Project title:	Brassicas: forecasting light leaf spot and powdery mildew in vegetable Brassica crops based on "in field" detection of airborne spores
Project number:	FV 333
Project leader:	Dr Roy Kennedy
Year of project:	Annual
Date of report:	31 December 2010
Locations:	University of Worcester
Industry Representative:	Andy Richardson
Project commenced:	01 August 2008
Completion:	31 August 2011
Key words:	Brassicas, disease, light leaf spot, *Pyrenopeziza Brassicae*, powdery mildew, *Erysiphe cruciferarum*, fungicide, pesticide, spore detection, Brussels sprouts, lateral flow device, disease prediction

Signed on behalf of: **National Pollen and Aerobiology Research Unit**
University of Worcester

Signature: …………………………………………   Date: ………………………

Name: Professor Roy Kennedy
Director and Head of Department

CONTENTS **Page**

GROWER SUMMARY

SCIENCE SECTION

GROWER SUMMARY

Headline

Detection tests for conidia of Brassica powdery mildew and light leaf spot have been developed and tested.

Background and expected deliverables

Light leaf spot and powdery mildew on Brussels sprout crops are important pathogens which can cause blemishes on the Brussels sprout button. Controlling light leaf spot infection within Brussels sprout crops in Northern Britain, where the pathogen is commonly found, is difficult given the time periods required for crop production. Many fungicides used to control ringspot and dark leaf spot can be used effectively to control light leaf spot. In contrast little information exists on the epidemiology of powdery mildew (*Erysiphe cruceriferium*) in vegetable Brassica crops. It is likely that this pathogen may become more important in drier areas where vegetable Brassicas are produced.

Application of fungicides with the active ingredients Boscalid and Pyraclostrobin (Signum) gave good control of light leaf spot infection in Brussels sprout crops provided that they could be applied at the time when light leaf spot ascospores were present in the air. Results from previous studies show that light leaf spot ascospores appear in the air in large enough levels to be problematical only during discrete periods. Light leaf spot inoculum may be present at other times but occurs at too low concentrations to become problematical in developing sprout crops. By using air sampling allied with rapid antibody based techniques periods of light leaf spot risk in Brussels sprouts crops can be identified. Using air sampling methods described by Wakeham *et al.* (2004, 2010) the risk of light leaf spot infection could be successfully assessed within crops of Brussels sprouts at Arbroath in a laboratory test.

Sprays of fungicide could be applied in response to peaks in airborne numbers of ascospores of light leaf spot. However if inoculum testing is to be used widely there will be a requirement for "in field" tests operated by the grower or consultant. This project will develop tests which can be used directly by the grower or consultant to determine the presence of pathogenic inoculum of light leaf spot and powdery mildew early in disease development within the crop (pre symptom)

The expected deliverables from this project are:

- Better detection of powdery mildew and light leaf spot in the field before disease is visible in the crop.

- Detection tests which can be used "in field" to determine the level of risk to the vegetable Brassica crop posed by these pathogens.

- Improved use of Signum within vegetable Brassica production systems and the reduced likelihood of tebuconazole resistance within light leaf spot populations (already been reported).

- Monoclonal antibodies which recognise powdery mildew conidia and light leaf spot ascospores.

Summary of the project and main conclusions

A competitive lateral flow device (Figure 1) has been developed which can detect the number of powdery mildew spores in a test sample (when used in conjunction with an electronic reader). However visual readings on tests were possible only at higher concentrations of conidia. Further work will be carried out to optimize the assay format to increase visual test sensitivity whilst retaining good discrimination. The test was validated in a glasshouse in the presence of plants infected with powdery mildew.

Figure 1 A semi-quantitative competitive lateral flow device with powdery mildew conidial numbers tested between 0 – 4800.

A competitive lateral flow for the detection of ascosporic inoculum of *Pyrenopeziza Brassicae* (light leaf spot) has been developed and tested in a commercial Brassica crop field crop in the presence of monitored light leaf spot inoculum. Using this system shows potential to designate the likely onset of disease occurrence of light leaf spot in field crops of

Brussels sprouts. An MTIST spore sampler was operated as a reference trap to validate quantitative readings made using the developed light leaf spot lateral flow system.

Financial benefits

- The usage of the "in field" test for powdery mildew will improve the control of this pathogen in field crops.

- More information will be available on the appropriate timing for application of Signum to vegetable Brassica crops. This will improve the efficacy of this expensive chemical especially in production of vegetable Brassicas in Northern Britain.

Action points for growers

The specific action points for growers at this stage in the project are:

- Growers can help validate the 'in field' test to determine when light leaf spot and powdery mildew are present in their crop.

- The tests can be used with existing inoculum tests for ringspot ascospores and dark leaf spot conidia.

- Tests can also be used in conjunction with disease forecasts.

SCIENCE SECTION

Introduction

Light Leaf Spot

Many fungal pathogens occur on vegetable Brassica crops and these can be difficult to control despite the usage of fungicidal sprays. In Scotland and parts of Northern England the light leaf spot (*Pyrenopeziza Brassicae*) is the predominant pathogen affecting this crop. Light leaf spot occurs most frequently on oilseed rape in Northern England and Scotland. *Pyrenopeziza Brassicae* (anamorph *Cylindrosporium concentricum*), the pathogen causing light leaf spot is an important disease of winter oilseed rape in Germany (Amelung & Daebeler, 1991), France (Brun *et al.,* 1979), Poland (Karolewski, 1999) and the UK (Fitt *et al.,* 1997). Light leaf spot is a polycyclic disease, which infects oilseed rape leaves, stems, flowers and pods during the course of the season between sowing in autumn and harvest in summer (McCartney & Lacey, 1990; Gilles *et al.,* 2000b). The fungus produces ascospores, formed in apothecia on dead tissue, and conidia, formed in acervuli on living tissue. Ascospores and conidia are morphologically similar, when observed under a light microscope (Rawlinson *et al.,* 1978). Ascospores are hyaline, cylindrical, 0-1 septate, 15.5-15.5 x 2.5-3µm and conidia are hyaline, cylindrical, aseptate, 10-16 x 3-4µm. Ascospores play an important role in initiating epidemics in the autumn, when they are released from infected oilseed rape debris (Gilles *et al.,* 2001b) as it dries after overnight dew or rainfall (McCartney & Lacey, 1990) and dispersed by wind (McCartney *et al.,* 1986). Conidia travel only short distances by splash dispersal (Fatemi & Fitt, 1983) and are responsible for secondary spread of the disease during autumn and winter (Evans *et al.,* 1999). Infection resulting from ascospores affect crops of Brussels sprouts grown in these regions which are are under a great risk of developing light leaf spot epidemics. The disease is thought to be transmitted from oilseed rape to Brussels sprouts. Preliminary evidence for this was found by Gladders (1984), who observed that light leaf spot was more common in Brussels sprout crops adjacent to oilseed rape crops than in more distant crops.The Brussels sprout crop is at risk over considerable periods of time which is an additional problem if light leaf spot is to be controlled. Brussels sprout crops in Scotland are planted in May and can remain until harvest in March of the following year. This means that the plants must be protected against light leaf spot for a considerable period of time. The wind-dispersed ascospores of *P. Brassicae* are likely to be responsible for transmitting light leaf spot to Brussels sprouts (Gilles, Kennedy, Jackson & Steinmetz, 2002). These ascospores are produced on leaf debris underneath oilseed rape crops in spring/early summer when sprout crops are

transplanted to the field and on stem and pod debris after harvest of the oilseed rape crops during late summer/autumn when sprout crops have been fully established (McCartney & Lacey 1990). Volunteer oilseed rape plants may play an important part in the epidemiology of light leaf spot on Brussels sprout crops in Scotland. The crop is protected over the growing period using approved fungicides. Fungicides containing Tebuconazole (Nativo) have full label approval for control of light leaf spot in sprout crops. Other diseases must be controlled using products such as those containing Difenconazole (Plover) however these are less effective in controlling light leaf spot. The occurrence of air-borne ascospores of *P. Brassicae* within Brussels sprouts and its use in anti-resistance strategies for controlling light leaf spot epidemics has been collected in Scotland from 2003 until present.

The infection of oilseed rape plants by *P. Brassicae* ascospores is influenced by environmental factors such as temperature and leaf wetness duration. Infection conditions for conidia have been examined in controlled environment experiments by assessing leaf symptoms (chlorotic areas; *P. Brassicae* does not produce necrotic lesions in controlled environments) or sporulation (production of new conidia). In controlled environment experiments at $12^{o}C$ and $18^{o}C$, chlorotic areas developed on the fourth, fifth and sixth leaves of oilseed rape (cv. Cobra) inoculated with suspensions of conidia, when leaf wetness duration after inoculation was 16 to 48 h, but not when it was 0 to 13 h (Figueroa *et al.,* 1995b). Sporulation developed at temperatures from 4 to $20^{o}C$ (but not at $24^{o}C$); sporulation occurred when leaf wetness duration was > 6 h at $20^{o}C$, 16 or $12^{o}C$ and > 10 h at $8^{o}C$, 6 or $4^{o}C$ (Gilles *et al.,* 2000a). With *P. Brassicae* ascospores inoculated on to oilseed rape leaves (cv. Bristol), sporulation was observed at $16^{o}C$ after 48 h of wetness duration (Gilles *et al.,* 2001b).

In experiments on oilseed rape, the shortest latent period (c. 10 days) for conidial infection was at $16^{o}C$ after 24 h wetness duration and the latent period increased as temperature increased to $20^{o}C$ or decreased to $4^{o}C$ (Gilles *et al.,* 2000a). When plants were moved immediately after inoculation (with conidia) to field conditions at weekly intervals from September to February (under different sets of fluctuating conditions), the latent period ranged from 15 to 40 days (Figueroa *et al.,* 1995b). However, when the latent period was expressed in accumulated temperature ($>0^{o}C$), the range was much less, from 150 to 250 degree-days. Little is known about the relative effectiveness of ascospores or conidial inoculum in causing a successful infection (which produces new spores). Recent controlled environment work on the infectivity of ascospores and conidia on oilseed rape and Brussels sprouts leaves suggests that ascospores may be more infective than conidia.

Powdery Mildew

Powdery Mildew on Brassicas is caused by the fungal plant pathogen *Erysiphe cruciferarum* and can infect any above ground plant part of the vegetable *Brassicae* plant (Figure. 2).
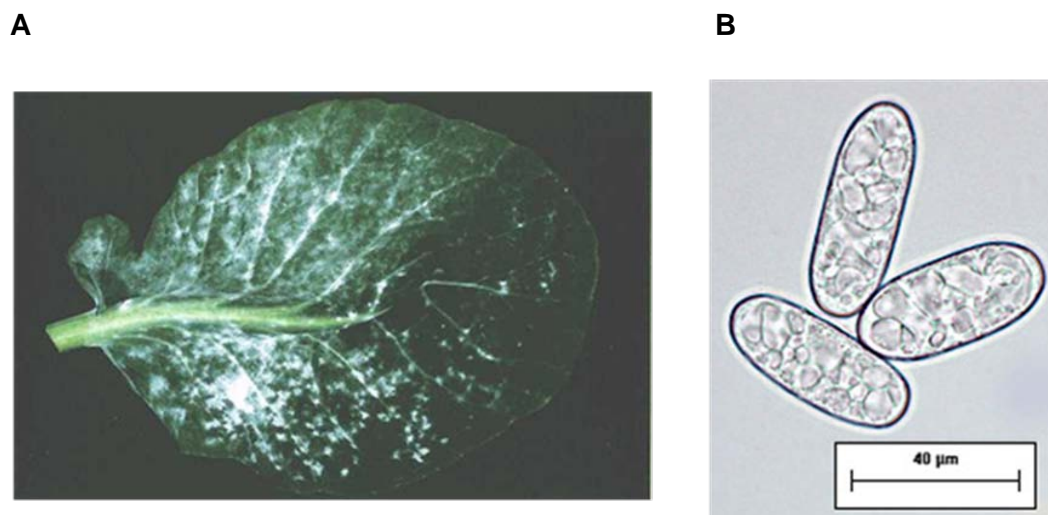
A   **B**

Figure 2. (A) Powdery mildew infection on leaves (B) powdery mildew conidia

Powdery mildew reduces plant growth and yield in *Brassica* crops and in seed production a reduction in the quantity and quality is observed. Most horticultural Brassicas are susceptible to infection by *Erysiphe cruciferarum* and these include Brussels sprouts, cabbage, Chinese cabbage, kohlrabi, broccoli, kale, mustard, collards, cauliflower, radish, and horse radish. Infection of vegetable Brassica crops is unaffected by the powdery mildew occurring on oilseed rape crops as this is caused by *Erysiphe polygoni.* On Brussels sprouts the disease develops from late August onwards initially infecting all types of foliage but becoming more prevalent on axillary buds (buttons). The presence of the disease on the sprout buttons can downgrade their value especially if cold weather occurs which gives rise to melanisation of the mycelium rendering a speckled appearance on the button. *Erysiphe cruceriferium* can enable the entry of secondary organisms on infected tissues such as grey mould (*Botrytis cinerea*). Periods of moisture stress may render plants more susceptible to powdery mildew infection. Powdery mildew is highly airborne despite its relatively large dimensions (Figure. 2). Small numbers of conidia can be wind dispersed over large distances.

To date there is little information on the requirements for powdery mildew development in vegetable Brassica crops. *Erysiphe cruciferarum* can infect and develop over a wide range of environmental conditions however on vegetable Brassicas it appears to be favoured by

dry conditions and, these usually only occur during early summer. The airborne concentration of inoculum required for disease development in the crop is unknown however it is likely that threshold levels during June, July and August are key in the degree of damage that this pathogen causes. Serious disease epidemics may result only from the influx of substantial amounts of inoculum into the crop although the epidemiology of the disease in the crop is poorly understood. The occurrence of older tissues where powdery mildew development is more favoured, during autumn and winter may act as a bridge for the pathogen to occur on Brussels sprouts buttons. However it is also possible that powdery mildew penetration into axillary buds may also lead to button infection. Airborne inoculum of *E. cruciferarum* will play a vital role in the initial development of powdery mildew in Brassica crops. The present study aims to develop a system to monitor field aerosols and, using an on-site lateral flow immunoassay test, identify periods when *E. cruciferarum* is present at a level to initiate disease. Information of target inoculum level and field environmental data should provide a platform to determine risk of exposed field Brassica crops to *E. cruciferarum*. Improved management of the disease and reduced applications of the fungicides applied should be achieved as determined for ringspot on vegetable Brassicas (Wakeham & Kennedy, 2010).

There have been relatively few recent investigations involving *Erysiphe cruciferium*. These structures are typical of the powdery mildew *Oidium* subgenus *Pseudoidium*, anamorph of the genus *Erysiphe* (Jee *et al.,* 2007). Periods of moisture stress may render plants more susceptible to powdery mildew infection. It was demonstrated that exposure of Brassica powdery mildew conidia to free water did not affect conidial germination but decreased infection. Percentage infection was inversely proportional to the period of time exposed to free water (Crowton & Kennedy, 1999). Effective fungicidal sprays (Nativo) are approved which can be applied to control the disease. Information about the availability of powdery mildew inoculum would be useful in control regimes. Measuring inoculum in crops is possible using lateral flow devices.

Immunological tests (Lateral Flow Devices)

The technical basis of the lateral flow immunoassasy test (lfd) was derived from the latex agglutination assay (Plotz and Singer, 1956). However establishment of the technology for the lateral flow test was not available until the late 1980's. Pioneering work in the development of a 'home test' for determination of human pregnancy assisted this technology to the wider market place, enabling complex laboratory processes to be carried out on-site by non-laboratory personnel. The simplicity of the design, requiring addition only of the

sample and, the compact and portable capability of the test, make it popular for development of a wide range of assay tests.

Lateral flow assays are only one type of rapid assay which can be employed to quantify target particles or molecules. However they are now commonly and widely used for detection purposes. They rely upon the specific reaction of sensitised coloured particulates. Antibodies (polyclonal or monoclonal) raised to a specific target spore, are bound to these coloured particles. These sensitised particles (latex or immunogold particulates are generally used) are then applied using an immersion procedure on to a release pad, to produce a stable particle reservoir for release on to a nitro-cellulose-based membrane. In a standard lateral flow test two lines of reagents are immobilised on to the membrane using a sophisticated reagent dispenser. The constituents of these lines will vary from test to test but commonly only two types of formats are adopted.

In a competitive assay format the test line comprises of homologous antigen (target spore components) and the other, the control, is a line of anti-species antibodies. The release pad and membrane are assembled together with an absorbent pad into a plastic housing as illustrated below (Figure 3). The fluid sample is added to the well, releasing the specific antibody bound coloured particles, which then begin to flow across the membrane. If the target antigens (light leaf spot and powdery mildew spore components) are present in the sample extract, antibody binding will occur to produce a coloured particulate conjugated antibody -antigen complex. Any antibody conjugated coloured particles that fail to bind to target antigen will attach to the immobilised test line as they traverse the membrane. If present at a high enough concentration, a visible line of deposited coloured particulates will form at the test line. The anti-species antibody will capture excess sensitised antibody / coloured particles to produce a control line, providing a visible confirmation of antibody / particulate flow. Sufficient antigen target presence (powdery mildew conidia or light leaf spot ascospores), would induce complete inhibition of antibody attachment to the test line, a result that is indicated by a single line of coloured particle deposition (the control line). This would result in one visible line on the device indicating a positive result. Two lines of equal colour intensity indicate a negative result.
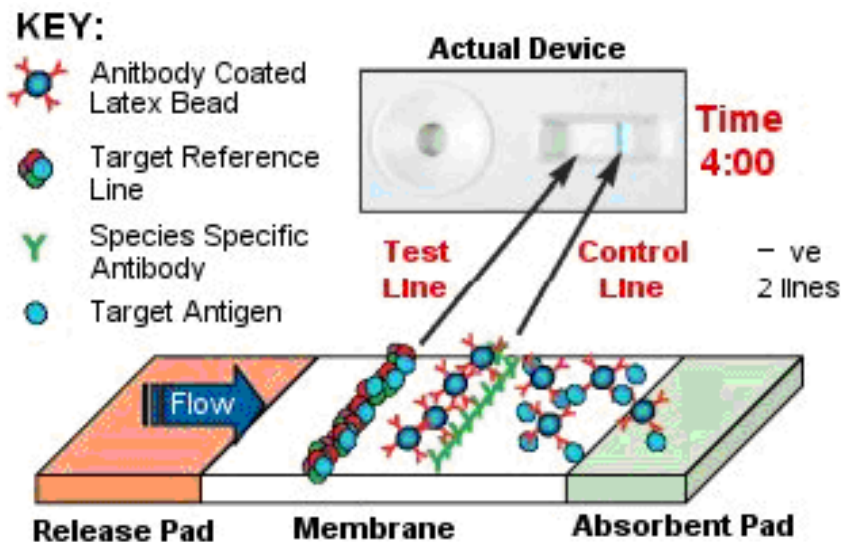
Figure 3. The Competitive lateral flow assay format.

The competitive lateral flow format can produce a semi-quantifiable test. Use of reader technology allows the line intensity to be recorded, and therefore the level of particulate accumulation to be calculated using reflectance photometry.

Epidemiological advantages of testing for detecting inoculum

The economics of production of many vegetable Brassica crops vary as do the effect of disease on the marketability of those crops. Areas of vegetable production are usually concentrated in specific areas where the soil is suitable for production. This means that crops at different stages or of different types are often side by side in close proximity to one another. There is ample opportunity for crop to crop spread of disease. The situation is further complicated by the ownership of different crops within the production area. Many growers and producers will have different crop protection regimes applied to different crop types but these may not be suitable for neighbouring crops owned and managed by other growers.

Given these constraints there are major periods when inoculum at high levels is present within crops but those crops are largely disease free. The grower will have no information other than weather on which to base his decisions because his crop is largely disease free and he has no information on the risk posed to his crop by surrounding control practices. This often accounts for disease levels in crops moving from a very low level to a very high level in a short period of time. At certain times of year (e.g. harvest and during harvesting of oilseed rape crops and autumn cauliflower crops) long season vegetable Brassica crops are very susceptible to increased disease risks and the grower is vulnerable to outside

pressures on his crop which he has no way of measuring consistently. By using "in field" inoculum tests developed within this project the grower can measure these risks in his locality both at the spatial and temporal scale. This will enable disease risk to be correctly measured and dealt with in many instances using appropriate fungicides.

The application of these lateral flow tests has expanded beyond clinical diagnostics to areas as diverse as veterinary, agriculture, bio-warfare, food, environmental health and safety, industrial testing, as well as newer areas such as molecular diagnostics and theranostics. Different configurations of the lateral flow assay exist however all require the basic elements of a solid membrane phase, a fluid transport and, a test specific labelled antibody. In year two of this study a competitive lateral flow assay format was used to develop a prototype field test for semi-quantification of trapped airborne inoculum of *E. cruciferarum and Pyrenopeziza Brassicae*. A competitive lateral flow device (clfd), in the absence of a target sample (*E. cruciferarum* conidia or *P. Brassicae* ascospores), will give rise to the formation of a test line. Rate of test line depletion will relate directly to target levels in the test sample. In the competitive format, the test line depletion is generally measured using a portable optical device (Figure 4).

Figure 4. Lateral flow reader device

Materials and Methods

Production and collection of spore material

Erysiphe cruciferarum (Brassica powdery mildew)

Production of powdery mildew.

E. cruciferarum sporulating leaf material, identified on field grown Brassica crops at Wellesbourne, was collected. New leaf material was inoculated by brushing infected leaves gently over the foliage of ten glass house grown Brussels sprout plants cv. Revenge. The plants were placed in a glasshouse compartment at 12-14°C and routinely monitored for powdery mildew disease symptoms. Following leaf sporulation and identification of *E. cruciferarum* by bright field microscopy, the powdery mildew infected plants were used as a source of inoculum to infect further Brussels sprout plants. In this way a continuous source of sporulating leaf material of *E. cruciferarum* was maintained.

Collection of powdery mildew

An MTIST air sampling device (Burkard Manufacturing Co., Rickmansworth, UK) was used to check, production and airborne transport of powdery mildew conidia. Within the sampler, the airflow is channelled through 32 trumpet-shaped nozzles each directed at the base of a microtitre well. The sampler contained four microtitre strips (catologue no. 469957, Nunc Immunodiagnostics, Life Technologies Ltd, Paisley, Scotland) each containing eight wells. Air flow through the sampler was estimated in still air by measuring the air speed at different points across the inlet manifold using a hot film anemometer (Air velocity transducer model number 8460, TSI Incorporated, St Paul, MN, USA) and integrating over the area of the inlet. In the tests reported here, the volume flow rate through the device was measured at 57-litre air min^{-1}.

Enumeration of the collected powdery mildew spores

The numbers of *E. cruciferarum* spores (powdery mildew) impacted on the base of each microtitre well was counted by using a Nikon model TMS inverted binocular microscope (x 200). Spore concentrations were calculated from the numbers of spores trapped in each of the microtitre wells and the volume of air sampled by the MTIST spore trap.

Pyrenopeziza Brassicae (Brassica light leaf spot)

Production of *Pyrenopeziza Brassicae* ascospores

To produce conidia, leaves of oilseed rape plants cv. Bristol at GS 1,6-1,7 were sprayed with a suspension of conidia (0.5×10^6 conidia per ml^{-1}) obtained from leaves of plants in Rothamsted field experiments, using an aerosol sprayer. Directly after inoculation, plants were covered with polyethylene bags for 48 h. After 18 days post inoculation, conidia were washed from the leaves by shaking them in distilled water. The collected conidial suspension was adjusted to 1×10^4 spores ml^{-1} using a haemocytometer slide (Weber Scientific International Limited, Teddington, UK). To produce ascospores, leaves of oilseed rape plants cv. Bristol were inoculated with a suspension of conidia. After the leaves had senesced, they were collected and dried at 20°C for 24 h. The petioles were removed from the leaves and soaked in rain water for 16 h. The wetted petioles were placed in plastic boxes (12.5 cm x 8 cm) on three layers of filter paper (Whatman no. 1; Whatman International Limited, Maidstone, Kent, UK) and wetted with 7 ml of rain water. After 18-22 days of incubation at 13°C in darkness, petioles with mature apothecia containing ascospores were observed.

Production of immunogen for antibody production

Erysiphe cruciferarum (powdery mildew)

Collection of *E. cruciferarum* spores from leaf surfaces

Conidia of Brassica powdery mildew were collected from a freshly sporulating leaf using a hand held Burkard cyclone air sampler (Burkard Manufacturing Co., Rickmansworth, Herts, UK). The collected spore material was suspended in 10ml of chilled phosphate buffered saline (PBS, pH 7.2) and held at 0-4ºC while a conidial count was taken using a haemocytometer. The spore suspension was adjusted in PBS to a combined concentration of 1×10^5 conidia ml^{-1}. To release spore material the conidia were then mechanically disrupted (3 x 25 seconds at a speed setting of 5.5) using a Fast Prep device (Qbiogine FP120, BIO101, Anachem Ltd, UK) according to manufacturers guidelines. The sample was then microfuged (MSE Microcentaur) at 13 x g for 5 mins and the soluble fraction retained.

Immunogen Preparation

The collected soluble material was separated by filtration in to two molecular weight fractions of >30 and < 30 Kilo Daltons (Kda). To the <30 KDa sample an additional dialysis procedure was carried out to remove material of molecular weight <2 KDa. After which the two samples

were assessed for protein concentration and each adjusted to 1mg ml^{-1}. The immunogen preparation at >30 KDa was coded as Immunogen Preparation A and the conidial material at <30 > 2KDa was coded Immunogen Preparation B

Pyrenopeziza Brassicae (light leaf spot)

Immunogen preparation

Ascosporic inoculum of *P. Brassicae* was produced as described above, collected in PBS and adjusted to 1x10^5 spore's ml^{-1}. Collected spores were mechanically disrupted (1 x 25 seconds at a speed setting of 4) using a Fast Prep device (Qbiogine FP120, BIO101, Anachem Ltd, UK) according to manufacturers guidelines. A 1ml sample was then mixed with three monoclonal antibody cell lines (MAbs), each of which had previously been raised to ascosporic inoculum of *M. brassicicola*, at a ratio of 1 part *P. Brassicae* 3 parts MAb mixture. The spore / antibody sample was gently mixed over a 1 h period at room temperature (18ºC). To remove unbound antibody the sample was microfuged at 6000 rpm for 6 minutes and the pellet gently resuspended in PBS. The spore preparation was washed a further two times in PBS as described and suspended to a final volume of 1ml. The sample was coded as Immunogen preparation C.

Antibody production

Spore preparations A, B (*E. cruciferarum*) and C (*P. Brassicae)*

Immunization of mice

Three female Balb C mice then each received 50µl of immunogen preparation A (100µg protein / 50µl) mixed with an equal volume of Titermax adjuvant. Three additional mice each received 50µl of immunogen preparation B and a further 3 mice preparation C. The mice were immunised twice more at 14 day intervals but this time without adjuvant. Collected tail bleeds were screened using a standard plate-trapped antigen enzyme-linked immunosorbent assay (PTA-ELISA) and a mouse was identified which exhibited antibodies highly reactive to conida of *E. cruciferarum* and an additional mouse to *P. Brassicae*. Of the two mice selected a final pre-fusion boost was administered to each and the spleens were removed four days later. For each mouse a fusion was then carried out where antibody producing B cells isolated from the spleen were fused *in vitro* with a lymphoid tumour cell (myeloma). The cell hybrids (hyridomas) were fed on days 3, 6, and 10. Cell tissue culture supernatants (TCS) were screened by PTA ELISA 14 days after the cell fusion for the presence of antibodies which bound to either conidial epitopes of *E. cruciferarum* or ascosporic material of *P. Brassicae.*

Hybriodoma Antibody Screening

Plate trapped antigen ELISA (PTA ELISA)

Polysorp microtitre well strips were used (Nunc, Roskilde, Denmark; Cat. No. 469957) and a 100µl of *E. cruciferarum* soluble conidial washings (Immunogen preparation A) in 0.01M PBS was aliquoted in to each of 300 wells (10µg protein per well). The strips were incubated overnight in an enclosed chamber at 4ºC. Unbound material was removed and the microtitre wells were washed once more with 200µl PBS. The microtitre wells were blocked with 200µl of 1% casein buffer (1% (w/v) casein PBS) and incubated at 37ºC for 45 min. Residual blocking buffer was removed and wells were washed once for one min each with 200µl PBSTwC (PBS, 0.05% Tween 20 and 0.1% Casein). A 100 µl amount of fusion hybridoma tissue culture supernatant mixed with PBSTwC was added to each well. Following incubation in a Wellwarm incubator at 18ºC for 45 mins wells were washed three times for one minute each with 200µl PBSTwC. A DAKO duet amplification system (K-0492; DAKO Ltd, Cambridge) was then used to amplify bound antibodies and the reaction viewed by colour development using 100µl/well 3,3',5,5'tetramethylbenzidene substrate (www.sigmaaldrich.com Cat. T-3405 and P-4922). The reaction was stopped by adding 25µl of a 20% 1 M H_2SO_4 solution to each well and absorbance values were recorded using a Biohit BP800 ELISA plate reader (Alpha Laboratories, Hampshire).

Following incubation as above, wells were washed three times for one min each with 200µl PBSTincTw. After which a Protein-A Horseradish peroxidase conjugate was applied to each well (100µl) and the microtitre stips were incubated for 45 mins at 37ºC. Wells were washed as described above and 100µl of 3,3', 5,5'- tetramethylbenzidene substrate (catalogue no. T-3405 and P-Sigma 4922 Sigma) was added to each well. The reaction was stopped by adding 25µl of a 20% 1M H_2SO_4 solution to each well. Absorbance at 450nm was determined with a Biohit BP800 ELISA plate reader (Alpha Laboratories, 40 Parham Drive, Eastleigh, Hampshire, UK).

Immunofluorescence (IF)

Twenty µl of a 1×10^4 *E. cruciferarum* spore suspension was aliquoted to each of 300 wells of multiglass slides. Following overnight drying any unbound spore material was removed with a PBSTwC wash. Material remaining bound to the multiwell glass slide was incubated with hybridoma tissue culture supernatants (TCS; 20µl/well) for a period of 30 mins at room temperature. A counterstain of Evans blue and Eriochrome black was incorporated within the TCS antibody suspension to quench *E. cruciferarum* autofluorescence. Each multiwell then

received a wash as described above and following air drying were incubated with an anti-mouse antibody which had been conjugated to fluorescein isothanyacyte dye. A counter-stain was again included to ensure quenching of conidial autofluorescence. Incubation was carried out at room temperature in darkness to prevent photo-bleaching of the conjugated antibody. The processed microwells received a final wash of PBSTwC and after air drying were mounted and viewed by episcopic fluorescence for the presence of antibody / fluoroscein tagged conidia of *E. cruciferarum*. Hybridomas, which were identified as producing antibodies reactive to *E. cruciferarum* using either PTA-ELISA or immunofluorescence were selected and cloned once.

Reactivity screening of selected powdery mildew hybridomas

To determine the specificity of the selected *E. cruciferarum* hybridoma cell lines a range of obligate airborne transmissible fungal species, commonly found in horticulture production, were screened by PTA-ELISA and IF. These included *Bremia lactucae* (lettuce downy mildew), *Hyaloperonospora Brassicae* (downy mildew on Brassicas), *Peronospora destructor* (onion downy mildew) and *Albugo candida* (white blister on Brassicas). In addition, tests were then carried out on the airborne stage of the tomato powdery mildew pathogen *Oidium neolycopersici*. The fungal spores of each species were collected directly from host material using a cyclone sampler and prepared for PTA-ELISA and IF as described previously.

Validation of lateral flow devices for detection of powdery mildew conidia

The Burkard cyclone tube sampler was used by on site validation of *E. cruciferarum*. Daily air samples were collected using a Burkard 8-day cyclone multi-tube sampler (Figure 5) in a controlled glass-house environment and over a three week period. Disease free and infected powdery mildew infected Brussels sprout plants were introduced in to the glass-house on day four. To determine trapped spore numbers in the collected air samples, a sample from each was taken and viewed by bright field microscopy. The remaining sample was assessed for *E. cruciferarum* conidial presence using the developed competitive lateral flow prototype. As a control a set of standards of known *E. cruciferarum* conidial numbers was included within the test process. Using an electronic reader competitive lateral flow test line density values were recorded ten minutes after the sample was applied to each of the tests.

Figure 5. Burkard 8-day cyclone multi-tube sampler

Validation of lateral flow devices for detection of light leaf spot ascospores.

Detection of light leaf spot in air samples using ELISA

Field exposed microtitre strips were blocked with 200µl of 1% Casein buffer (1% (w/v) casein PBS) and incubated at 37ºC for 45 min. Residual blocking buffer was removed and wells were washed four times for one min each with 200µl PBS, 0.05 % Tween 20 and 0.1% Casein (PBSTw C). Wells 1-4 of each strip then received 100 µl of polyclonal Ab EMA (raised at Warwick HRI to *P. Brassicae*), with the remaining wells of 5-8 each receiving 100µl of PBS. 0.05% Tween 20 and 0.1% Casein. Following incubation in a Wellwarm shaker incubater (30ºC) for a period of 45 mins as above, wells were washed three times for one min each with 200µl PBSTincTw. A DAKO duet amplification system was used (DAKO Ltd, Angel Drive, Ely, Cambridge, UK; Cat no. K0492) to amplify the signal generated by bound tissue culture supernatant antibodies. Wells were washed as described above and 100µl of 3,3',5,5'- tetramethylbenzidene substrate (Sigma, Poole, Dorset, UK; Cat. No. T-3405 and P-4922) was then added to each well. The reaction was stopped by adding 25µl of

a 20% 1M H_2SO_4 solution to each well. Absorbance at 450nm was determined with a Biohit BP800 ELISA plate reader (Alpha Laboratories, 40 Parham Drive, Eastleigh, Hampshire, UK).

Competitive lateral flow device for powdery mildew conidia or light leaf spot ascospores

Lateral flows for dark leaf spot comprised of a Millipore 135 HiFlow™ cellulose ester membrane direct cast on to 2ml Mylar backing (Cat No. SHF2400225, Millipore Corp, USA.), an absorbent pad (Cat No. GBOO4, Schleicer and Schuell, Germany) and a sample pad (Cat No. T5NM, Millipore Corp., USA). Following lateral flow construction control lines of an anti-mouse serum were sprayed directly on to the membrane surface using a flat bed air jet dispenser (Biodot Ltd, The Kingley Centre, West Sussex, UK). A collected *P. Brassicae* soluble mycelial suspension was adjusted to a protein concentration of 500µg ml^{-1} in PBS and applied as a test line to the membrane using the flat bed air jet dispenser. Membranes were air dried at 35ºC for a period of 4 hours. The test and control line labelled lateral flows were cut in to 4 mm strips and each strip housed within a plastic case (Schleicer and Schuell, Germany). A volume of 500 µl purified IgM monoclonal antibody, produced at University of Worcester to *P. Brassicae* (coded UW 268) or *E. Cruciferarum* (coded UW EMA 254) was mixed with 375µl of a goat anti-mouse IgM 40nm gold conjugate (Code BA GAMM 40, British Biocell International, Cardiff, UK) and made up to 2ml in phosphate buffered saline buffer (PBS) and incubated on a roller incubator for 3 hours. The antibody bound gold beads were collected by centrifugation (4000 xg) and resuspended to a final volume of 1.625ml in University of Worcester application buffer (20mM Sodium phosphate buffer, 100Mm Sodium Chloride, 0.25% Trehalose, 0.1% Sucrose, pH 7.2). Each sample pad of each lateral flow device had 60µl of the antibody gold conjugate solution added before air drying at 27ºC. Following air-drying the lateral flows devices were mounted within a plastic housing device (Schleicer and Schuell).

Detection and quantification of collected spore samples using lateral flow devices

Approximately 200µl of extraction buffer was added to each of the collected eppindorf vessels (cyclone spore sampler). Tubes were agitated using a Gallenkamp Spinmix, for 3 minutes at high speed. A 60µl aliquot of each spore suspension was then applied to a sample pad of an individual competitive lateral flow device. Determination of test line development was made by visual assessment and, using an ESE QUANT hand held reader. Each device was scanned on two occasions using the ESE QUANT hand held reader.

Monitoring Light Leaf spot at commercial sites in Scotland

Monitoring airborne inoculum of light leaf spot in a crop of Brussels sprouts in Fife and East Lothian, Scotland

A commercial crop of Brussels sprouts (c.v. Petrus), was monitored continuously over a period of 3 month for the presence of light leaf spot ascospores in air samples at Fife, Scotland. Air samples were taken using a Burkard cyclone sampler and a microtitre immuno-spore trap (MTIST) operated over 7 day periods. At the East Lothian site only the microtitre immuno-spore trap (MTIST) was operated in a crop of Savoy cabbage. The collection vessels were replaced in the trap at weekly time periods (by the agronomist) with clean vessels. Prior to field exposure the microstrips for the MTIST trap were stored at 4ºC in a sealed container. Air flow through the MTIST sampler was estimated in still air by measuring the air speed at different points across the inlet manifold using a hot film anemometer (air velocity transducer model number 8460, TSI Incorporated, St Paul, MN, USA) and integrating over the area of the inlet. In the tests reported here, the volume flow rate through the device was measured at 57-litre min^{-1}. The MTIST sampler and the cyclone sampler were operated daily for 12 H periods (06:00H – 18:00H) as previous studies had shown that spores of light leaf spot were present in air samples during daylight hours.

Results

Year two results

Development of a lateral flow device for detection of powdery mildew conidia and for light leaf spot of Brassicas

Powdery mildew lateral flow development

In year 1 six monoclonal antibody cell lines, each producing antibodies which recognize the airborne conidial stage of *Erysiphe cruciferarum* were produced. In year 2, an additional seven cell lines were identified from the three remaining mice which had been immunized with soluble conidial material of > 30 Kilo Daltons *E. cruciferarum*. Selected cell lines were tested for reactivity to an extended range of airborne fungal spores and their suitability for inclusion within a lateral flow test determined. A competitive lateral flow (Figure 10.) prototype was developed for the semi-quantitative measurement of *E. cruciferarum* conidia (airborne spores).
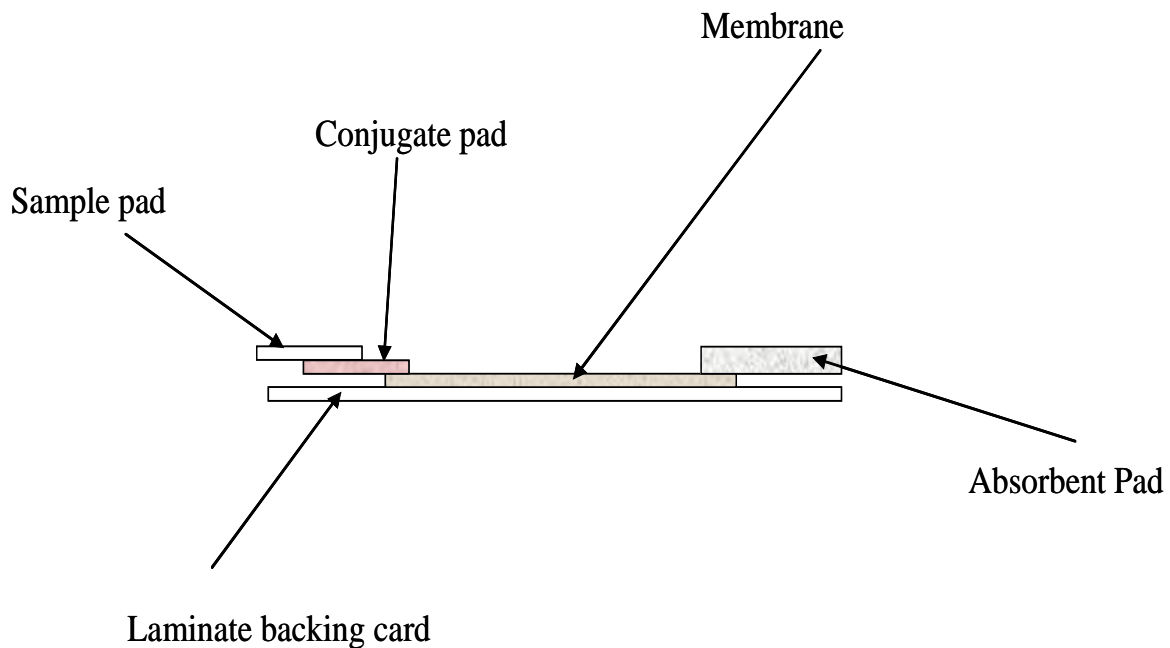

Figure 10 Components of a lateral flow device

Reactivity of Powdery Mildew monoclonal antibodies to other powdery mildew and other pathogen present in Brassica crops under field conditions.

Thirteen monoclonal antibody cell lines produced from the five successive fusions, cell line 2H10B2D4 (coded EMA 254) demonstrated characteristics for
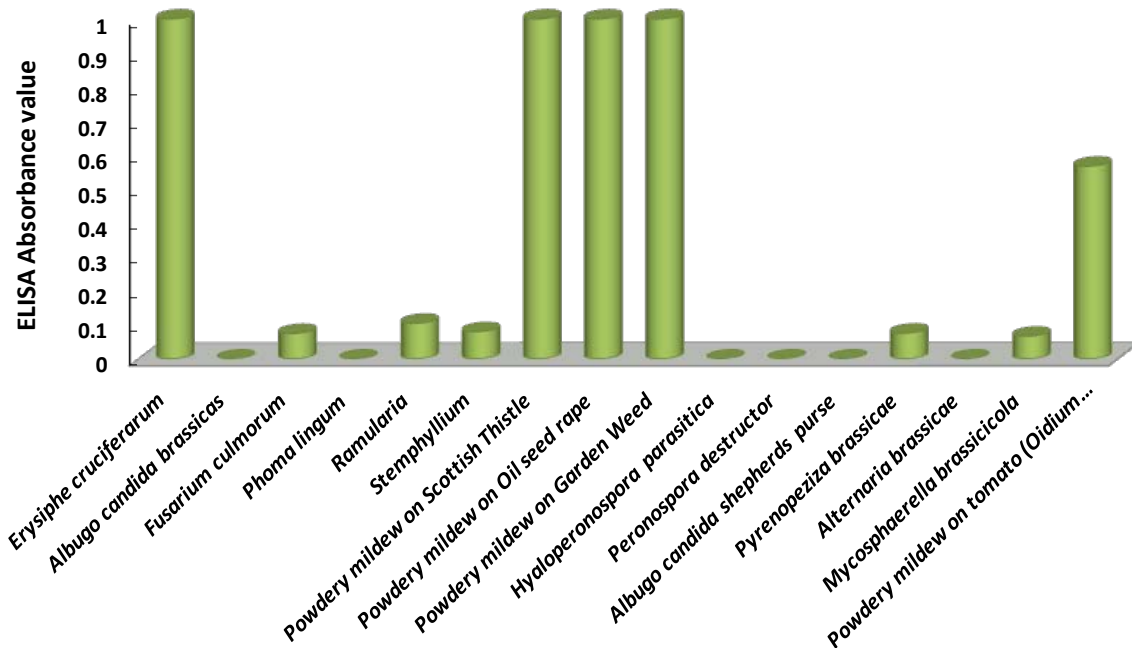

Figure 11 Reactivity of EMA 254 to a range of powdery mildew species and fungi commonly found in vegetable Brassica crops.

use in a competitive lateral flow format. Reactivity tests thus far determine that the selected monoclonal antibody cell line (MAb) reacts to powdery mildew found on a number of hosts but negative to all other fungal species tested (Figure 11). A competitive lateral flow prototype was produced using EMA 254 which proved the most sensitive for estimation of *Erysiphe cruciferarum* conidial numbers. A semi-quantitative competitive lateral flow device gave a visible test line formation when powdery mildew conidial numbers were at or below 300 (Figure 12). In glass house tests where 24hr collected aerosols were monitored for *E. cruciferarum* conidial presence, the competitive lateral flow (clfd) prototype of EMA 254 provided a correlation of $r^2 = 0.8821$ for observed conidial numbers and test line clfd optical density readings. The control standard of known *E. cruciferarum* conidial numbers, ranging between 2400 and 37 conidia in a doubling dilution series, resulted in a correlation of $r^2 = 0.9421$ (Figure 13).

Figure 12 A semi-quantitative competitive lateral flow device with powdery mildew conidial numbers tested between 0 – 4800.
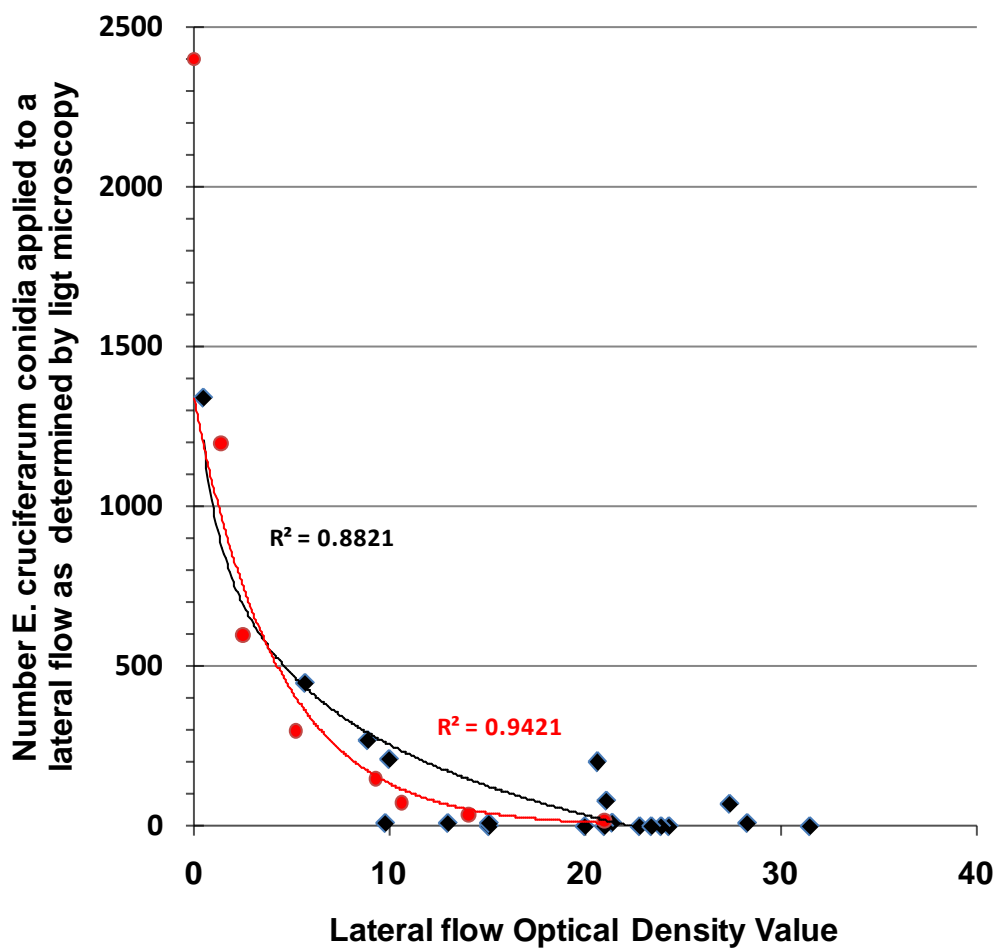

Figure 13 Glass house tests using the powdery mildew competitive lateral flow (clfd) prototype for estimation of *E. cruciferarum* conidial numbers.

Summary

The developed competitive lateral flow device enabled quantitative readings to be made of *Erysiphe cruciferarum* numbers when an electronic reader device was used. However test visual readings only enabled samples in excess of 1x 10^4 conidia ml^{-1} to be identified. Further work will be carried out to optimize the assay format to increase visual test sensitivity whilst retaining good discrimination using an electronic reader.

Monitoring Light Leaf spot at two commercial sites in Scotland

Monitoring light leaf spot ascospores in air samples using ELISA in commercial crops is shown in Figure 14 (East Lothian) and Figure 15 (Fife). Light leaf spot was detected at both sites however the estimated numbers of light leaf spot ascospores at the Fife site were higher (Figure 15). Weekly samples with an ELISA value of greater than 0.1 were considered as high risk periods on susceptible commercial crops. The pattern of light leaf spot risk differed between sites. Maximum light leaf spot risk at Fife occurred during the 8 – 14 September 2010. However at East Lothian risk of light leaf spot increased significantly from the 20 September 2010 onwards although the weekly monitoring time periods at both sites differed and could not be directly compared.
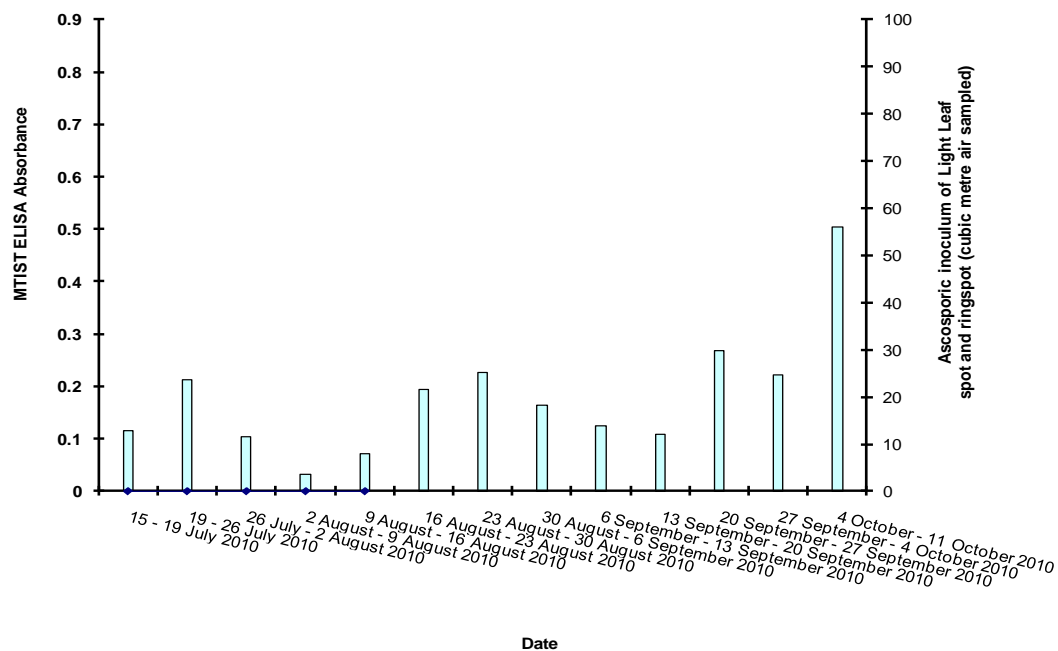

Figure 14 Light leaf spot ascospores in air samples at East Lothian (July 2010 – October 2010)
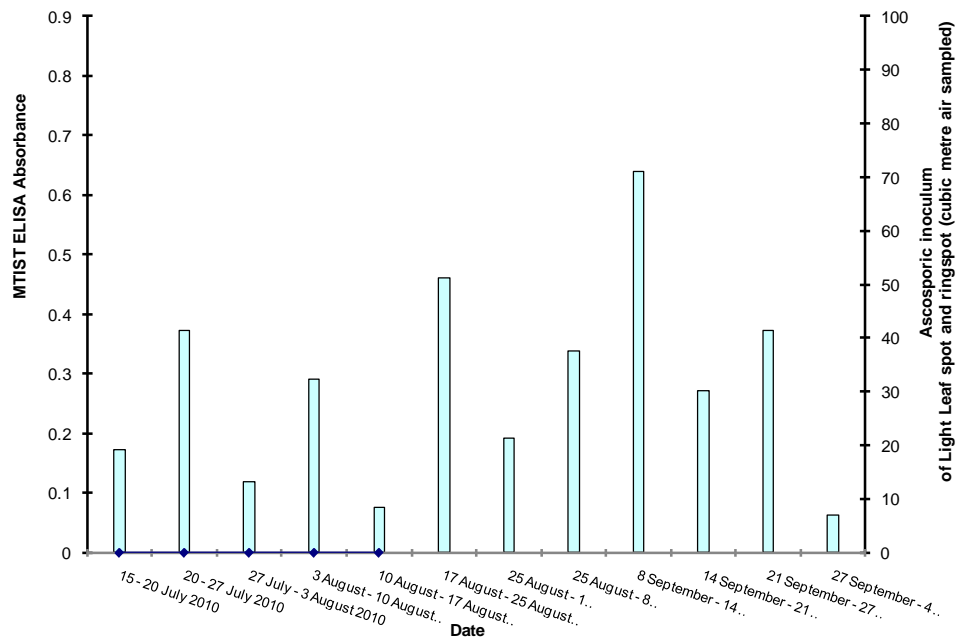
Figure 15 Light leaf spot ascospores in air samples at Fife (Crail) (July 2010 – October 2010)

Developing a light leaf spot lateral flow device

In the development of a lateral flow prototype for semi-quantitative analysis of light leaf spot ascospore inoculum it was determined that a test line application of 1mg ml^{-1} homologous antigen applied at a rate of 15mm s^{-1} 180 Hi Flow membrane (www.millipore.com) was optimal. Test line depletion of the light leaf spot lateral flow prototype was determined using an ESE Quant hand held reader (www.qiagen.com). Of the field exposed cyclone air sampling periods tested a peak in ascosporic inoculum of *P. Brassicae* was recorded in early to mid September with levels in continual reduction on either side of this (Fig. 16). With the exception of the sampling period at the end of August a similar profile was observed when MTIST trapped air samples were analysed by ELISA. The results Figure 16 show that in these limited tests the light leaf spot lateral flow device indicated a peak in light leaf spot ascospores numbers during early September 2011. This corresponded with the results from the MTIST trap as processed by ELISA. The sampling regime between the two traps was different and the litres of air sampled also differed between the two systems. Light leaf spot prediction using the ELISA system gave an estimate of the peak from the 8 – 14 September

2011. The lateral flow device in conjunction with the cyclone trap estimated the peak in light leaf spot ascospores from the 8 – 14 September 2011. The variation in results may be due to the differences in the two traps. All material is collected in one collection vessel with the cyclone trap at a low sampling rate. However the MTIST trap samples into 32 different wells with a higher air sampling rate.
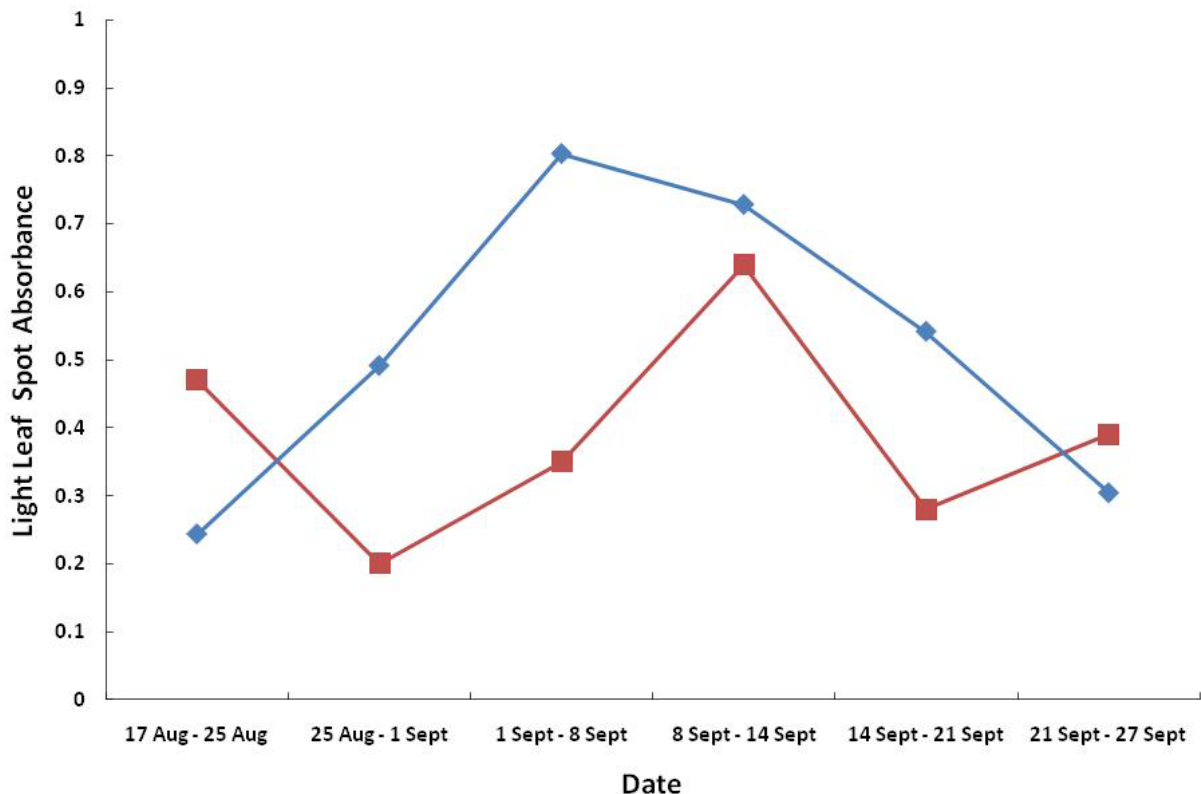

Figure 16 Comparison of detection of light leaf spot in air samples collected in Fife using lateral flow (♦) and ELISA (■).

Summary

A competitive lateral flow for the detection of ascosporic inoculum of *Pyrenopeziza Brassicae* (light leaf spot) has been developed and tested in a commercial Brassica crop field crop in the presence of monitored transmissive inoculum of *P. Brassicae*. Using this system shows potential to designate the likely onset of disease occurrence of light leaf spot in field crops of Brussels sprouts (Figure 16). An MTIST spore sampler was operated as a reference trap to validate quantitative readings made using the developed light leaf spot lateral flow system.

Discussion

Practical usage of the existing ringspot lateral flow tests under field conditions in commercial crops

In vegetable Brassica crops detecting pathogenic spores before they can infect crops has also been shown to be a useful approach in controlling air borne diseases (Kennedy, et.al., 2006). If the technique is to be of value practically then methods of detecting spores in the field are a necessary prerequisite for uptake. The system also needs to be economic in relation to the value of the crop and the cost of fungicide applications. Producing field devices which can detect ascospores of ringspot was successfully investigated within previous HDC contract FV233 and 233a. These devices require sensitivity testing in relation to the appearance of ringspot symptoms and validation under field conditions in conjunction with air sampling devices. Air samplers are available which can trap airborne particulates within vessels. These vessels can be sampled for ringspot ascospores using the 'in field' spore detection devices. The deployment of the tests can be determined using forecasts based on environmental risk of infection. Validation of these tests in the field requires environmental data from a weather station and a spore trap which, could be integrated with the weather station. At each test location both the environmental data and the air-borne spore risk can be assessed (the latter using the lateral flow device). For each test period the vial would be replaced with a fresh one and the sample in the removed vial tested. The results of the test would be visible as lines on the lateral flow device or could be assessed using a lateral flow reader device. By using the existing sensitivity, the system has the advantage of detecting the very earliest possibility of disease occurrence. The grower or consultant is able to estimate real disease risks which he can control using fungicides. The system could enhance the activity of protectant fungicides or even biological control agents. By applying fungicides quickly the grower will be in a situation where he can use protectants to control disease. Use of protectant fungicides could help reduce residues in the crop at harvest. The requirement for zero or low fungicide residues is of increasing importance in crop production.

Controlling Light Leaf Spot in Vegetable Brassica Crops using information from laboratory based inoculum tests

Controlling light leaf spot infection within Brussels sprout crops is difficult given the time periods required for crop production and the prevalence of light leaf spot in arable oilseed crops grown in adjacent localities. Results from previous studies show that light leaf spot

ascospores appear in the air in large enough levels to be problematical only during discrete periods. Light leaf spot inoculum may be present at other times but occurs at too low concentrations to become problematical in developing sprout crops. By using air sampling techniques allied with rapid antibody based techniques periods of light leaf spot risk in Brussels sprouts crops can be identified. Using air sampling techniques described by Wakeham *et al.* (2004, 2010) the risk of light leaf spot infection could be successfully assessed within crops of Brussels sprouts in Fife in a laboratory test using a polyclonal antibody technique. Sprays of tebuconazole could be applied in response to peaks in airborne numbers of light leaf spot. Tebuconazole is one of the few active ingredients available to vegetable Brassica growers which have activity against light leaf spot. For this reason Tebuconazole (Nativo) is widely used by vegetable Brassica growers to combat the potential for light leaf spot development within their crop. However, tests on isolates, taken from vegetable Brassica crops has shown the prevalence of isolates that could grow in the presence of 10ppm Tebuconazole. The tests were carried out using an agar plate technique adapted from Hermann and Gisi (1994). Light leaf spot sensitivity to triazoles varied between 0.1ppm and 20ppm in isolates of light leaf spot taken from arable Brassicas. A sensitivity to 10ppm tebuconazole by isolates from vegetable Brassicas grown at the site indicated the presence of tebuconazole insensitive isolates within Brussels sprout crops. Fungicides with the active ingredients of Boscalid and Pyraclostrobin (Signum) have relatively recently been given approval for use on vegetable Brassica crops. However application of Signum gave good control of light leaf spot infection in Brussels sprout crops provided that they could be applied at the time when light leaf spot ascospores were present in the air.

Control of light leaf spot on Brussels sprout crops also varies with cultivar. In Scotland very susceptible cultivars such as cv. Millenium could not be used successfully in control regimes. This has major implications in the control of light leaf spot in Brussels sprouts where a range of cultivars are grown with differing maturity dates during the season. Often cultivars are grown side by side in the same locality or field. Use of cultivars with high susceptibility to light leaf spot increases the risk of light leaf spot epidemics as the season progresses even when effective chemicals are used to control infection and these are applied when light leaf spot inoculum is present. The use of resistant cultivars such as cv. Petrus has been successful in producing disease free crops late in the growing season and keeping these crops disease free until final harvest in March the following year. Consideration should be given to separating cultivars in the field into different areas as this should reduce the risk. The use of inoculum tests as criteria for fungicide application appears to have prevented the build up of resistance within pathogen populations which could reduce control in these late maturing cultivars. However the inoculum testing system currently used is laboratory based

and requires specialised expertise for its successful operation. More rapid tests which could be used in the field directly by growers are required if more widespread uptake of techniques is to be achieved.

Controlling powdery mildew in Vegetable Brassica Crops

To date there is little information on the requirements for powdery mildew development in vegetable Brassica crops. The pathogen can infect and develop over a wide range of environmental conditions found in the field within crops. The epidemiology of the disease in the crop is poorly understood. It is possible that serious epidemics result only from the influx of substantial amounts of inoculum into the crop. Powdery mildew on vegetable Brassicas appears to be favoured by dry conditions and these usually only occur in vegetable Brassica crops during early summer. It's likely that development of disease in the crop above threshold levels during June, July and August is key in the degree of damage that this pathogen causes. The occurrence of older tissues where powdery mildew development is more favoured, during autumn and winter and may act as a bridge for the pathogen to occur on Brussels sprouts buttons. However it is also possible that powdery mildew penetration into axillary buds may also lead to button infection. This is more likely if large amounts of conidia are present within crops.

Development of "in field" tests for light leaf spot and powdery mildew inoculum

In this report field tests which react to conidia of light leaf spot and powdery mildew have been developed. Developing theses tests has formed the major part of the year two work on this project. Selectively reactive antibodies are required if rapid tests for inoculum detection are to be produced. This is the first step in the development of rapid "in field" tests for these pathogens. Control of plant pathogens could be improved if inoculum could be detected quickly in the field directly by the grower. Airborne inoculum plays a vital role in the development of epidemics caused by *Botrytis* leaf blight on onion crops (Carisse *et al.,* 2005). In this work, a linear relationship was found between number of lesions on plants and the air-borne *Botrytis* conidial concentration. Airborne conidial concentrations of 25 to 35 conidia m $^{-3}$ of air were associated with 2.5 lesions per leaf. When detection of *Botrytis* inoculum was used as a control criterion under field conditions it led to a reduction in fungicide usage of 75 and 56% in 2002 and 2003. A similar relationship between spore number and disease intensity has been reported for *Cercospora apii* on celery (Berger, 1969). In both these studies, microscopes were used to determine spore numbers from air samples. In vegetable Brassica crops detecting pathogenic spores before they can infect crops has also been shown to be a useful approach in controlling air borne diseases (Kennedy *et.al.,* 2006). This report details the successful

development of the 'in field' tests for powdery mildew conidia and light leaf spot ascospores If the technique is to be of value practically then methods of detecting spores in the field are a necessary prerequisite for uptake. Additionally the use of field tests can be determined by using them in conjunction with forecasts based on environmental risk of infection available with MORPH.

References

Amelung, D. & Daebeler, F. (1991). Occurrence of fertile apothecia and epidemiology of *Pyrenopeziza Brassicae* (Sutton et Rawlinson) in the German Democratic Republic. International Organisation for Biological Bulletin 14, 147-50.

Brun, H., Renard, M., Jouan, B., Tanguy, X. & Lamarque, C. (1979). Observations preliminaires sur quelques maladies du colza en France: *Sclerotinia sclerotiorum*, *Cylindrosporium concentricum*, *Ramularia armoraciae*. Science Agronomique Rennes, 7-17.

Carisse, O., McCartney, H.A., Gagnon, J.A. & Brodeur, L. (2005). Quantification of air-borne inoculum as an aid in the management of leaf blight of onion caused by *Botrytis squamosa*. Plant Disease 89, 726 – 733.

Crowton, O.W.B. & Kennedy, R. (1999). Effects of humidity and wetness duration on the germination and infection of *Erysiphe cruciferarum*. 1 st International powdery mildew conference, 29 August – 3 September 1999, Avignon, France.

Evans, N., Fitt, B.D.L., McCartney, H.A., Welham, S.J. & McRoberts, N. (1999). Investigating the horizontal spatial spread of light leaf spot (*Pyrenopeziza Brassicae*) of winter oilseed rape in the UK. Aspects of Applied Biology, Information Technology for Crop Protection 55, 31-6.

Fatemi, F. & Fitt, B.D.L (1983). Dispersal of *Pseudocercosporella herpotrichoides* and *Pyrenopeziza Brassicae* spores in splash droplets. Plant Pathology 32, 401-4.

Figueroa, L., Fitt, B.D.L., Welham, S.J., Shaw, M.W. & McCartney, H.A, (1995). Early development of light leaf spot (*Pyrenopeziza Brassicae*) on winter oilseed rape (*Brassica napus*) in relation to temperature and leaf wetness. Plant Pathology 44, 641-54.

Fitt, B.D.L., Gladders, P., Turner, J.A., Sutherland, K.G., Welham, S.J. & Davies, J.M.L. (1997). Prospects for developing a forecasting scheme to optimise use of fungicides for disease control of winter oilseed rape in the UK. Aspects of Applied Biology, Optimising Pesticide Applications 48, 135-42.

Gladders, P. (1984) Present and potential disease interactions between oilseed rape and vegetable Brassicas. Proceedings 1984 British Crop Protection Conference - Pests and Diseases pp 791-798.

Gilles, T., Kennedy, R., Jackson S. & Steinmetz, M. (2001). Studies on the role of ascospores of *Pyrenopeziza Brassicae* in transmitting light leaf spot from oilseed rape to Brussels sprouts. Proceedings of the Crop Protection Conference in Northern Britain 2002, Dundee 19 –20 th February 2002.

Gilles, T., Fitt, B.D.L., Kennedy, R., Welham, S.J. & Jeger, M.J. (2000a). Effects of temperature and wetness duration on conidial infection, latent period and asexual sporulation of *Pyrenopeziza Brassicae* on leaves of oilseed rape. Plant Pathology 49, 498-508.

Gilles, T., Evans, N., Fitt, B.D.L. & Jeger, M.J. (2000b). Epidemiology in relation to methods for forecasting light leaf spot (*Pyrenopeziza Brassicae*) severity on winter oilseed rape (*Brassica napus*) in the UK. European Journal of Plant Pathology 106, 593-605.

Hermann, D. & Gisi, U. (1994). Cross-resistance among DMI fungicides and sensitivity distributions of *Septoria tritici*. Proceedings of the Brighton Crop Protection Conference-Pests and Diseases, 487-492.

Jee, H.J., Shim, C.K., Choi, Y.J. & Shin, H.D. (2007) Powdery mildew caused by *Erysiphe cruciferarum* is found for the first time on Chinese cabbage in Korea. Plant Pathology 16.

Karolewski, Z. (1999). The occurrence of light leaf spot on winter oilseed rape in Western Poland in 1991-1996 and the characteristics of *Pyrenopeziza Brassicae* isolates. Phytopathologia Polonica 18, 113-21.

Kennedy, R., Wakeham, A.J., Byrne, K.G., Meyer, U.M. & Dewey, F.M. (2000). A new method to monitor airborne inoculum of the fungal plant pathogens *Mycosphaerella brassicicola* and *Botrytis cinerea*. Applied and Environmental Microbiology 66, 297 – 307.

Kennedy. R. & Wakeham, A.J. (2006). Impact of fungicide resistance on light leaf spot control on vegetable Brassicas in Scotland. Aspects of Applied Biology 78, p51 – 58.

McCartney, H.A. & Lacey, M.E. (1990). The production and release of ascospores of *Pyrenopeziza Brassicae* on oilseed rape. Plant Pathology 39, 17-32.

McCartney, H.A., Lacey, M.E. & Rawlinson, C.J. (1986). Dispersal of *Pyrenopeziza Brassicae* spores from an oil-seed rape crop. Journal of Agriculture Science, Cambridge 107, 299-305.

Rawlinson, C.J., Sutton, B.C. & Muthyalu, G. (1978). Taxonomy and biology of *Pyrenopeziza Brassicae* sp. nov. (*Cylindrosporium concentricum*), a pathogen of winter oilseed rape (*Brassica napus* ssp. *oleifera*). Transactions of the British Mycological Society 71, 425-39.

Wakeham, A.J., Kennedy, R. & McCartney, H.A. (2004). Using ELISA to monitor the collection and retention of a range of common airborne spore types in air-samples. Journal of Aerosol Science. 35, 835 – 850.

Kennedy, R., Wakeham, A.J. and Cullington, J.E. (1999). Production and immunodetection of ascospores of Mycosphaerella brassicicola: The ringspot pathogen of vegetable crucifers. Plant Path. 48: 297-307

Plotz, C.M. and Singer, J.M. (1956) The latex fixation test. Application to the serologic diagnosis of rheumatoid arthritis. Am. J. Med. 21 (6):888-892.

Wakeham, A.J., Kennedy, R. and McCartney, H.A (2004) Using ELISA to monitor the collection and retention of a range of common airborne spore types in air-samples. Journal of Aero. Sci. 35: 835-850.