	+	-	nt	+
Xanthomonas arboricola subsp. *fragariae*	PD 3164	+	-	nt	+

[a] NCPPB – National Collection of Plant Pathogenic Bacteria, CSL, Sand Hutton, York; PD – Culture Collection of Plant Pathogenic Bacteria (PD), Plant Protection Service, Wageningen, The Netherlands.
[b] (+) - positive, (-) –negative, (nt) – not tested

Table 1.2 *Xanthomonas fragariae* and *Xanthomonas arboricola* pv. *fragariae* dilution series, in water, and mean C_T values generated during real-time PCR assays

No. of *Xf* (or *Xaf*) cells ml^{-1}	Mean C_T value			
	Xf 16S PCR	*Xf hrpB* PCR	*Xf gyraseB* PCR	*Xaf pep* PCR
10^8	+ (16.1)[1]	+ (26.8)	+ (16.7)	+ (21.7)
10^7	+ (20.3)	+ (30.4)	+ (19.9)	+ (23.5)
10^6	+ (24.7)	+ (31.8)	+ (24.2)	+ (25.3)
10^5	+ (28.6)	+ (37.5)	+ (26.9)	+ (28.2)
10^4	+ (34.0)	-	+ (31.2)	+ (33.2)
10^3	+ (38.7)	-	+ (34.4)	+ (36.3)
10^2	-	-	+ (36.7)	-
10^1	-	-	-	-

[1] (+) - positive reaction; (-) – negative reaction. C_T value: PCR cycle number (in brackets) at which fluorescence is first detected during a 40-cycle PCR.

Table 1.3 *Xanthomonas fragariae* and *Xanthomonas arboricola* pv. *fragariae* dilution series, in strawberry leaf disc extracts, and mean C_T values generated during real-time PCR assays

No. of *Xf* (or *Xaf*) cells per leaf disc	Mean C_T value			
	Xf 16S PCR	*Xf hrpB* PCR	*Xf gyraseB* PCR	*Xaf pep* PCR
10^6	+ (25.13)[1]	+ (34.70)	+ (27.88)	+ (23.37)
10^5	+ (30.03)	+ (37.70)	+ (32.08)	+ (27.10)
10^4	+ (32.98)	-	+ (35.49)	+ (31.39)
10^3	+ (35.85)	-	+ (36.86)	+ (33.47)
10^2	-	-	-	-
10^1	-	-	-	-

[1] (+) - positive reaction; (-) – negative reaction. C_T value: PCR cycle number (in brackets) at which fluorescence is first detected during a 40-cycle PCR.

DISCUSSION

The results presented in this report demonstrate the development of effective assays for the reliable detection of *Xanthomonas fragariae* and *Xanthomonas arboricola* pv. *fragariae*. These assays include the first ever detection assay for *Xaf* and the first real-time PCR assay for the detection of *Xf*. In addition a reliable and cost effective protocol to extract and detect bacterial DNA from leaf samples has also been devised.

It was hoped that all the assays would detect only the pathogen from which each assay was designed, i.e. that the *Xf* 16S assay would only detect *Xf* strains and not any other bacteria. With the obvious exception of the *Xf* 16S assay this was largely the case with the *hrpB* assay demonstrating 100 % specificity to *Xf* strains and the *Xaf* pep assay only cross reacting with two other *Xanthomonas* strains (neither of which were *Xf*) from the panel of strains against which both assays were tested.

The *Xf* 16S assay was shown to be able to detect the majority of *Xanthomonas* strains which it was tested against. However this assay was retained for other work during the project as it was (a) much more sensitive than the specific *hrpB* assay and (b) could be used as a screening assay. Although this assay was shown to have poor specificity it was considered that the most likely *Xanthomonas* strains which it would come up against (on strawberry leaf samples) were either *Xf* or *Xaf.* Therefore it could be used as a screening assay to test one sample against both pathogens and then the other, more specific PCRs, could be used to identify which pathogen was present. Other work in the project showed that 10^3

cells per disc was a pathogen population level likely to be associated with latent infections. The *Xf hrpB* assay was shown to have a sensitivity of 10^5 cells per disc and thus, if applied as a stand alone test, could result in the possibility of false negative results, especially from latently infected leaf material.

The development of the *Xf gyraseB* assay late in the project should lead to replacement of the less reliable *Xf* 16S assay. This assay was shown to be as sensitive as the *Xf* 16S assay and also to be more specific, though further validation will be carried out to ensure that this assay does not cross-react with other *Xanthomonas* and closely related strains. With the *Xaf gyraseB* real-time PCR also being able to detect to 10^3 cells per disc this will give one assay for each pathogen able to detect latent infections in nursery material and, perhaps more importantly, nuclear stock material.

Standard operating procedures are available and CSL diagnosticians have been trained in their application. The staff time required to process one sample has been estimated at 3 minutes per disc, with a consumables cost of less than £2 per sample. This will enable high-throughput screening of plant samples in the CSL Molecular Technology Unit. A demonstration of the effectiveness of the real-time PCR assays was provided from samples received from two UK outbreaks of angular leaf spot. The *Xf* 16S and *Xf hrpB* assays were used to detect and identify *X. fragariae* in material received from these outbreaks. The *Xf* 16S PCR indicated that either *Xf* or *Xaf* was present in the samples and also established that the levels of the pathogen were above those known to be detectable by the *Xf hrpB assay.* This highly specific assay was then applied to the samples and, with the *Xaf pep* PCR being negative, proved that the pathogen causing the symptoms was *X. fragariae*.

OBJECTIVE 4. Initial pathogenesis studies on *Xanthomonas arboricola* pv. *fragariae*.

INTRODUCTION

Little is known about the epidemiology of *Xanthomonas arboricola* subsp. *fragariae*. Significant losses have not been reported in Italy, the only country so far known to have been affected by this pathogen. However, there is evidence that some losses in Italy may have been mistakenly attributed to *Xanthomonas fragariae*, as both pathogens can occur simultaneously on strawberry plants. To attempt to learn more about this pathogen, a series of artificial inoculation experiments were set up at CSL. With the exception of the hydroponic experiment (described below) these experiments were conducted before the development of the *Xaf pep* PCR assay.

MATERIALS and METHODS

Bacterial strains

Two *Xanthomonas arboricola* subsp. *fragariae* strains (NCPPB 4182, 4183) were cultured on nutrient agar (NA) at 28°C. Suspensions of each strain were prepared in sterile phosphate buffer.

Artificial Inoculation of strawberry plants with *Xaf*

Two inoculation methods (for each strain) were employed. For each method 20 plants (cv. Elsanta and Everest) were inoculated with sterile phosphate buffer, which served as a negative control or a 10^{8} cells mL^{-1} suspension. All plants were propagated and grown in glasshouse cubicles at CSL.

a) Syringe Infiltration
Xaf bacterial suspensions (10^{8} cells mL^{-1}) were drawn into a 5 mL syringe (without needle). The syringe aperture was placed firmly against the abaxial surface of a marked leaf. The syringe plunger was depressed carefully until a water-soaked area became visible. Care was taken not to injure the leaflet. Two leaves per plant were inoculated. Twenty plants per strain were inoculated. After inoculation plants were covered with clear polythene for one week to generate a humid environment thought to favour infection.

b) Puncture Inoculation
Xaf bacterial suspensions (10^{8} cells mL^{-1}) were drawn into a 5 mL syringe (with needle). Marked leaves were wounded by puncturing major veins, the blade, edge of blade, midrib and peduncle with the syringe needle. Bacterial suspension was then inoculated into the wounds by carefully depressing the syringe plunger to form droplets of suspension over each wound site. Two leaves per plant were inoculated. Twenty plants per strain were inoculated. After inoculation plants were covered with clear polythene for one week to generate a humid environment thought to favour infection.

c) Abrasion method
A *Xaf* bacterial suspension (10 mL of 10^{8} cells mL^{-1} suspension) was mixed with 0.1 g carberendum powder. A drop of this suspension was then placed onto the surface of a marked leaf and rubbed into the surface using a gloved hand. The experiment was repeated for several leaves on each plant. Ten plants per strain were inoculated. Plants were not covered in polythene after inoculation.

Hydroponic Experiment

Strawberry plants (cv. Everest) were propagated hydroponically in rockwool cubes (Fig 4.1) and fed with a standard plant nutrient solution. Three weeks after transfer to rockwool cubes plants were inoculated with suspensions from a decimal dilution series of *Xaf* in sterile distilled water (10^{7} cells per ml – 10^{1} cells per ml). Prior to inoculation plants had not been watered or fed for 3 days. Plants were

inoculated by pouring 200 mL of each suspension into rockwool cubes supporting the growth of each plant. Care was taken not to contaminate the leaves with any of the suspensions and at subsequent waterings care was also taken to prevent splash contamination of leaves. Two plants per concentration were inoculated. At weekly intervals (for 3 weeks) single leaves were removed from each plant. Discs were removed from each leaf and processed for real-time PCR analysis as described previously. Included in the real-time PCR was a set of leaf disc extracts containing known concentrations of *Xaf* in a decimal dilution series, ranging from 10^6 to 10^1 cells per disc. These samples were tested by the *Xaf pep* PCR assay for the presence of *Xanthomonas arboricola* pv. *fragariae*. Negative control plants, only inoculated with sterile distilled water, were included in this experiment.

Figure 4.1. Strawberry plants inoculated with *Xaf* in the hydroponic experiment (*Xf* inoculated plants also shown).

RESULTS

Artificial Inoculation of strawberry plants with *Xaf*

Possible symptoms were observed in leaves inoculated with the puncture inoculation technique (Fig 4.2). However, although similar symptoms did not appear on negative control plants, we were unable to re-isolate the pathogen from inoculated leaves and it is possible that the observed symptoms were caused by the inoculation technique, rather than *Xaf*. These slight symptoms compare unfavourably with *Xf* induced symptoms produced using the same inoculation method (Fig 4.3), where definite angular leaf spot symptoms were produced. No symptoms were observed in syringe infiltrated plants (despite this being the most successful inoculation method for *Xf*) or plants inoculated via the abrasion method.

Figure 4.2. Possible symptoms on leaves inoculated with *Xaf* NCPPB 4182 via the puncture inoculation method, 11 weeks post inoculation.

Figure 4.3 Angular leaf spot symptoms on puncture inoculated leaf (*Xf* strain NCPPB 1649), eight weeks post-inoculation.

Hydroponic Experiment

No bacterial leaf spot symptoms were observed on any of the strawberry plants during the experiment. At each of the three samplings individual leaves tested positive for the presence of *Xaf* (Table 4.1). No correlation was observed between the amount of initial inoculum and the occurrence of *Xaf* within strawberry leaves. C_T values from each positive disc indicated that levels of the pathogen were low, generally around the region of 10^3 cells per disc or lower, when compared with the mean C_T values derived from decimal dilution series of *Xaf* in leaf disc extracts.

Table 4.1 Real-time PCR analysis of leaves removed from *Xaf* inoculated strawberry plants in hydroponic experiment.

Harvested plant	1st sampling (7 days post inoculation)	2nd Sampling (14 days post inoculation)	3rd Sampling (21 days post inoculation)
10^7 (Leaf 1)[1]	-	-	-
10^7 (Leaf 2)	+ (37.42)[2]	+ (36.40)	-
10^6 (Leaf 1)	-	-	-
10^6 (Leaf 2)	-	+ (37.12)	-
10^5 (Leaf 1)	-	-	-
10^5 (Leaf 2)	-	+ (35.40)	-
10^4 (Leaf 1)	-	+ (35.40)	-
10^4 (Leaf 2)	-	+ (35.40)	-
10^3 (Leaf 1)	+ (35.68)	+ (36.37)	-
10^3 (Leaf 2)	-	+ (34.98)	-
10^2 (Leaf 1)	-	-	-
10^2 (Leaf 1)	-	-	-
10^1 (Leaf 1)	+ (36.42)	-	-
10^1 (Leaf 2)	-	-	+ (36.79)
-ve cont. (Plant 1)	-	-	-
-ve cont. (Plant 2)	-	-	-

[1] Individual leaves were removed from inoculated plants at each sampling. At each sampling a fresh leaf was harvested. Values indicate inoculum concentration applied to respective plants.
[2] (+) - positive reaction; (-) – negative reaction. C_T value: PCR cycle number (in brackets) at which fluorescence is first detected during a 40-cycle PCR.

Table 4.2 *Xanthomonas arboricola* pv. *fragariae* dilution series, in strawberry leaf disc extracts, and C_T values generated by the *Xaf pep* real-time PCR assay.

No. of *Xaf* cells per leaf disc	Mean C_T value *Xaf pep* PCR
10^6	+ (23.37) [1]
10^5	+ (27.10)
10^4	+ (31.39)
10^3	+ (33.47)
10^2	-
10^1	-

[1] (+) - positive reaction; (-) – negative reaction. C_T value: PCR cycle number (in brackets) at which fluorescence is first detected during a 40-cycle PCR.

DISCUSSION

The results from the artificial inoculation experiments indicate the difficulty of producing bacterial leaf blight symptoms under artificial conditions. In comparison it was possible to produce artificial symptoms with *Xf,* though it was not possible to induce symptoms away from the initial inoculation site – i.e. to produce a systemic infection mimicking a natural infection. At the time of the artificial inoculation experiments the *Xaf pep* PCR assay had not been developed and we were thus unable to determine whether *Xaf* cells did remain on the surface of the inoculated leaves. *Xaf* is considered relatively easy to isolate from infected material, at least when compared with the difficulty in isolating viable *Xf* cells, and thus as each inoculated leaf might have been expected to support a high population of viable *Xaf* cells, even in the absence of symptoms, we can assume that all the inoculation methods tested are not suitable for use as an artificial inoculation technique.

More success was met with the hydroponic experiment. In this trial the pathogen was detected in leaves inoculated with various concentrations of *Xaf*, with each plant being inoculated with 200 mL of suspension. Detected levels of the pathogen were relatively consistent for each positive leaf and did not correlate with the initial inoculum concentration, i.e. there were not higher levels of *Xaf* in plants inoculated with the 10^7 cells ml^{-1} suspension than those inoculated with the lesser concentrations. This indicates that strawberry plants are only able to take up a limited number of *Xaf* cells from the rhizosphere and that levels of the pathogen could not increase above a certain point within leaf tissues. In a parallel experiment, using *Xanthomonas fragariae* as the inoculum source, *Xf* was not detected in any leaves harvested from inoculated plants at any stage of the experiment.

As we have not been able to test *Xaf* leaf samples from a known outbreak we cannot correlate these concentrations with those observed in the hydroponically infected leaf material. However it is likely that higher levels of the pathogen are required before symptom expression can occur. In experiments conducted with *Xf,* symptoms were only observed in plants inoculated with suspensions containing 10^4 cells per ml^{-1} or above. Thus the levels of *Xaf* observed within the leaves harvested from the hydroponic experiment are unlikely to result in symptoms. However it is possible that mechanical damage to leaf tissues, harbouring an internal population of *Xaf*, could result in the pathogen escaping from the inside of the leaf onto the surface, increasing in number, and ultimately inducing bacterial blight symptoms. In addition this experiment also indicates that soil could also act as a method of spread for *Xaf*, in comparison to *Xf* which appeared to be unable to be taken up from the rhizopshere and into strawberry plants.

In the absence of a natural infection system we were unable to conduct trials to demonstrate whether significant economic losses could be induced by an outbreak of *Xanthomonas arboricola* pv. *fragariae* in the UK. All current reports suggest this pathogen is a lesser threat to UK production than *Xanthomonas fragariae*. However until a viable artificial inoculation method can be devised this theory will remain unproven.

Possible Future Work

The work on *Xaf* pathogenesis, and to a lesser extent similar studies carried out using *Xf*, demonstrate the difficulty in using artificial inoculation methods to produce symptoms similar to those seen in a natural infection. This holds for many other plant pathogens as well. Despite the presence of the pathogen on the leaf surface no symptoms were produced. If the mechanism for this avirulence, i.e. lack of proper attachment of bacteria to leaf cells, the absence of a specific environmental trigger, could be established this would lead to a better understanding of bacterial pathogenesis. In turn this could also lead to the better control methods, e.g. plants could be grown in the absence of a certain environmental factor or, if proper bacterial attachment was found to be a key factor in pathogenesis, plants could be treated with a compound (maybe even a detergent) which prevented attachment of the pathogen to leaf surfaces and consequently the appearance of symptoms.

References

Stoger A, Ruppitsch W. 2004. A rapid and sensitive method for the detection of *Xanthomonas fragariae*, causal agent of angular leaf spot disease in strawberry plants. *Journal of Microbiological Methods* **58**: 281-284.

References to published material

9. This section should be used to record links (hypertext links where possible) or references to other published material generated by, or relating to this project.

Angular Leaf Spot and bacterial leaf blight – two new notifiable strawberry plant diseases. HDC Factsheet 03/05. John Elphinstone.

Bacterial Angular Leaf Spot and Bacterial Leaf Blight of Strawberry. Poster at 2005 PHSI conference, January11-13, 2005. Stratford-upon-Avon