DEPARTMENT for ENVIRONMENT, FOOD and RURAL AFFAIRS **CSG 15**

Research and Development

Final Project Report

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Project title	Investigation of quarantine and emerging fungal pathogens in support of plant health pest risk analysis, management and policy
DEFRA project code	**PH0171**
Contractor organisation and location	Central Science Laboratory, Defra Sand Hutton York, YO41 1LZ
Total DEFRA project costs	£ 169,159
Project start date	30/03/01
Project end date	31/03/04

Executive summary (maximum 2 sides A4)

The project dealt with aspects of three fungal pathogens of quarantine significance: diagnostics for *Tilletia indica* (Karnal bunt of wheat); biology of pathogenic isolates of *Alternaria alternata* (apple blotch and pear black spot); and management and biology of *Stegophora ulmea* (elm black spot).

Tilletia indica was added to the EC Directive after being introduced into the USA in 1996 as this represented a new, significant pathway of entry into the UK and EU. The provisional Pest Risk Analysis (PRA) for the UK and EU indicated that *T. indica* could establish and cause economic damage. To date, several EU countries have intercepted the pathogen from Mexico, India, and possibly the USA. *T. indica* is difficult to distinguish from other tuberculate-spored species which can contaminate grain, e.g. *T. walkeri,* (ryegrass bunt) and *T. horrida* (rice smut). Confirmatory DNA tests take 2-3 weeks because cultures currently have to be produced from germinated teliospores. However, teliospores do not always germinate due to dormancy or non-viability. More rapid, reliable diagnostic methods were urgently needed as such slow diagnostic processes complicate decisions on action, disrupt milling schedules and incur significant storage costs for importers when consignments are held under notice.

Data on isolates of *T. indica* (5), *T. walkeri* (2) and *T. horrida* (3) were collected. For each isolate, 200 teliospores were characterised by size (diameter), ornamentation (0–3 scale) and colour (1–7 scale). Analysis of mean size using power analysis determined that the mean of a sample could be accurately estimated at the 5% significance level with only 50 spores, making it possible to differentiate the three species by this character with this number of spores. However, mean size alone might not be considered a reliable character because of the risk of species occurring together in a sample. Since the species showed differences in colour and ornamentation, all three parameters (size, colour and ornamentation) were combined in a multivariate analysis to produce a model using 80% of the teliospores from each isolate; the model was validated on the remaining 20%. The model identified single spores with a 72% mean accuracy. It identified single *T. indica* teliospores (76%) more accurately than *T. walkeri* (61%) or *T. horrida* (71%) teliospores. When the model was evaluated on additional isolates of *T. indica* (4) and *T. Horrida* (3), and one isolate of each species previously used in the model development, the model performed better for *T. horrida* isolates (97% average accuracy) and slightly less well than before for *T. indica* isolates (63% average accuracy); it

identified the control isolates originally used to develop the model with a higher accuracy than previously (88, 86 an 82% accuracy for the single *T. indica*, *T. walkeri* and *T. horrida* isolates respectively).

Collaboration with the University of Hull (UH) developed promising image analysis software for identifying 'bleached' teliospores. The UH software enabled image acquisition, image recognition and subsequent image analysis. In a preliminary analysis of 9 parameters, 96% of bleached spores were identified correctly. Using principal component analysis, the number of parameters was reduced to two. These two parameters correctly identified 97% of bleached teliospores. The ratio of the inner and outer spore diameter was also identified as a potentially important discriminator for these two species. The image analysis software was therefore shown to have potential but needs further development and evaluation.

In experiments to develop a rapid, sensitive and reliable molecular confirmation test, a simple method was developed for use on single germinated teliospores at 5–7 days (compared with previous test times of 2-3 weeks). The method involved excising germinated teliospores from agar plates using a fine needle and placing them directly into TaqMan® PCR reaction tubes containing master mix. Molecular identification of single germinated teliospores bearing basidiospores was achieved with an 80% success rate. This rate increased with the number of germinated teliospores tested: 2 spores (90%); 3 spores (95%); 5 spores (100%); and 10 spores (100%).

The work showed that it is possible to identify teliospores by morphology if relatively small numbers (>10) are present in grain, contrary to the general belief that larger numbers (hundreds) are required. This was also demonstrated in a ring test of the CSL-produced EU *Tilletia indica* Diagnostic Protocol. PCR directly on germinated teliospores can speed up the molecular confirmation process by 1–2 weeks as it does not rely on culturing from the basidiospores.

Pathogenic isolates of *Alternaria alternata* are economically damaging pathogens. Leaf infections and defoliation reduce fruit yield and encourage premature fruit drop; fruit infections seriously affect quality. The pathogens are listed as 'non-European species of *A. alternata* pathogenic to apple, pear and *Cydonia'*. The classification of these fungi remains unresolved being referred to as either true species which are morphologically different (e.g. *A. mali* on apple and *A. gaisen* on Asian pear) or strains of *A. alternata* separated by the production of host specific toxins which differentiate them from the ubiquitous saprophytic forms of *A. alternata*. In addition to the quarantine-listed apple and pear pathogens, morphologically similar toxin producing pathogens have emerged on other hosts, e.g. on strawberry, tomato, citrus, kiwifruit, walnut, rosemary and lavender (the latter two are PHSI interceptions). There are also new reports of strains that are not entirely host specific. For example, *A. mali* may infect Asian pear varieties (e.g. Nijisseiki), as well as apple; isolates from strawberry may also infect some pear cultivars. More information was therefore needed on the identity and host specificity of these pathogens to make informed decisions about their risk.

Apple and pear isolates were tested on a range of other plant species. The pathogenicity studies showed that they were not as host specific as previously believed but could also infect and cause necrotic symptoms on a wide range of other host plants. For apple isolates, walnut, apricot, quince and tomato, lettuce and lavender all showed degrees of susceptibility; the UK apple variety Cox's Orange was also more susceptible than the highly susceptible apple variety Red Chief. For the pear isolates, apricot, walnut, quince, lettuce, tomato, rosemary and strawberry were also susceptible to some of isolates; the UK variety Conference was as susceptible as the highly susceptible Asian pear variety Nijisseiki. These wider host ranges are a significant finding and will contribute to a review of the pest risk analysis.

Apple and pear varieties were also shown by these studies to be susceptible to various degrees to pathogenic isolates of *Alternaria alternata* originating from a variety of other plant species, e.g. apricot, strawberry, lettuce, tomato and walnut. All apple and pear varieties were susceptible to at least two isolates from other hosts; the majority of apple and pear varieties were susceptible to at least 5 isolates. Isolates from strawberry, lettuce, apricot and walnut caused infection over the widest range of apple varieties; isolates from strawberry and lettuce caused infection over the widest range of pear varieties.

Molecular DNA fingerprinting (AFLP) work on all of the isolates used in these studies revealed some evidence of grouping for some isolates, e.g. both tomato isolates grouped together, as did both strawberry isolates. However, isolates from some hosts were distributed through all or several groups, e.g. apple and pear, although one group was composed almost entirely of apple isolates. The mixed composition of each group, containing isolates from various hosts, could explain the variation in host range and infectivity found from the pathogenicity studies.

Stegophora ulmea was first intercepted by the PHSI in 1999 on bonsai elms imported from China. Statutory action was taken, a PRA was completed and the organism discussed at the EC Plant Health Standing Committee with a view to listing as an EC quarantine organism. The summary PRA highlighted areas where more information was needed to assist containment/eradication action, e.g. on the pathogen's lifecycle and on alternative fungicides which could support statutory action with minimum impact on the trade.

Isolates of the fungus were obtained both from bonsai elms inspected by the PHSI originating in China and by importation under licence of naturally infected elm tree leaves from the USA. The macroconidial stage (*Gloeosporium ulmicolum*) formed readily, primarily on the youngest leaves, and the microconidial stage (*Asteroma ulmea*) formed within the same fungal stroma as the macroconidia. When attached leaves were inoculated, only the youngest expanded leaves or unexpanded leaves developing from active buds were infected; there was no evidence of infection of dormant buds. Knowledge that only the youngest leaves are primarily susceptible could help growers reduce risk by initially pruning out such growth. This has been applied in practice by one grower, where removal of the first long shoots or 'whips' on new plants has helped reduce disease incidence. Further information concerning the lifecycle was obtained during studies with the sexual stage (*Stegophora ulmea*). Although infection by sexual spores resulted in the formation of the asexual conidial stage, the sexual stage (ascocarps) of *S. ulmea* were never observed even following extensive incubation of naturally or artificially bonsai leaves. This suggests that, in the UK, native elm trees are unlikely to be at risk from infection by aerially dispersed sexual spores from bonsai plants grown under protection. Finally, the currently recommended fungicide, prochloraz (as Octave), was found to be fully effective at protecting new growth, but was not totally effective as an eradicant. Propiconazole (as Bumper) was an equally effective protectant but was a more effective eradicant than prochloraz. This offers a potentially more effective alternative treatment to prochloraz.

Scientific report (maximum 20 sides A4)

The project dealt with aspects of three fungal pathogens of quarantine significance: diagnostics for *Tilletia indica* (Karnal bunt of wheat); biology of pathogenic isolates of *Alternaria alternata* (apple blotch and pear black spot); and management and biology of *Stegophora ulmea* (elm black spot).

Objective 1. Diagnostics for *Tilletia indica* (Karnal bunt of wheat)

Diagnosis of *T. indica* teliospores detected in wash tests (Peterson *et al*., 2000; Inman *et al*., 2003, *http://www.csl.gov.uk/prodserv/know/diagpro*) is complicated by the fact that wheat grain can be contaminated by 'look-alike' species, namely *Tilletia walkeri* (ryegrass bunt) and *Tilletia horrida* (rice smut), (Aggarwal *et al*., 1990; Castlebury, 1998; Castlebury and Carris, 1999; Cunfer and Castlebury, 1999; Milbrath *et al*., 1998; Frederick *et al*., 2000). Other tuberculate-spored ('warty') *Tilletia* species can also look morphologically similar to *T. indica* (Durán & Fischer, 1961; Durán 1987), but only *T. walkeri* and *T. horrida* are known contaminants of wheat. Although morphological diagnosis is possible when there are large numbers of teliospores present (NAPPO, 1999), it is considered difficult with only a few spores as there is significant overlap in the characteristics of each species (Figure 1a–c; Inman *et al.*, 2003). Molecular methods can confirm presumptive identifications (Frederick *et al*., 2000; Pimentel *et al*., 1998), but these take 2–3 weeks because teliospores must be allowed to germinate to produce cultures for DNA extraction (Figure1d–e); although there have been reports of successful PCR on ungerminated spores (McDonald *et al*., 1999), these are not repeatable (G. White, Agriculture Canada, pers. comm.). Such long diagnostic timescales are not compatible with trade in grain because consignments are impounded until the diagnosis is confirmed: this seriously disrupts milling schedules, incurs significant storage costs and complicates decisions on action by Defra. This was particularly highlighted during an interception in February 2000 in the UK on US grain which was subsequently confirmed as *T. walkeri* by PCR after the existing 3-week molecular confirmation process. There was therefore a need for more rapid, reliable diagnostic methods.

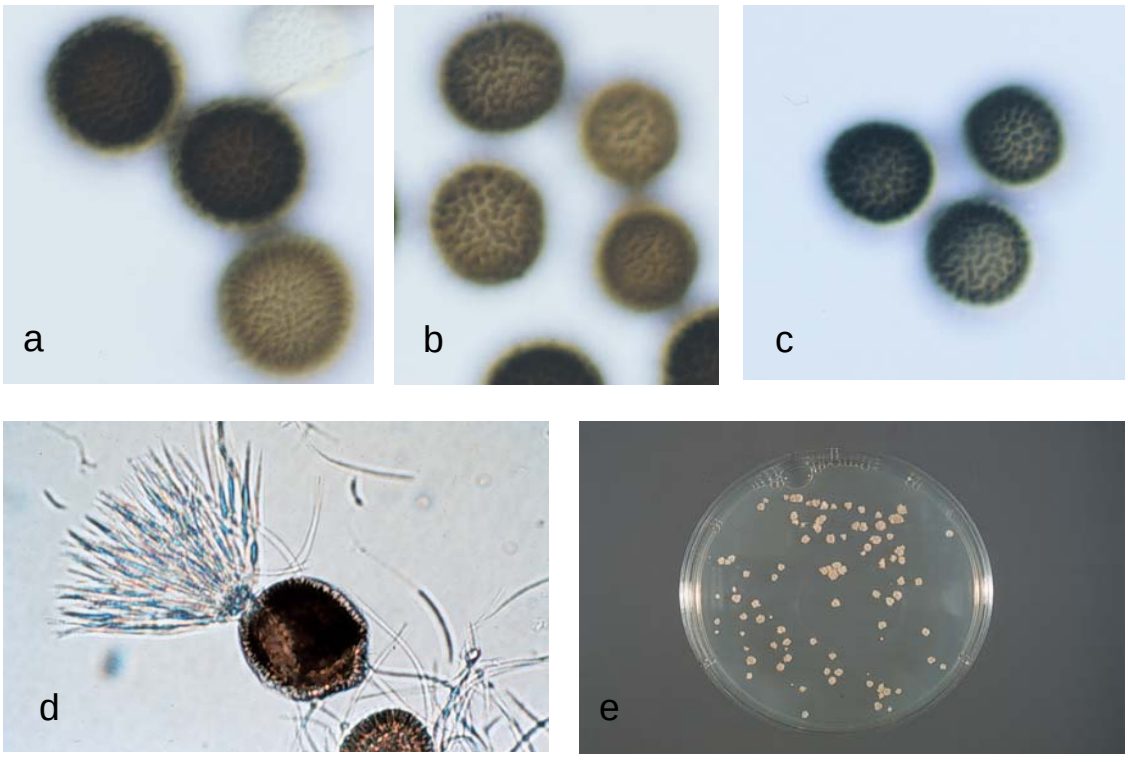

Figure 1. Teliospores of (a) *Tilletia indica*, (b) *T. walkeri* and (c) *T. horrida*; (d) germinated teliospore of *T. indica* showing basidiospore 'brush' after 1-week's incubation; (e) 1-week-old colonies on *T. indica* on agar.

Morphological methods

Analysis of morphological parameters: Isolates of *T. indica* were obtained, plus two isolates of *T. walkeri* and five of *T. horrida*; availability of *T. walkeri* isolates was markedly limited. Teliospores of selected isolates (*T. indica* x 5; *T. walkeri* x 2; *T. horrida* x 3), were recovered from bunted grains by disruption in water with a small amount of detergent. The suspensions were then applied to the same sieves that are used in the standard EU wash-test protocol, i.e. a 53 μm sieve and a 20 μm sieve. Teliospores were recovered from the 20μm sieve, centrifuged and the final pellet re-suspended in a small amount of distilled water for immediate characterisation. NB. The mountant used can significantly affect size parameters (Castlebury & Carris, 1999; Aggarwal *et al.,* 1990); water was used as this is the mountant recommended in the EU protocol as it does not affect teliospore viability and subsequent confirmation by molecular methods. For each isolate, 200 teliospores were characterised by size, (diameter), colour (1-7 scale) and ornamentation (0-3 scale).

There was significant overlap in size between the three species but differences were identified in both the maximum size and the mean size. *T. indica* isolates had a mean size of 34.5–40.9 μm and a maximum size of 42.5–53.0 μm; *T. walkeri* isolates had a mean size of 30.4–30.7 μm and a maximum size of 37.5 μm, with only one spore reaching 45 μm; *T. horrida* isolates had a mean size of 26.2–27.8 μm with a maximum size of 35 μm (Table 1). An analysis of mean size was done using power analysis to determine the number of spores required to give confident discrimination of the three species. The analysis indicated that 50 spores would be sufficient to separately identify the species, but not isolates of the same species, with a 95% confidence assuming an unmixed species composition. Colour and ornamentation parameters could, however, have the potential to increase confidence when combined with size parameters. Significantly, *T. walkeri* and *T. horrida* had no black, opaque spores while all *T. indica* isolates had some black, opaque spores, though the proportion varied with isolate from 5–62% of spores (5, 10, 19, 57, 62% for each of the five isolates respectively). With ornamentation, 77–98 % of *T. walkeri* spores had ridges (ridging to a highly significant degree); 59–88% of *T. indica* spores had ridges (ridging to less developed degree); while 36%–86% of *T. horrida* spores had ridging (ridging, when present, was not usually highly developed).

Species	Isolate	Mean (μm)	s.d.	Min (μm)	Max (μm)
Tilletia indica	Ti-10-USA	34.48	4.500	22.5	42.5
Tilletia indica	Ti-11-SA	34.70	4.022	25.0	42.5
Tilletia indica	Ti-08-Ind	35.51	4.338	22.5	47.5
Tilletia indica	Ti-05-Pak	38.28	3.408	27.5	50.0
Tilletia indica	Ti-03-Mex	40.93	4.937	25.0	53.0
Tilletia walkeri	Tw-02-LC	30.44	3.415	20.0	37.5
Tilletia walkeri	Tw-01-GP	30.73	3.144	22.5	37.5 (–45.0*)
Tilletia horrida	Th-01-Cal	26.24	1.863	22.5	32.5
Tilletia horrida	Th-12-Ark	27.83	2.046	22.5	35.0
Tilletia horrida	Th-Ark-con	27.55	2.337	25.0	35.0

Table 1. Size characteristics (mean and standard deviation; range) of isolates of *Tilletia indica*, *Tilletia walkeri* and *Tilletia horrida* based on measurements of 200 teliospores per isolate, mounted in water after sieving though a 53 μm sieve and collection on a 20 μm sieve. (*) , one teliospore only with this size.

Size, colour and ornamentation parameters were combined in a multivariate analysis with the aim of developing a model. After an initial analysis, an additional category for ornamentation was introduced; when ornamentation could not be discerned due to the darkness of the spore, an ornamentation score of 4 was assigned. A balanced sub-sample of 20% of the initial data set (20% of each isolate) was selected randomly and reserved for testing of the fitted model. Using the remaining 80% of each sample, a Principal Component Analysis (PCA) was undertaken to determine the variables that give the best separation into species. Using the variable set size, colour and ridges, the first two principal components accounted for 86% of the variance (this was greater than any of the other combinations of variables tested). This combination of variables was therefore used to determine the Linear Discriminant Analyses (LDA) equations. LDA was undertaken on the

80%-sample. The equations generated were then used on the 20%-samples to predict species membership and hence to test the effectiveness of the model.

The model identified single spores with a 72% mean accuracy; if 10 spores were tested there was a 96% probability of the model correctly identifying at least 5 spores correctly. It identified single *T. indica* teliospores (76%) more accurately than *T. walkeri* (61%) or *T. horrida* (71%) spores. For *T. indica*, 15% of spores were misidentified by the model as *T. walkeri* and 8% as *T. horrida*. For *T. walkeri*, 16% of spores were misidentified as *T. indica* and 24% as *T. horrida*. For *T. horrida*, 28% of spores were misidentified as *T. walkeri*, but only 2% as *T. indica*. Finally, the standard Minitab LDA was applied to the entire data set to generate the equations for the final model. This analysis was based on the Mahalanobis distance and assumed all groups to have the same covariance matrix. For any observation, the group with the smallest Mahalanobis distance will have the largest linear discriminant function. The species identification is given by the linear discriminant equation that gives the greatest value. The model equations were:

1) *T.horrida* : –25.37 + 1.623 (Di_μm) + 1.853 (Colour) – 1.524 (Ridges)
2) *T.indica* : –44.598 + 2.219 (Di_μm) + 1.657 (Colour) – 1.082 (Ridges)
3) *T.walkeri* : –30.015 + 1.809 (Di_μm) + 1.435 (Colour) – 0.886 (Ridges)

The model was evaluated further on additional isolates of some species (*T. indica* x 4; *T. horrida* x 3) which had not been used in the development of the model; one original isolate from each species was also included as a comparative check (Table 2). For the new *T. indica* isolates: the model correctly identified on average 63% of teliospores (range 50–68%); misidentifications were on average evenly distributed between *T. walkeri* (17%) and *T. horrida* (17%); 88% of teliospores were correctly identified for the *T. indica* comparative control isolate (used in the original model development), but this higher accuracy was due to it being a larger-spored isolate (mean 39 μm compared with 33–36 μm for the new test isolates). For the *T. horrida* isolates: the model correctly identified 97% of teliospores; all misidentifications were ascribed to *T. walkeri*; 82% of teliospores were correctly identified for the *T. horrida* comparative control isolate, but this lower accuracy was due to it being a larger-spored isolate (mean 28 μm compared with 24–25 μm for the test isolates). No additional *T. walkeri* isolates could be obtained, but an 86% accuracy of identification was achieved with an isolate used in the original model development. In general, the model performed better for *T. horrida* isolates and slightly less well with the new *T. indica* isolates compared with those isolates on which the model had been developed.

Species	Isolate	No. spores	Mean	Range	Model predictions (% spores)		
					T. indica	*T. walkeri*	*T. horrida*
Test isolates							
T. indica	Ti-4-Mex	50	33.4	25–50	50	22	28
T. indica	Ti-6-Mex	50	34.8	25–43	68	16	16
T. indica	Ti-6-Pak	50	35.6	28–45	66	10	24
T. indica	Ti-7-Ind	50	35.5	28–45	68	18	14
Mean					**63**	17	17
T. horrida	Th-A-Cal	50	24.5	20–29	0	4	96
T. horrida	Th-C-Ark	50	25.1	20–30	0	2	98
T. horrida	Th-E-Cal	49	24.7	21–33	0	2	98
Mean					0	3	**97**
Model isolates							
T. indica	Ti-3-Mex	50	39.8	30–50	88	8	4
T. walkeri	Tw-1-LC	50	29.7	25–38	2	86	12
T. horrida	Th-12-Ark	50	28.4	28–30	0	18	82

Table 2. Evaluation of the predictive model against four isolates of *Tilletia indica,* 3 isolates of *Tilletia horrida* and one isolate of each species (*T. indica, T. walkeri, T. horrida*) used in the initial model development.

Development of image analysis approaches (CSL collaboration with University of Hull): In preliminary studies before the project began, bleaching teliospores was identified as having potential for diagnosis as it revealed additional morphological characters, notably the spore profile and spore inner wall. It was proposed that these additional morphological characters, as well as other spore characteristics (size, area, etc.), could be potentially exploited within an image analysis system to discriminate species.

Bunted seeds were placed in water to extract teliospores of each *Tilletia* species. The water was then sieved through a 100 μm membrane to remove larger debris and the teliospore suspensions were centrifuged at 1000 x *g* for 3 minutes. The supernatant was removed and 10% domestic bleach (*ca.* 0.5% active NaOCl) was added to the teliospore pellets. After bleaching for 10 minutes, the samples were centrifuged again and the pellet washed twice with distilled water. The teliospores were then pelleted again by centrifugation and the teliospores re-suspended in either a minimal amount of water or in lactoglycerol-trypan blue stain. In the case of the latter, teliospores were stained for a minimum of 2 hours, then pelleted by centrifugation, washed in water and then re-suspended in a minimal amount of water (*ca*. 100 μl). Teliospores were either viewed with normal light microscopy at x400 magnification, or with differential contrast (DC) microscopy (Figure 2). Unbleached teliospores were also examined for comparison. Images were obtained using a JVC TK-1270 colour video camera and a snapper card and snapper tool (Datacell V2). The images were saved in either TIFF or JPEG format and converted later into Windows BITMAP files.

The image processing software was written in Borland C++ (Thomas Bernard and Dr. David Chesmore*****, University of Hull) which is a high level programming language running under the Windows operating system. It was decided to write custom software as this would give maximum flexibility and allow the development of algorithms not currently implemented in commercial image processing packages. The software was designed to automatically locate and measure all spores in an image (Windows BITMAP format) and produce a text file containing all measurement parameters for the spores in an image. The first task in this process was to convert images into greyscale since colour was not employed in this project. Once converted, the optimal threshold for discriminating between the spores and background was calculated and each spore located by scanning the image from the top left to the bottom right. The location process involved determining whether a particular pixel belonged to a spore or to the background. The area of each spore was also taken into account and any objects detected with areas smaller than a defined threshold were rejected as debris. Once each spore was located, it's contour and other parameters were calculated and the spore labelled. This process continued until all spores had been recognised and the bottom of the image reached. A total of nine parameters were measured for each spore: (1) perimeter (contour); (2) number of white pixels left inside the spore after thresholding; (3) surface area of spore (all pixels inside the spore after thresholding); (4) average value of the size of the spines; (5) number of spines; (6) shortest radius from spore centre to border; (7) longest radius from spore centre to border; (8) aspect ratio (max/min diameter); (8) smoothness of the outline.

Initial analysis with limited numbers of *T. indica* and *T. walkeri* teliospores suggested differences between the two species, e.g. surface area, aspect ratio and smoothness of the outline. Images of bleached teliospores obtained using DC microscopy produced more consistent and reliable results than images obtained by normal light microscopy, or images of bleached-stained spores, or unbleached spores. In order to maximise the discrimination between species, it was decided to apply principal components analysis (PCA) to the nine parameters using data from bleached spores viewed with DC microscopy. PCA is a process of dimensionality reduction where the nine dimensions in this problem are reduced to two or three by determining which of the dimensions parameters show most sensitivity to the two species. Figure 3 shows the results for *T. walkeri* and *T. indica* showing good discrimination although there is some overlap: 97% of bleached teliospores were correctly identified by just two parameters. Further analysis was carried out by examining the ratios of the spores' internal to external diameters visible after bleaching. This appears to be as successful at separating the two species (Figure 4) as PCA and is mathematically much simpler. In conclusion, the use of image processing for rapidly discriminating between *T. walkeri* and *T. indica* has been shown to be feasible. Two methods – PCA and internal/external spore diameters of bleached spores – have been tested and have potential for discriminating between the two species. However, much work remains to be done, specifically:

inclusion of PCA and spore diameter calculations into the main software package; further evaluation using additional isolates, including *T. horrida*, and larger numbers of spores.

***** Now Univeristy of York

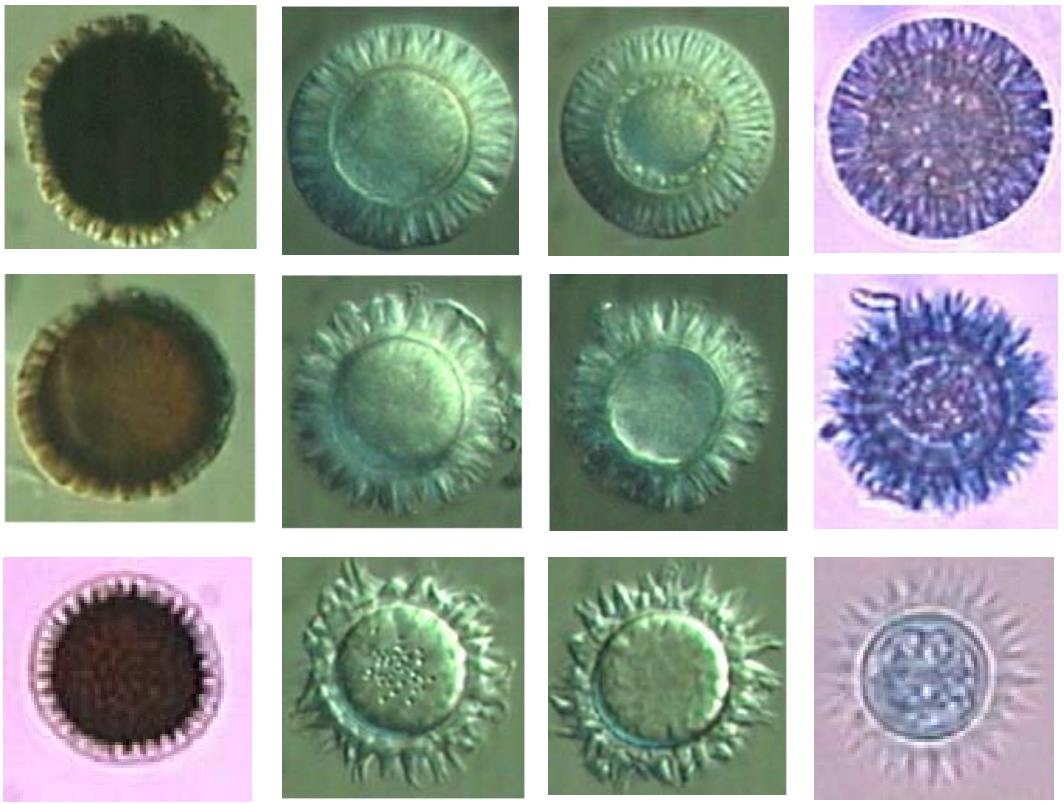

Figure 2. Unbleached teliospores (left column), bleached teliospores viewed with differential contrast (middle columns) and bleached-stained teliospores (right column) of *Tilletia indica* (top row), *T. walkeri* (middle row) and *T. horrida* (bottom row).

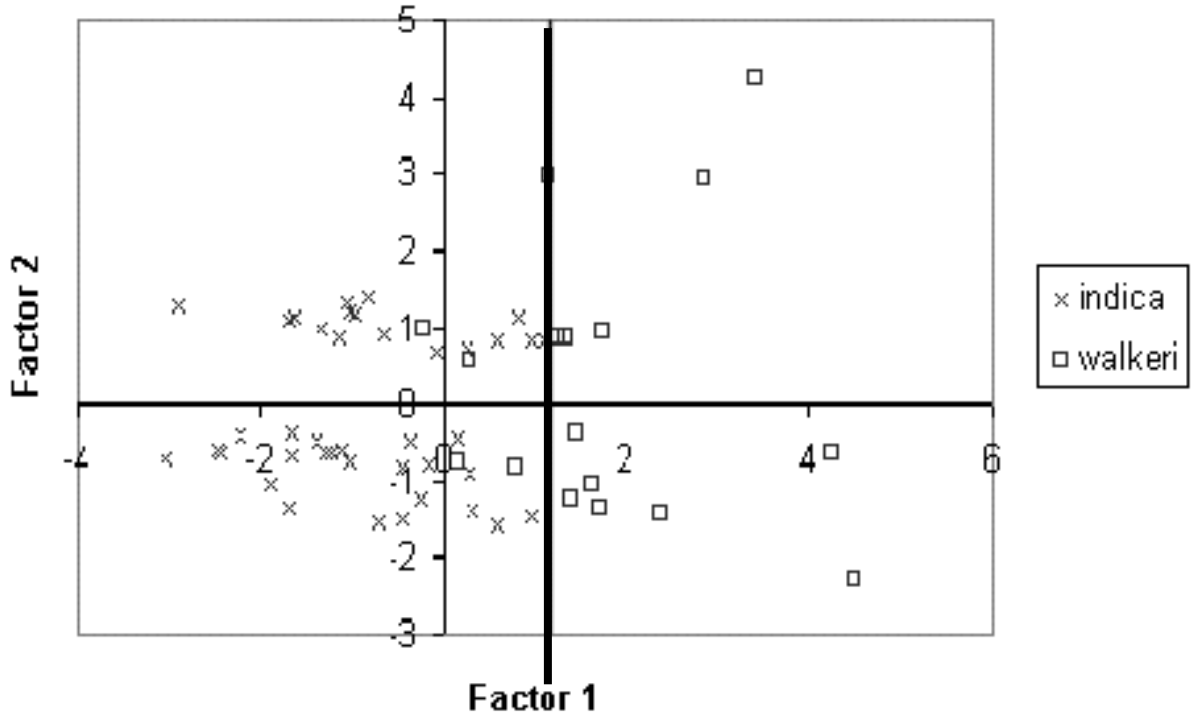

Figure 3. Principal components analysis results plotted as Factor 1 versus Factor 2 for bleached teliospores of *Tilletia indica* and *T. walkeri*. (T. Bernard & D. Chesmore, University of Hull; A. Inman & R. Bowyer, CSL).

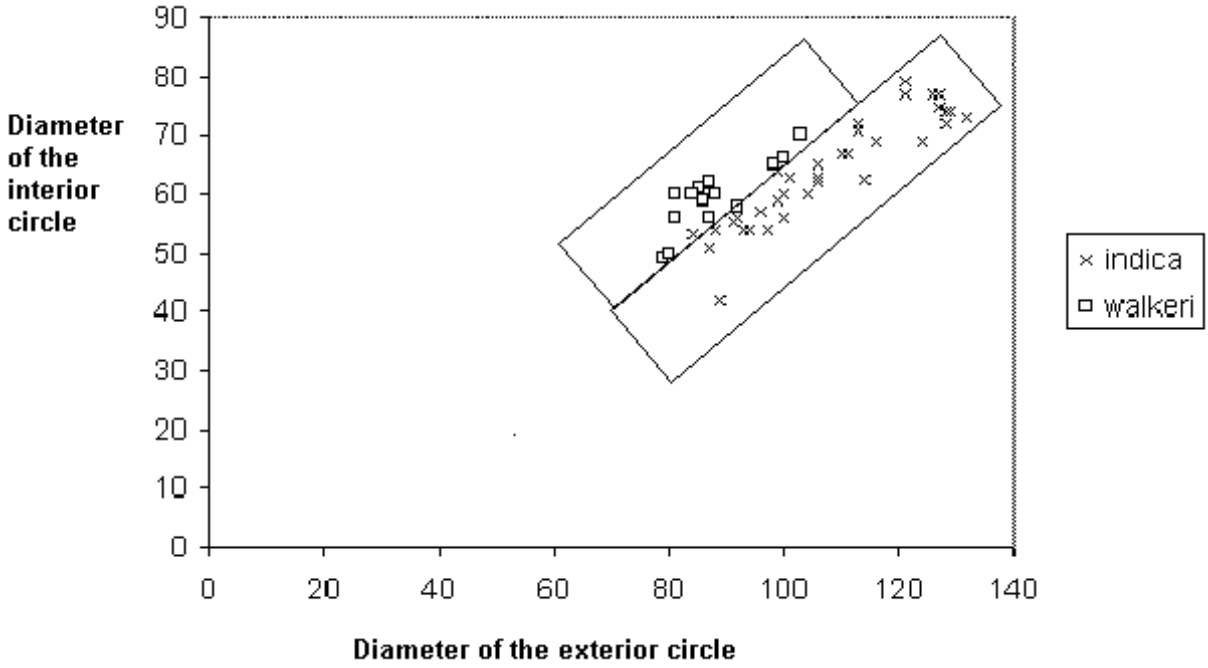

Figure 4. Potential separation using bleached teliospores of *Tilletia indica* and *T. walkeri* based on measurements of internal spore diameter and external spore diameter. (T. Bernard & D. Chesmore, University of Hull; A. Inman & R. Bowyer, CSL).

Molecular methods

Molecular methods for confirmatory identification of tuberculate-spored *Tilletia* species detected in wash tests currently require germination of the teliospore/s and production of mycelial cultures from the resultant basidiospores (Frederick *et al.*, 2000). This approach has been incorporated into the EU-recommended Diagnostic Protocol for *Tilletia indica* (Inman *et al.*, 2003; due for publication in *EPPO Bulletin*, 2004) which was produced by CSL and ring tested by CSL alongside other EU laboratories. The teliospore germination process can take up to 7–14–(21) days for teliospores to produce a promycelium (basidium) with mature basidiospores. It then takes a further 4–7 days to produce cultures on potato dextrose broth for subsequent molecular analysis using species-specific primers and/or sequencing or PCR-RFLP. Previous work had established that PCR on ungerminated teliospores was difficult and was not reproducible even with large numbers of teliospores (McDonald *et al.*, 2000; G. Peterson, USDA-ARS, Fort Detrick pers. comm.; K. Hughes, CSL, York, pers. comm.). This project aimed to develop methods for reliable molecular identification of single germinated teliospores. To this end it investigated: germination systems; approaches for efficient recovery of germination products; and efficient DNA extraction methods.

To investigate these aspects, teliospores of *T. indica* were recovered from bunted grains, soaked overnight in water and then put through a decontamination step using acid electrolysed water (Bonde *et al.*,1999.; EU Diagnostic Protocol, 2003). The resulting teliospore suspension was put through a dilution series and 10µl drops of suspension were 'spot-plated' onto either 2% water agar (WA) or 5 x 5 mm sterile polyester membranes on WA plates (5 replicate spots per plate); the dilutions corresponded to ca. 10–30 (low), 50-80 (medium) and 120–200 (high) teliospores per 10 µl drop. Plates were incubated over a time course of either 0, 1, 2, 4, 6, 8 or 12 days at 20°C under a 12 hour white light day-length regime. At each time point, the number of teliospores that were ungerminated or germinated in each 'spot' was assessed; the plates were placed into a -80°C freezer for use in subsequent investigations. For plates on which teliospores had been placed on membranes, individual membranes were removed from the agar and placed into individual sterile microcentrifuge tubes and stored at -80°C, again for subsequent processing.

In an initial experiment, samples from the 6-day sampling point were used (when ca. 25% of teliospores had produced promycelia or basidiospores) to investigate recovery by washing with 100 μl water, followed by DNA extraction using various extraction methods. For the membranes, water was added directly onto membranes stored in microcentrifuge tubes and the water flushed numerous times over the membrane. For the agar plates, individual 'spots' were excised, placed into microcentrifuge tubes and then washed with 100μl water, as described above for the membranes. The following extraction methods were then trialled on replicate samples: bead-beating; boiling for 10 mins or for 20 mins; or use of a DNA extraction kit. Samples were also taken at 12 days, when more teliospores (*ca.* 45%) had produced mature basidiospores or colonies with secondary sporidia. These were suspended in 500 μl of water and subjected to freeze-boil cycles. Aliquots (5 μl) were taken from the 100 μl suspension and pipetted in reaction tubes with master mix for TaqMan PCR. No amplification was detected in any of the sample extracts, either at day 4 or day 12, most probably due to the large dilution effect. The extraction approach was therefore refined and germinating teliospores were recovered from plates using reduced volumes of water. This involved using 20 μl aliquots of sterile distilled water and flushing the aliquots repeatedly onto the 'spot' of teliospores on the agar or on membranes placed in a sterile Petri dishes and repeatedly flushing the aliquot over the inoculation 'spot'. This method recovered teliospores, though not with a particularly high efficiency. Only 33% of teliospores were recovered from the agar and 19% from the membranes. Although teliospore recovery was not particularly high, the method was used to evaluate PCR identification of germinating teliospores at each sample point in the time course experiment as it was anticipated that the germination products (basidiospores, mycelium and secondary sporidia) might, if present, be more efficiently recovered than the teliospores themselves. At each time point, 20 μl aliquots were used to recover teliospores and any germination products from the germination substrates (agar or membrane; 3 replicates per time point per substrate). Each 20 μl of suspension was then placed directly into an individual PCR reaction tube containing the PCR master mix for TaqMan PCR. For samples using 'spotted' agar plates, no PCR positives were obtained, presumably because it was harder to recover the products from this substrate: when teliospores are germinated on the agar, promycelia can germinate into the agar; similarly mycelial colonies adhere to the agar surface and are unlikely to be recovered by washing. For the membranes, positive PCR results were not recorded for samples taken at day 0, 1 or 2 when teliospores had not produced any germination products (promycelia or basidiospores). However, PCR positives were recorded at days 4, 8 and 12 when germination products were present (approx. 5%, 38% and 45% teliospore germination, respectively).

In a subsequent preliminary experiment, sensitivity of the TaqMan assay was evaluated at an early stage of germination using 1 or 2 germinated teliospores (3 replicates of each) recovered from membranes after only 4 days incubation. For single teliospores, one of the three replicates produced a positive TaqMan result. For two teliospores, two of three replicates produced a positive TaqMan result. The experiment was repeated with a larger number of germinated teliospores but this time plated directly on 2% water agar. After 6 days incubation at 20°C, individual teliospores with basidiospores were picked-off the plates with a fine needle and placed into TaqMan PCR tubes containing 5 μl sterile distilled water (SDW). Replicate tubes were prepared with differing numbers of germinated teliospores in them as follows: 1 and 2 teliospores (20 replicates of each); 3, 5 and 10 teliospores (10 replicates of each). PCR TaqMan mastermix was added to the tubes and the samples tested by the standard TaqMan assay (50 cycles), together with positive controls (DNA extracted and purified from cultures) and SDW negative controls . Positive results were recorded for samples with CT values of <45 cycles as follows (Table 3): 16/20 (80%) of the 1-spore samples ; 18/20 for the 2-spore samples; 9/10 for the 3-spore samples; 10/10 for both the 5- and 10-spore samples. Mean CT values decreased with increasing numbers of spores, as did the range of values, whilst the final ΔRN value increased with increasing numbers of teliospores.

In conclusion, morphological diagnostic approaches have been developed and a simple, rapid and sensitive method for molecular confirmation of single germinated teliospores produced.

No. of germinated teliospores	No. positive samples/Total (CT >45)	% positive samples (CT >45)	Mean CT values for positive samples	Range of CT values for positive samples	Mean ΔRN for positive samples	Range ΔRN for positive samples
1	16/20	80	36.6	32.9–41.1	0.75	0.20–1.24
2	18/20	90	34.9	32.6–38.8	1.00	0.52–1.22
3	9/10	90	32.9	31.6–37.0	1.10	0.80–1.29
5	10/10	100	33.0	31.1–38.4	1.06	0.90–1.33
10	10/10	100	30.6	29.6–31.4	1.25	1.18–1.39
Positive control DNA	2/2	100	25.1	25.1–25.1	1.59	1.56–1.61
Negative control	0/4	0	NA	NA	NA	NA

Table 3: TaqMan PCR results for differing numbers of germinated teliospores (with basidiospore 'brush') recovered from 2% water agar plates after 6 days incubation at 20°C: The CT value corresponds to the number of PCR cycles accrued before the fluorescence value exceeds the baseline value; ΔRN is the TaqMan fluorescence value, which increases with the amount of PCR product.

Objective 2. Risks associated with pathogenic isolates of *Alternaria alternata*

Alternaria blotches of apple and pear are economically damaging diseases. Leaf infections and defoliation reduce fruit yield and encourage premature fruit drop; fruit infections seriously affect quality. The pathogens (EC, II/AI) are listed as 'non-European species of *A. alternata* pathogenic to apple, pear and *Cydonia'*. The classification of these fungi remains unresolved being referred to as either true species (Roberts, 1924; Simmons, 1999) which are morphologically different (*A. mali* on apple and *A. gaisen* on Asian pear) or strains of *A. alternata* separated by the production of host specific toxins (Kohmoto *et al.*, 1974) which differentiate them from the ubiquitous saprophytic forms of *A. alternata*. These pathogens occur in many countries exporting fruit to Europe and also in limited parts of the EPPO region, e.g. on apple in Yugoslavia, and on Asian pear in France and Italy. In addition to the quarantine apple and pear pathogens, morphologically similar toxin producing pathogens have emerged (Otani *et al*., 1995) on: strawberry, tomato, citrus, kiwifruit, English walnut (Italy), rosemary and lavender (the latter two are PHSI interceptions from Israel). However, there are now reports of strains that are not entirely host specific (Maeno, 1984; Otani *et al.,*1995). For example, *A. mali* may infect Asian pear varieties (e.g. Nijisseiki), as well as apple. Isolates from strawberry may also infect some pear cultivars. More information was needed on the identity and host specificity of these pathogens to make informed decisions about their risk to crops.

Develop pathogenicity tests

A thorough literature survey was carried out to develop and optimise a pathogenicity test suitable for the range of host plants under investigation. Based on this literature study, the following parameters were tested in order to establish the most effective method to use with a range of pathogenic *Alternaria alternata* isolates and their corresponding susceptible host plant: (1) inoculum concentration; (2) detached vs. attached leaves; (3) method of inoculum application; (4) upper vs. lower leaf surface; (5) leaf age; (6) wounded vs. unwounded leaves; and (7) incubation conditions and time before assessment.

Based on the above preliminary tests, the most optimal method to enable a range of host plants to be tested was defined as follows. Test isolates were grown on potato dextrose agar (PDA) at 18^{o}C with a 16-hour daylength for 14 days. Spores were harvested from plates using sterile distilled water (SDW) and diluted to a final concentration of $2x10^5$ – $1x10^6$ spores per ml. Young, but fully expanded, leaves were selected and detached from plants for testing. Leaves were placed adaxial surface uppermost onto dampened tissue paper in a damp chamber. For each test isolate, five 10 µl drops of conidial suspension were placed on to each of two unwounded upper leaf surfaces (5 per leaf) or, for plant species with larger leaves, ten 10 µl drops onto a single leaf. The lid (previously misted with water) was then placed onto the box and sealed. The pathogenicity

test chamber was incubated at approximately 20^{o}C with a 16-hour daylength for 14 days. The maximum lesion diameter was measured and recorded from each inoculation point (Figure 5).

Figure 5. Apple leaf artificially inoculated with a pathogenic isolate of *Alternaria alternata* from apple (syn. *A. mali*) using the optimal pathogenicity protocol. Note brown necrotic lesions at each inoculation point after 14-day's incubation.

Investigate host range of isolates from apple and Asian pear

The host range of 17 pathogenic *A. alternata* isolates from apple, and 5 from Asian pear, was tested using the methodology described above. Apple isolates were selected from Europe (5), South Africa (1) and the USA (11). Asian pear isolates were from Europe (3), Korea (1) and an unknown origin (1). The following wider range of plant species was selected: *Malus* var. Red Chief (control host for apple isolates), *Pyrus* var. Nijisseiki (control host for pear isolates), *Prunus armeniaca* (apricot), *Lycopersicon* (tomato), *Fragaria* (strawberry), C*itrus x tangelo* (minneola), *Citrus jambhiri* (rough lemon), *Cydonia* (quince), *Lactuca* (lettuce)*, Juglans* (walnut), *Malus* var. Cox's Orange (apple), *Rosmarinus* (rosemary) and *Lavendula* (lavender). Negative controls were sterile distilled water and a saprophytic isolate of *A. alternata* which was the type specimen for the species. Mean lesion diameter was calculated for the range of isolates on each plant species tested after 14 days incubation.

Investigation of host range of pathogenic *A. alternata* isolates from apple: The 17 isolates from apple caused lesions not only on the control apple variety 'Red chief', but also on a range of other plant taxa (Figure 6 & 7). The lesions that developed on walnut, quince and the apple variety Cox's Orange were, in fact, more extensive than those on the highly susceptible control apple variety 'Red chief'. Apricot, tomato, lettuce and, to a very much lesser extent, lavender were also shown to have limited susceptibility to some, or all, of the apple isolates. No infection was observed on minneola, rosemary, rough lemon or strawberry. No lesions were observed on SDW negative controls on any of the test plants. Lesions were, however, present when leaves of walnut and apple variety Cox's Orange and to a small extent, apricot, tomato and lettuce were challenged with the saprophytic type *Alternaria alternata* isolate. However, in each of these instances, there were considerably larger lesions when the leaves were challenged with the pathogenic apple isolates. Due to the lesion development using the saprophytic *A. alternata* isolate, it would be advisable to confirm the results (particularly from walnut and apple variety Cox's Orange) on attached leaves *in vivo*.

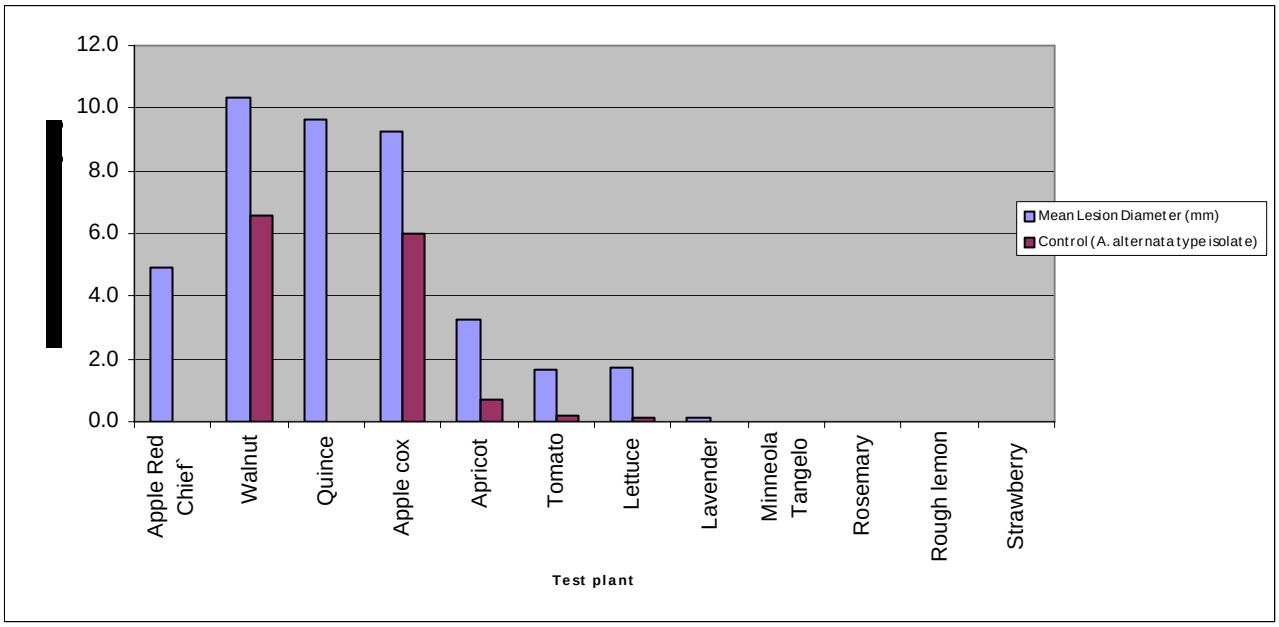

Figure 6. Infection (mean lesion diameter across 17 pathogenic isolates of *Alternaria alternata* from apple) on a range of other plant species.

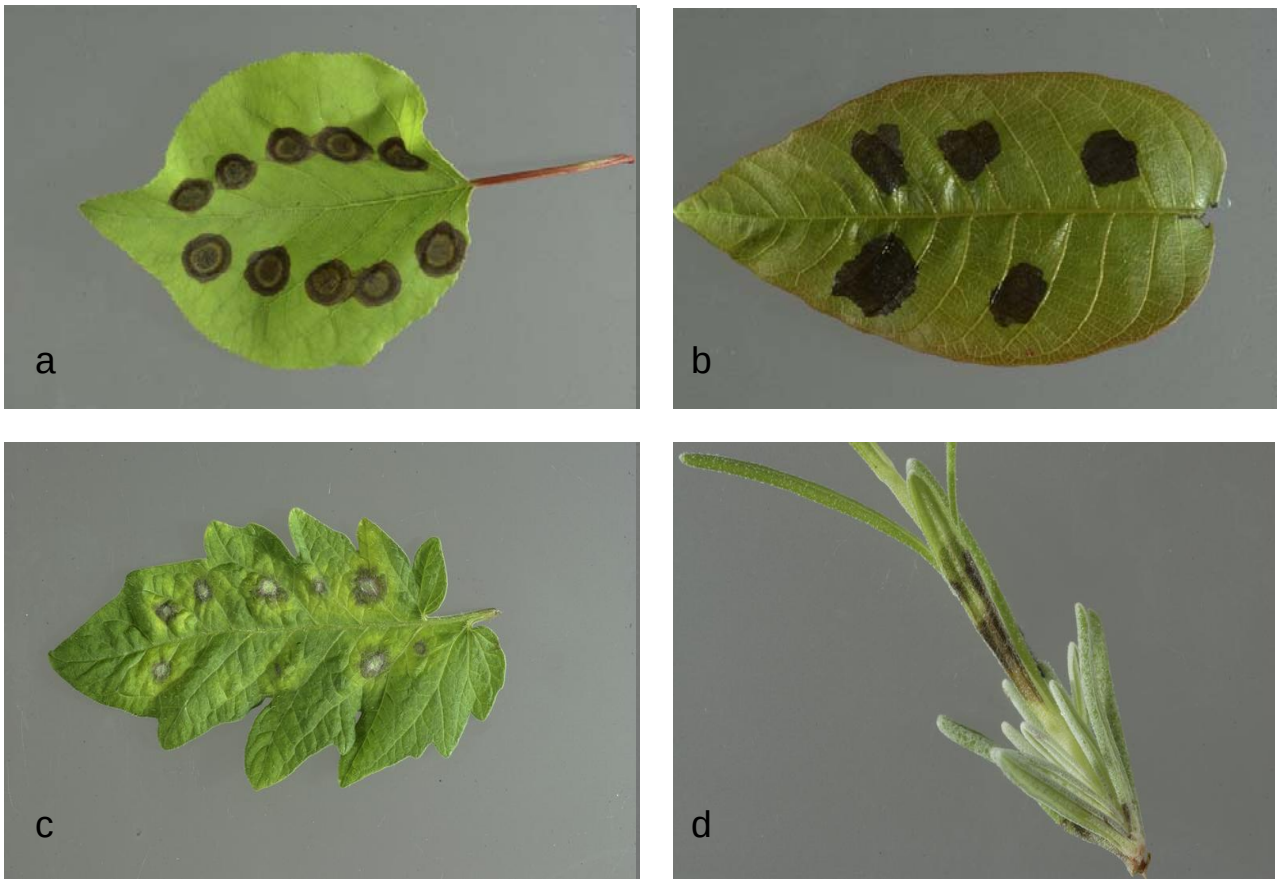

Figure 7. Lesion development on (a) apricot, (b) walnut, (c) tomato and (d) lavender 14 days after inoculation with a pathogenic *Alternaria alternata* isolate (syn. *A. mali*) from apple.

Investigation of host range using isolates from Asian pear: The five pathogenic isolates of *A. alternata* from pear infected the known susceptible pear variety Nijissieki, producing necrotic lesions averaging 4 mm in diameter after 14 days. Infection also occurred on walnut, apricot, the pear variety Conference, lettuce and quince and, to a much lesser degree, tomato, rosemary and strawberry (Figure 8). Necrotic symptoms were not observed on minneola, rough lemon, lavender or with the SDW control. The saprophytic *A. alternata* type isolate again produced necrotic lesions on walnut, apricot, lettuce and tomato, so the pathogenicity of the pear isolates should be confirmed using an *in vivo* attached leaf assay for these plant species.

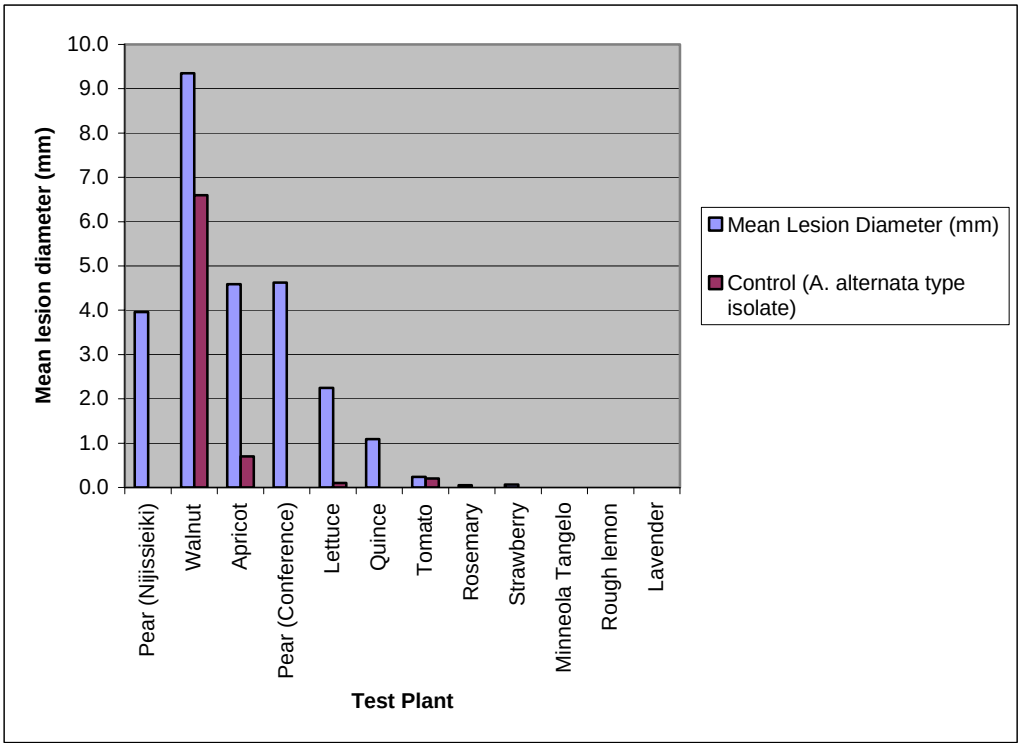

Figure 8. Infection (mean lesion diameter across all 5 pathogenic *Alternaria alternata* from pear) on a range of other plant species.

In summary, pathogenic isolates from both apple and Asian pear were found to infect and cause necrotic lesions on a range of alternative plant genera, namely walnut, quince, apricot, tomato, lettuce and lavender. In addition, widely grown UK varieties of apple (Cox's Orange) and pear (Conference) were also shown to be susceptible to apple and Asian pear isolates of *A. alternata* respectively.

Determine pathogenicity to apple and pear for isolates from other hosts

Six pathogenic isolates of *A. alternata* were obtained from the following hosts: strawberry variety Don, strawberry var. Miss, apricot, tomato (2 separate isolates) and lettuce. These were tested using the standard pathogenicity test on a range of apple varieties (Red Chief, Firm Gold, Cox's Orange, Bramley, Akame, Empire and Winter Banana), European pear varieties (Conference and Comice) and Asian pear (variety Nijisseiki).

The majority of apple varieties tested (Figure 9) were susceptible to between 5 and 7 of the seven isolates obtained as pathogenic isolates from other hosts. Only the apple varieties Empire and Winter Banana were resistant to the majority of isolates. For Empire, necrotic lesions were only observed with two of the pathogenic *A. alternata* isolates (from lettuce and strawberry var. Miss). Similarly, for Winter Banana, only two isolates (lettuce and apricot) produced necrotic lesions. All internal positive control inoculations produced lesions of various sizes on their respective host plants; the low level of necrosis observed on the tomato plants with tomato isolates could be due to susceptibility differences between tomato varieties. No lesions were observed with the SDW negative control or with the saprophytic *A. alternata* type isolate.

For European pear (Figure 10), the *A. alternata* isolate from strawberry var. Miss infected the greatest number of European pear varieties, including the commonly UK-grown variety Conference. Pear variety Comice was infected with the greatest range of isolates. No European pear variety was resistant to all of the *A. alternata* isolates from the other hosts. All control inoculations produced lesions of various sizes on their respective host plants. No necrosis was observed when the leaves were inoculated with SDW or a suspension of the saprophytic, type isolate of *A. alternata*.

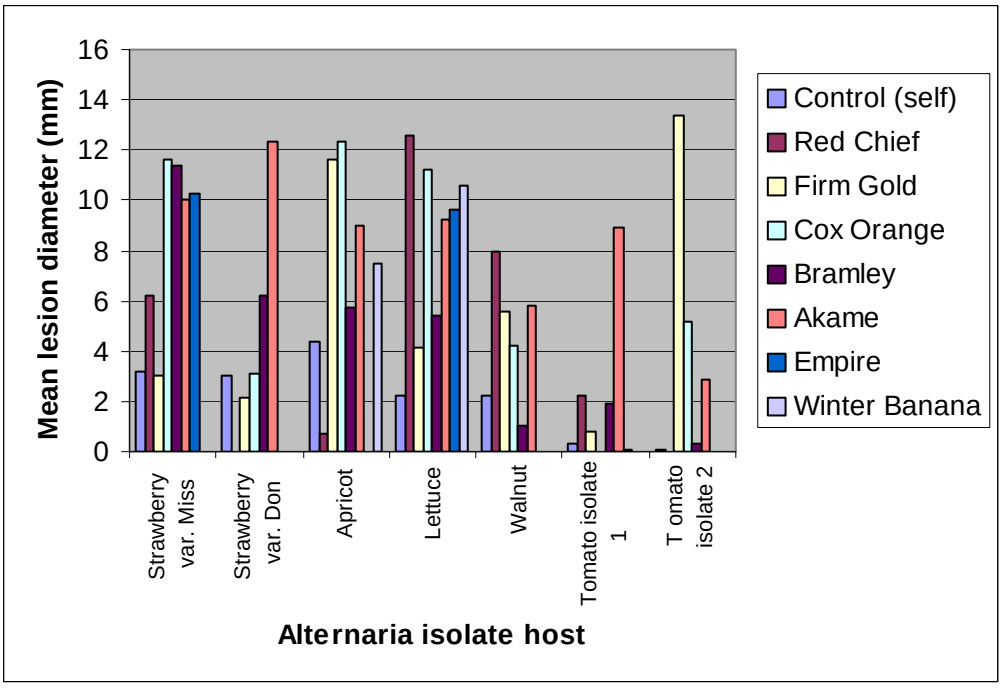

Figure 9. Infection (mean lesion development) of apple varieties by a range of *Alternaria alternata* isolates that are pathogenic on a range of other hosts.

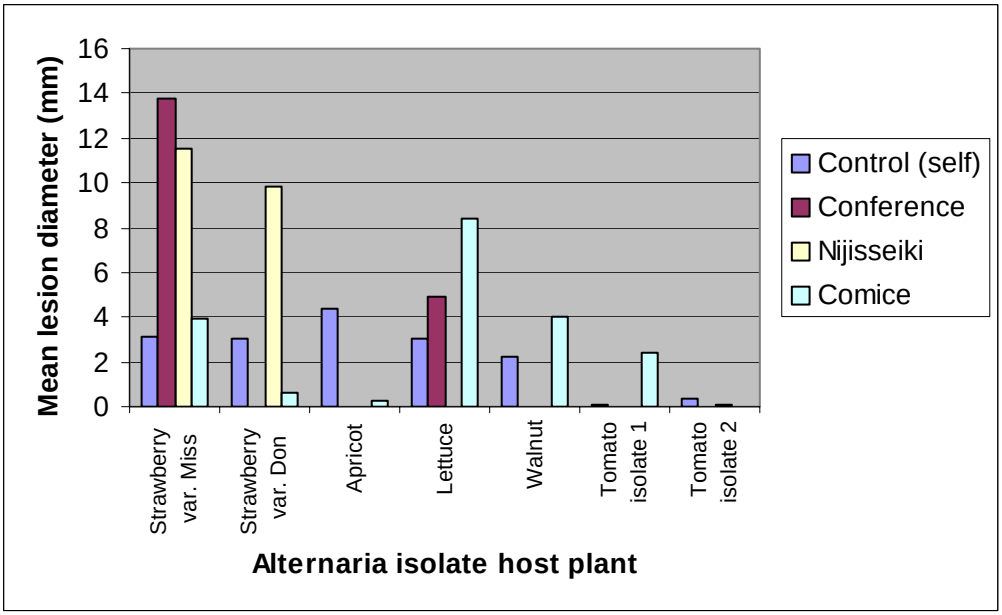

Figure 10. Infection (mean lesion development) of European pear varieties (Conference & Comice) and Asian pear (Nijisseiki) by a range of *Alternaria alternata* isolates that are pathogenic on a range of other hosts.

Further work was carried out on each of the isolates to determine any relationships at the molecular level. A DNA fingerprint (AFLP) method was applied (Bock *et al*., 2002) using primer set Alt-AFLP-EF2 and Alt-AFLP-TR2. Each of the isolates was compared and grouped according to the resulting fingerprint pattern (Figure 11). *Rhyncosporium secalis* isolates were used as outlying control isolates.

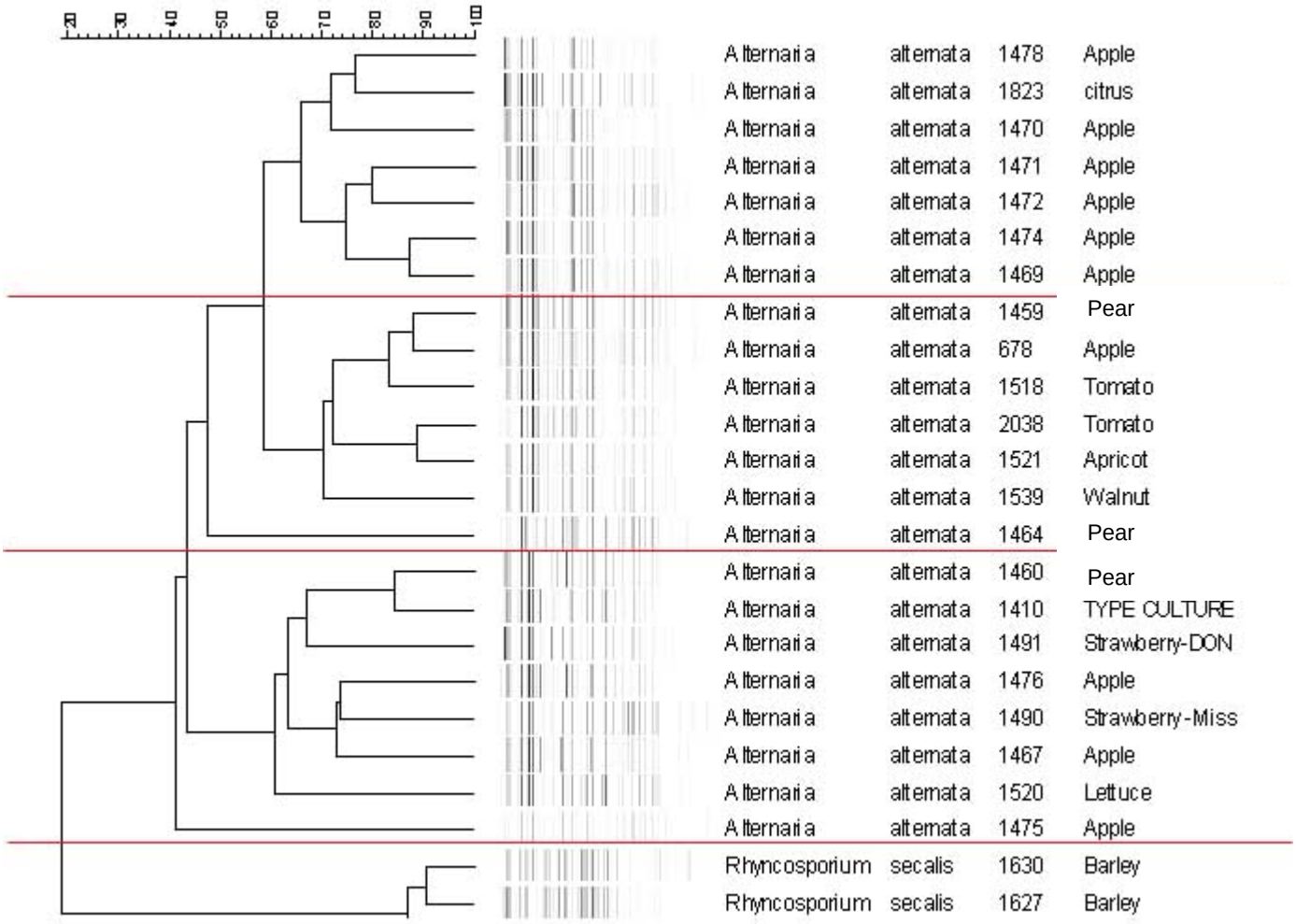

Figure 11. AFLP dendrogram for pathogenic *Alternaria alternata* isolates from apple and Asian pear and from other hosts used in pathogenicity tests compared to the saprophytic type isolate of *A. alternata*; *Rhyncosporium secalis* isolates are included as outlying control isolates.

The dendrogram can be divided into four main groupings. The outlying control *R. secalis* isolates stand alone in one group. Most of *A. alternata* isolates from apple (syn. *A. mali*) grouped together by themselves and with a single isolate from *Citrus* (not host-range tested). However, various other apple isolates could be found in two of the other groupings mixed with the remaining isolates, including the saprophytic *A. alternata* type culture. *A. alternata* isolates from pear (syn. *A. gaisen*) appeared to also fall into several groups. Both the tomato isolates grouped together, as did both of the strawberry isolates. These data could help explain the variation in host range and infectivity found from the pathogenicity studies.

Re-evaluate risks to apple and pear

Pathogenic *A. alternata* isolates from a range of hosts were shown not to be as host specific as previously believed, but were able to infect and cause lesions on a range of apple and European pear varieties. These findings highlight the potential risks from these pathogenic isolates to apple and pear, and to other potential hosts. These data will be utilised in a PRA to establish risks to UK-grown apple and pear varieties.

Objective 3. Risk management for *Stegophora ulmea* (elm black spot)

Stegophora ulmea was first intercepted by the PHSI in 1999 on bonsai *Ulmus* imported from China. Statutory action was taken and a summary Pest Risk Analysis (PRA) was completed. It has since been intercepted in all subsequent years. The pathogen is now an EPPO A1 organism and is being considered for EC I/AI listing by the EC Plant Health Standing Committee. In North America, where the fungus is widespread, *S. ulmea* is the most important pathogen of elm, after Dutch elm disease, causing damaging defoliation of many species including those grown as bonsais, amenity and forestry trees, as well as ornamental Japanese *Zelkova*. North-eastern Chinese sources of Siberian elm (*U. pumila*) are virtually destroyed by this fungus in elm nurseries. The summary PRA highlighted areas where more information was needed on the pathogen's lifecycle to assist containment/eradication policies, e.g.: investigations to determine if the fungus can overwinter in dormant buds, particularly on bonsai elm; the effect of leaf age on primary infection (McGranahan & Smalley, 1984a-b; Pomerleau, 1938); the potential for the pathogen to produce its sexual stage (teleomorph) under protection as this produces aerially dispersed spores (only the macroconidial stage has been found on material intercepted in the UK). There was also a need to evaluate alternative recommendations on statutory action. Currently, infected plants are destroyed and the remaining plants treated with prochloraz causing significant economic loss to growers. The effectiveness of existing and alternative chemical treatments should also be evaluated.

Develop culturing and storage methodologies

Isolates of the fungus were obtained from two sources (Asia and North America) but principally from material submitted by the PHSI during routine plant health inspections on consignments of bonsai elms (commonly *Ulmus parvifolia*) from China. Leaves were incubated in humid conditions at *ca.* 20°C until mature fungal fruiting bodies were present containing numerous asexual spores (macroconidia). These were picked out from stroma with a sterile needle and transferred aseptically to agar containing antibiotics (streptomycin and penicillin). Plates were incubated in the laboratory until a typical colony was observed. Colonies could not be initiated from single spores but only from small clumps of conidia, as encountered by previous researchers. The initial colony was then transferred onto a range of media (tap water agar (TWA), potato dextrose agar (PDA), PDA + streptomycin and penicillin, oat meal agar (OMA), and oat water soluble agar (OWS). Using this method seven isolates were successfully obtained from bonsai elm material originating in Asia obtained by the PHSI. All isolates were very slow growing (*ca.* 2 mm per month; McGranahan & Smalley 1984b), but after 4-5 weeks produced characteristic hard, black, crust-like stroma. Of the five media investigated, OMA was the best for growth and for production of macroconidial asexual spores (of the synanamorph *Gloeosporium ulmicolum*).

In addition to samples from the PHSI, naturally infected elm leaves were obtained under licence from Prof. Smalley in the USA (University of Wisconsin-Madison). On this material only the sexual stage (*Stegophora ulmea*) was present but direct isolation was unsuccessful. However, an ascospore suspension was prepared and used to infect bonsai elms (*Ulmus parvifolia* was used throughout the study) resulting in the formation of the macroconidial stage (*G. ulmicolum*). An isolate of the fungus was obtained from the macroconidial stage on the infected leaves. This work confirmed previous research that *S. ulmea* is a particularly difficult organism to obtain in culture; so much so that early workers had thought it was an obligate parasite.

Initial success using macroconidia from fresh cultures to initiate infection could not be repeated due to rapid loss of spore viability; macroconidia produced from sub-cultures were always sterile. This problem was not unexpected due to previous experience at CSL with another similar very slow growing fungus, *Diplocarpon rosae* (rose black spot), where viability was lost following storage of isolates for several months. Alternative methods for storing the pathogen were investigated. Firstly, eight isolates were stored as agar blocks in sterile water at 4°C, a method used routinely for maintaining fungal culture collections. After *ca.* 6 months, the isolates were sub-cultured but failed to re-grow. Secondly, naturally infected leaves obtained from the USA with the sexual stage were air-dried; after *ca.* 6 months storage ascospores did not infect leaves. Finally, infected leaves with mature stromata with macroconidia were stored at -20°C; these samples maintained their

viability. To ensure availability of inoculum for subsequent studies, naturally and artificially infected leaves with macroconidial stromata were therefore frozen at -20ºC.

Determine if the sexual stage and microconidial stages develop on protected bonsai

Cultures were incubated on a range of media as described previously to ascertain if either the microconidial or sexual stage would form *in vitro*. After 5–month's incubation on OMA, the microconidial stage (*Asteroma ulmeum*) developed within the stroma of the macroconidial stage (*G. ulmicolum*). The same phenomenon has been observed *in planta* from both naturally and artificially infected leaf material. However, on no occasion was the sexual stage observed *in vitro* or following incubation of naturally or artificially infected bonsai elm leaves under conditions similar to those for bonsai plants grown under protection.

Naturally infected elm leaves containing the teleomorph of the pathogen were obtained under licence from the USA. This facilitated study of the sexual stage (which has never been observed on imported bonsais either in the UK or NL). An ascospore suspension was sprayed onto susceptible bonsai elms in a quarantine controlled environment room and incubated at 16ºC with 100% RH for 24 hours, then at 16ºC with 75% RH and an 18-hour daylength. Following ascospore inoculation, characteristic symptoms and macroconidial stomata were observed after 14 days; the microconidial stage was also observed. After 4–5 weeks, infected leaves fell off; attached and detached leaves with lesions were incubated in moist chamber to determine if the teleomorph would form or not under these conditions. Leaf spots contained stromata morphologically identical to those of *G. ulmicolum* on infected bonsai from Asia and conformed to the published description of the fungus. The sexual stage (teleomorph) was not observed on the lesions produced by this American isolate with known ability to naturally produce its sexual stage outside. This again indicated that the sexual stage, which produces aerially dispersed ascospores, is unlikely to form on bonsai under protection, minimising the risk of spread to outside, native elms.

In summary, both the microconidial (*A. ulmeum*; considered a non-infective spermatial stage) and the macroconidial (*G. ulmicolum*) stages were observed *in vitro* and *in planta* (both naturally and following artificial inoculation). The sexual stage (*S. ulmea*) was not observed *in vitro* or following incubation of naturally or artificially infected elm leaves. Information has been provided to CSL and Forest Research Plant Health Consultants in order to update the PRA for *S. ulmea*.

Determine the effect of leaf age on infection

Bonsai elm trees were grown at 17°C with 75% RH and a 16-hour daylength in a quarantine CE room and leaves removed to leave just one leaf per stem at the following nodal positions: 0 (terminal bud), 1 (the first leaf after the terminal bud), 2, 3, 4, 5 or 6. The leaves were inoculated with a macroconidial suspension harvested from either fresh or frozen leaf material ($1x10^6$ spores/ml) and leaves assessed for disease (percentage leaf area infected). In the initial experiment (Experiment 1), the leaves at nodal positions 1, 2 and 3 were heavily infected (50–95% leaf area diseased). A low level of infection was also observed on older leaves at nodal positions 4, 5 and 6, but the disease rarely developed significantly (Table 4). However, when this experiment was repeated on three further occasions no infection was observed on leaves at nodal position 4 or older (Table 4).

In summary, infection was achieved by inoculation of a macroconidial spore suspension harvested from either fresh or frozen leaf material. Symptoms were first seen after 11 days, were conspicuous after 14 days and resulted in premature leaf fall after several weeks. The youngest leaves were the most susceptible on almost all occasions. Some older leaves were susceptible but only to a very limited extent.

	% Leaf area diseased in relation to nodal position (leaf age increasing with increasing nodal position)							
	-1 unfurled leaf	0 leaf from terminal bud	1 first leaf	2 second leaf	3 third leaf	4 fourth leaf	5 fifth leaf	6 sixth leaf
Experiment 1								
Plant A	-	-	50	45	65	1 (5)*	1 (2)*	0 (1) *
Plant B	-	-	90	50	75	0 (10)*	1	0
Experiment 2								
Plant A	-	-	30	0	0	0	0	0
Plant B	-	-	40	0	0	0	0	0
Experiment 3								
Plant A	-	-	100	100	80	0	0	0
Plant B	-	-	40	95	0	0	0	0
Experiment 4								
Plant A	4	0	25	2	1	0	0	0
Plant B	0	0	0	0	0	0	0	0
Plant C	0	0	1	20	2	0	0	0
Plant D	0	0	0	0	0	0	0	0
Plant E	0	0	5	2	20	0	0	0
Plant F	0	30	1	1	0	0	0	0
Plant G	0	0	0	10	0	0	0	0
Plant H	0	0	1	0	0	0	0	0
Plant I	0	0	1	0	0	0	0	0
Plant J	0	0	0	0	0	0	0	0

Table 4. Susceptibility of leaves of bonsai *Ulmus parvifolia* plants to *Stegophora ulmea* after two weeks incubation at 17°C in relation to leaf age for four separate experiments. *For Experiment 1, higher disease incidence was observed at nodal position 4 after 3 weeks (data in brackets) than at 2 weeks; but a similar increase was not observed between weeks 2 and 3 for older leaves at nodal positions 5 or 6.

Determine if the pathogen survives in dormant buds

Due to the very slow growth of the fungus it was not possible to isolate from artificially inoculated dormant buds or stems to check for the presence of the fungus, nor from buds or stems naturally exposed to inoculum. Therefore, this objective could only be investigated through observations of naturally and artificially infected bonsai elms. Naturally infected trees were obtained from the PHSI following confirmation of the disease and maintained in conditions conducive for disease development. In addition to this, trees which had been artificially infected with the fungus by spray inoculation of shoots and leaves were also monitored regularly through the project for the development of new symptoms related to leaves developing from buds after abscission of infected leaves. On no occasion following leaf fall were further symptoms of elm black spot observed when new leaves emerged from dormant buds exposed to the inoculum or associated with infected, abscised leaves. Although symptoms have been reported on young stems in America the disease was never observed on stems of naturally or artificially inoculated bonsai elms in this current study.

In summary, it was not possible by isolation to determine directly whether dormant buds harboured the fungus due to the slow growth of the pathogen. However, there was no evidence in this study that buds or stems were either directly or indirectly infected. Typically, bonsai elm trees are despatched from China by sea in refrigerated containers to maintain plant dormancy. During the 3–4 weeks transit period, leaves may be present but these frequently fall off during unpacking. Once in the UK, the plants are usually placed out on

benches under protection and leaves take about 2–3 weeks to appear again on the plants, depending on the time of the year. The short interval between plants arriving and inspection by the PHSI coupled with the absence of the organism in the UK indicates that the fungus must be present on arrival. As a foliar pathogen, it is most likely to be associated with buds or stems (external contamination or possible internal infection), although infection from leaf debris or contaminated packaging material cannot be ruled. Leaves within an active bud and just about to unfurl were found to be susceptible in this present study, so it is also possible that these young, unexpanded leaves could also be cryptically infected.

Determine the effectiveness of a range of fungicides for protectant and eradicant activity

A literature search was performed to identify any suitable fungicides for control of *S. ulmea* in addition to the existing statutory recommendation of prochloraz (e.g. Octave). Two further products were identified and tested alongside prochloraz:

Active ingredient	**Product**	**Activity**
Penconazole	Topas	Protectant conazole
Propiconazole	Bumper	Systemic, curative and protectant conazole
Prochloraz	Octave	Broad spectrum protectant and eradicant conazole

Bonsai elm trees with no recent history of fungicide treatment were obtained and maintained under standard conditions conducive for infection and disease development as described above. Fungicides were applied either prior to inoculation or post-inoculation to investigate, respectively, the protectant and eradicant activity of the three products. All trees were inoculated on the same day (Day 0) and sprayed with fungicide either 2 or 4 days before inoculation or 2, 4, 6 or 8 days after inoculation. Ten branches per tree were tagged at nodal position 6 and then 5ml of $1x10^6$ spores/ml of a fresh macroconidial suspension applied evenly using a mini-hand held sprayer to all leaves on the branch. Positive controls (inoculated with macroconidia only and no fungicide applied) and negative controls (sprayed with sterile distilled water only but fungicide applied) were also set up. Trees were then sealed in a large plastic bag, spayed inside with water, for 24 hours to aid infection. Fungicides were made up freshly on each spray occasion and applied to ensure there was no spray drift. Leaves were assessed for disease 14 days after inoculation and any signs of phytotoxicity were also recorded. Disease was assessed using the scale developed by Trumbower (1933), as follows:

0 = 0–5 spots; **1** = 6–60 spots; **2** = 61–180 spots; **3** = 181–300 spots; **4** = 301–420 spots & **5** = 421–540 spots

No symptoms were observed for the negative control (distilled water) but typical symptoms were observed with the positive control (no fungicide application) on leaves at nodal position 0, 1 and -1 (the first new leaf to emerge from the bud). All three fungicides significantly reduced levels of infection in comparison with the positive control when applied as either a protectant or eradicant fungicide (Figure 12). For all treatments, mottled leaves (which eventually fell off) were noted on many of the inoculated/fungicide treated trees but not in the negative controls treated with fungicides, suggesting it was not due to phytotoxicity. It was concluded that there was no phytotoxicity.

Propiconazole (as Bumper) was the most effective fungicide, both as a protectant and an eradicant as no symptoms were observed on any of the fungicide treated plants (Figure 12). This correlates with studies in the USA where propiconazole (as Alamo) was used to control Dutch elm disease, but also significantly reduced elm black spot (Delbridge *et al*., 1996). Prochloraz (as Octave), the currently recommended product, was effective as a protectant but was not completely effective as an eradicant because a very small amount of infection was seen on inoculated, treated leaves (Figure 12). Topas was the least effective, both as a protectant and as an eradicant (Figure 12).

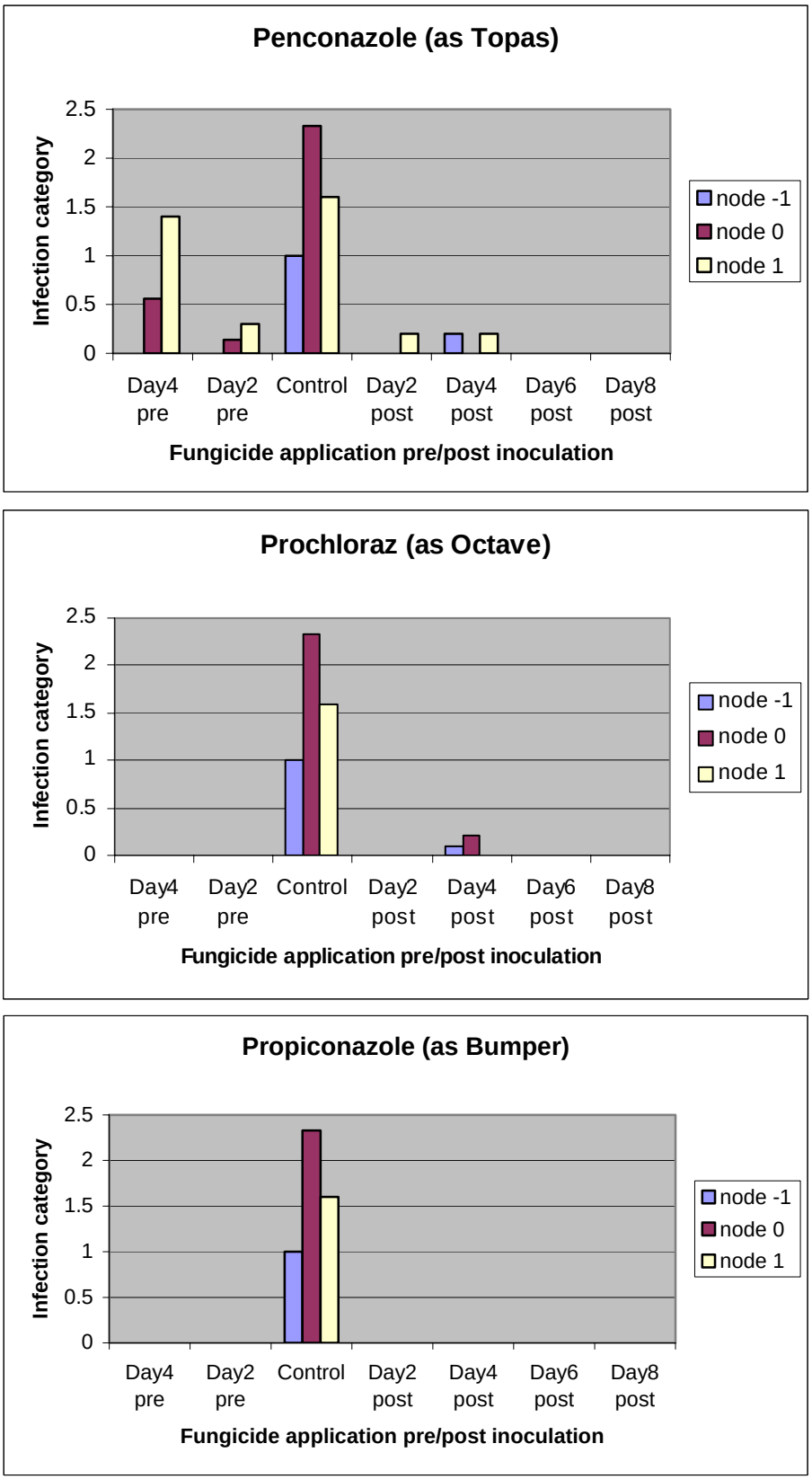

Figure 12. Protectant and eradicant ability of propiconazole (as Bumper), prochloraz (as Octave) and penconazole (as Topas) when applied to young bonsai elm leaves (leaves at nodes –1, 0 and 1), either 2 or 4 days before inoculation, or 2, 4, 6 or 8 days after inoculation with conidia of *S. ulmea*, in relation to untreated but inoculated controls.

Infected leaves were also harvested from this experiment to further test the eradicative capability of the three fungicides. Six heavily infected leaves (category 5) from the positive control tree were sprayed evenly with each fungicide and incubated for 24 or 48 hours before the macroconidia were washed off. These were then used to inoculate leaves of healthy trees as described previously to determine spore viability and infectivity; leaves were assessed for symptoms after 14 days incubation. Once again propiconazole (as Bumper) was the most effective, being completely effective after both 24 and 48 hours. Prochloraz (as Octave) was the next most effective, taking 48 hours to be totally effective. Penconazole (as Topas) was the least effective (Figure 13).

In summary, the current PHSI recommended fungicide prochloraz (as Octave) is a good protectant and eradicant but propiconazole (as Bumper) was more effective. This data and advice has been provided to Eradication and Containment Plant Health Consultants at CSL, and Defra Plant Health Division and PHSI HQ.

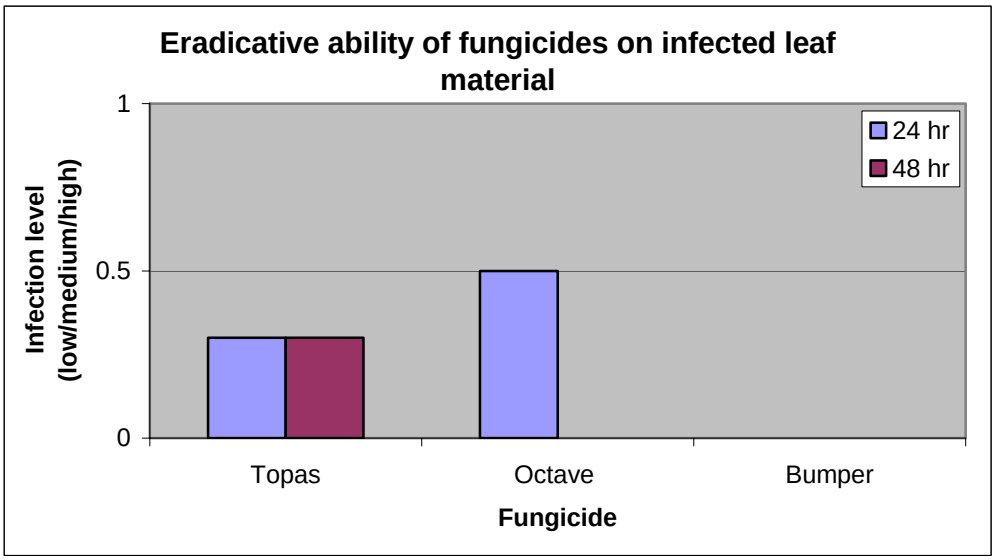

Figure 13. Effectiveness of propiconazole (as Bumper), prochloraz (as Octave) and penconazole (as Topas) as an eradicant when applied to macroconidial stromata on infected leaves; data relates to viability of treated macroconidia in stromata 24 or 48 hours after application of fungicides, expressed as infectivity when inoculated onto leaves on bonsai elm.

BENEFITS AND IMPLEMENTATION

Diagnostics for *Tilletia indica* (Karnal bunt) and look-alike species

- The diagnostic research has enabled Defra to make rapid, informed decisions on action when interceptions occur. This reduces costs to importers/millers which result from disruption to trade or milling schedules which occur when imported grain is held pending diagnosis. The methods have also supported contingency planning.
- The research data has been incorporated into the CSL-produced EU Recommended Protocol for *Tilletia indica* and this is used routinely by CSL diagnosticians monitoring imported grain. CSL's expertise in this area was recognised internationally when its diagnosticians were invited to act as an independent laboratory in a recent trade dispute when suspect *Tilletia* spores were found in exported grain.
- Development of an image analysis skills base will have a longer-term benefit as this new technology is developed further for fungal plant pathogens with new equipment recently purchased at CSL. The *Tilletia* image analysis work is also currently being progressed by an MSC student at York University with Dr. David Chesmore, in collaboration with CSL.

Risks associated with pathogenic isolates of *Alternaria alternata*

- The results have provided an evaluation of risks posed to UK crops, especially apple and pear, by existing and emerging pathogenic strains. This will contribute to recommendations and policy on interceptions of pathogenic strains of *A. alternata* on imported plants.
- The data has been provided to CSL pest risk analysts for updating their assessments of risk and their development of risk management approaches.

Risk management for *Stegophora ulmea* (elm black spot)

- The epidemiological and control work will be used to justify or amend existing recommendations on statutory action for interceptions.
- Management options have been identified which might be used to prevent spread from nursery bonsai to native elms with minimal impact on the trade.
- The data on the pathogen's life cycle will also contribute to the PRA for *S. ulmea*, allowing a revised and more comprehensive evaluation of risk based on targeted scientific investigation.

PAPERS/PROCEEDINGS

Chesmore D, Bernard T, Inman A & Bowyer R (2002). Image analysis for the identification of the quarantine pathogen *Tilletia indica. EPPO Bulletin* 33, 495–499.

Inman A, Bowyer R & Chesmore D (2003). Morphological identification, including image analysis, for *Tilletia indica* and look-alike species. Proceedings of the 8th International Congress of Plant Pathology, Christchurch, New Zealand, 3-7 February 2003. Abstract 7.5.

Inman A, Bowyer R, Hughes K & Barnes A (2003). EU Recommended Protocol for the Diagnosis of *Tilletia indica*. http://www.csl.gov.uk/prodserv/know/diagpro/ and *EPPO Bulletin* (in press).

PRESENTATIONS

- A review of the *Tilletia* diagnostic R&D progress was presented at a Karnal Bunt Contingency Planning Meeting held with DEFRA Plant Health Division, PHSI, CSL and representatives from the wheat trade (GAFTA) and the milling industry (NABIM) at CSL (April 2002).
- Aspects of the project's R&D on *Tilletia* diagnostics, *Stegophora ulmea* and *Alternaria* was presented at: Annual PHSI Technical Refresher Courses held at CSL; Annual Meetings of the UK Phytodiagnostics Group; Annual Meetings of European Mycological Network; UK Forest Pathology Group, September 2001.
- Information on elm black spot was provided to PHSI for a presentation to the North West Bonsai Association.
- A poster was presented at the PHSI Technical Conference (February 2002) on the *Tilletia* diagnostic R&D.