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Project number:	FV 333
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AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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GROWER SUMMARY

Headline

Detection tests for spores 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 a problem only during discrete periods. Light leaf spot inoculum may be present at other times but occurs at too low concentrations to become a problem 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 using 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 has developed 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

Test kits have been developed that are able to identify the disease of Brassica powdery mildew (Figure 1) and light leaf spot. These kits are called lateral flow devices and they work by reacting with the disease to provide a test line reading. The depth of test line colour provides information on the disease level present. When no test line is observed (Figure 1: Spore concentration of >300) the crop is at high disease risk.

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

Brassica light leaf spot and powdery mildew disease is spread by spores in the air. The spores can be collected using a cyclone air sampler (Figure 2). The lateral flow test kits were used with the air sampler to identify airborne disease spore concentration. Visual readings were possible but only at high spore concentrations of spores. Using an electronic reader to provide digital test readout provided the potential for a semi-quantitative system to measure disease inoculum concentration.

Figure 2. Burkard multi-vial 8 day air cyclone sampler

In commercial Brassica cropping systems a Microtiter immunospore trap was operated as a reference trap to validate the quantitative readings made using the developed light leaf spot and powdery mildew lateral flow system. Air sampling systems show the potential to designate the likely onset of disease occurrence in field Brassicas.

Financial benefits

- The usage of the "in field" test for Brassica powdery mildew and light leaf spot will improve the control of these pathogens in field crops.
- When used in conjunction with Brassica spot™ (environmental weather disease forecast) the improved spray timing for application of Signum to vegetable Brassica crops should be achieved. This will improve the efficacy of this chemical especially in production of vegetable Brassicas in Northern Britain.

Action points for growers

- The 'in field' test kits can be requested from Roy Kennedy to determine when light leaf spot and powdery mildew are present the crops. Contact Roy Kennedy on 01905 855 255 or r.kennedy@worc.ac.uk to request the kit.
- Other ''in field tests'' are available for ringspot and dark leaf spot.
- Tests can also be used in conjunction with disease forecasts. Contact Roy Kennedy on 01905 855 255 or r.kennedy@worc.ac.uk

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 sprouts crops in Scotland are planted in May and can remain until harvest in mid April 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 *et al* 2001b). 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.,* 1995). 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.,* 1995). However, when the latent period was expressed in accumulated temperature (>0ºC), 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. 3a). Powdery mildew reduces plant growth and yield in *Brassica* crops and in seed production

A **B**

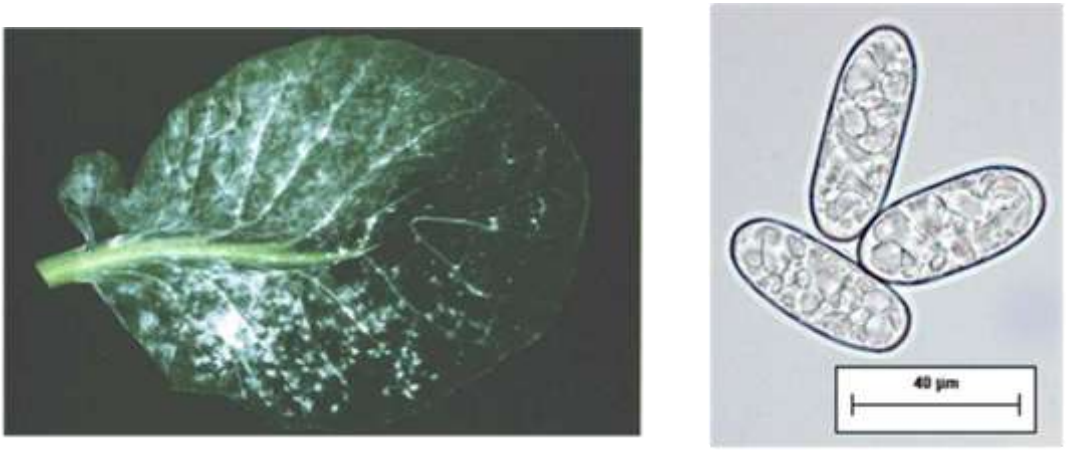

Figure 3. **(A)** Powdery mildew infection on leaves **(B)** powdery mildew conidia

a reduction in the quantity and quality is also 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 (Fig. 3). 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 is 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*. Chains of conidia 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 devices such as 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 (Fig. 4). 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.

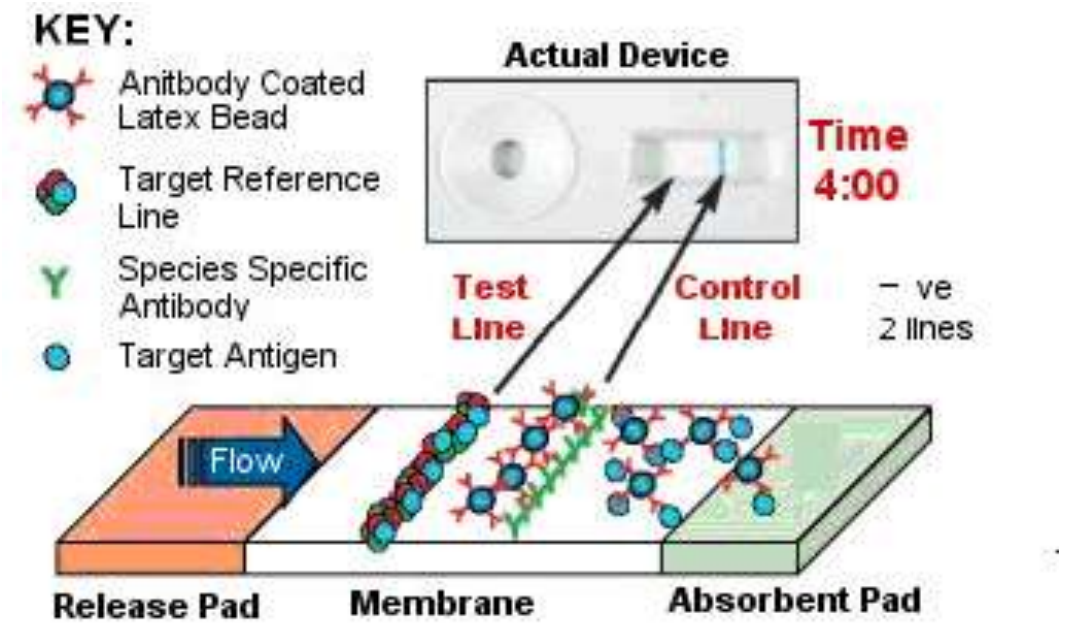

Figure 4. Competitive lateral flow assay format.

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 disease 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 (Fig. 5).

Figure 5. ESE Quant portable lateral flow reader

3. SUMMARY OF YEAR 1

WORKPLAN

(a)

The work in year one concentrated on the production of antibody probes for use in the development of 'in field' test kits to detect aerosols of disease inoculum of light leaf spot and Brassica powdery mildew. The air sampling procedures examined for their use in the assay system were the Microtiter immunospore trap (MTIST) and the multi-tube cyclone air sampler (Fig 6 a and b).

(b)

Figure 6. The multi-tube cyclone (a) and the Microtiter immunospore air sampler (b).

Selection of the antibody probes for use with the air sampling systems was then assessed for their reactivity to other airborne biological material. Monoclonal antisera which provided the greatest level of specificity to their target disease organism: *Pyrennopeziza Brassicae* (light leaf spot) and *Erysiphe cruciferarum* (Brassica powdery mildew) were identified and the respective hybridoma cell lines were twice cloned and allocated a unique Mab code.

MATERIALS AND METHODS

PRODUCTION OF IMMUNOGEN FOR ANTIBODY PRODUCTION

***Erysiphe cruciferarum* (Brassica 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 $1x10^5$ 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. 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 (Kennedy *et al.,* 1999) and adjusted to $1x10^5$ spore's ml^{-1}. The 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.

MONOCLONAL ANTIBODY PRODUCTION

Immunization of mice with spore preparations of A, B (*E. cruciferarum*) and C (*P. Brassicae)*. Three female Balb C mice 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 mixed with an equal volume of Titermax adjuvant 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 an SP2 carcinoma cell line. The hybrid cell lines (hyridomas) were fed on day 7. 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 600 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 (HTCS) supernatant mixed with PBSTwC was added to each well. The HTC supernatants associated to Mice which had received immunogen of *E. cruciferarum* (types A and B). 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 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_2S0_4 solution to each well and absorbance values were recorded using a Biohit BP800 ELISA plate reader (Alpha Laboratories, Hampshire).

The process was repeated with the microtitre wells coated with 100µl of soluble *P. Brassicae* ascosporic inoculum and the HTCS supernatants from type C immunized mice.

*Immunofluorescence (IF)***.** Twenty µl of a $1x10^4$ ml^{-1} *E. cruciferarum* spore suspension was aliquoted to each of 300 multi well glass 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 HTCS antibody suspension to quench *E. cruciferarum* autofluorescence. Each multiwell 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 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.

The process was repeated but with the glass multi well slides coated with 100µl of *P. Brassicae* ascosporic inoculum and the HTCS supernatants from type C immunized mice.

REACTIVITY SCREENING OF THE SELECTED ANTIBODY HYBRIDOMA CELL LINES

Obligate fungal species commonly found in horticulture production were screened by PTA-ELISA and IF for reactivity with the selected monoclonal antibody cell lines. The spore types were : *Bremia lactucae* (lettuce downy mildew), *Hyaloperonospora Brassicae* (downy mildew on Brassicas), *Peronospora destructor* (onion downy mildew), *Albugo candida* (white blister on Brassicas) and *Oidium neolycopersici (*powdery mildew on tomatoes). 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. Antibody hybridoma cell lines which exhibited non or low level reactivity to these fungal spore types were cloned twice and included in an expanded PTA-ELISA reactivity test.

RESULTS

ANTIBODY PRODUCTION

***Erysiphe cruciferarum* (Brassica powdery mildew)**

Immunisation of mice with E. cruciferarum spore fractions. A mouse which had received Immunogen preparation B died prior to tail bleed analysis. The remaining mice each produced an immune response to *Erysiphe cruciferarum* immunogen preparations A and B when tail bleeds were tested by PTA-ELISA (Figs. 7a, b).

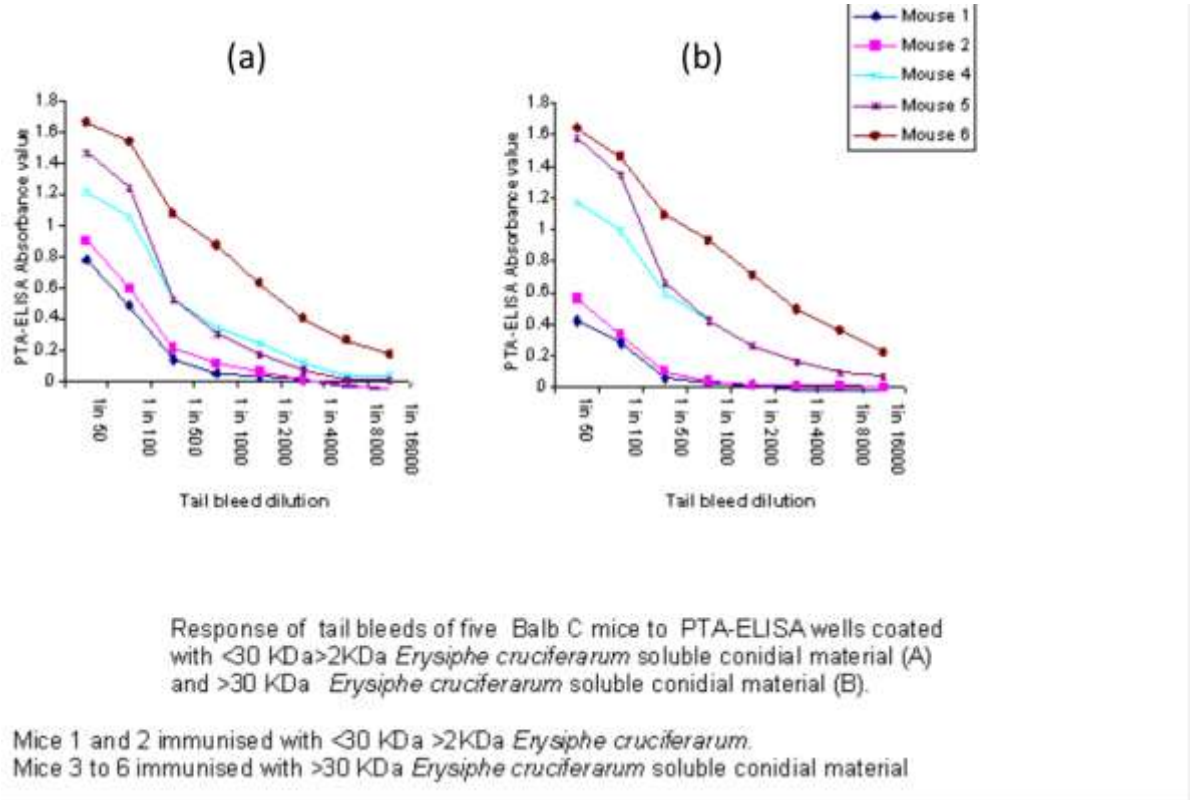

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Figure 7. Mouse tail bleeds as tested by PTA ELISA to *Erysiphe cruciferarum* conidial soluble fractions

***Pyrenopeziza Brassicae* (Brassica light leaf spot)**

Immunisation of mice with P. Brassicae ascosporic material.

The mice each produced an immune response to immunogen preparation C when tail bleeds were tested by PTA-ELISA (Fig 8).

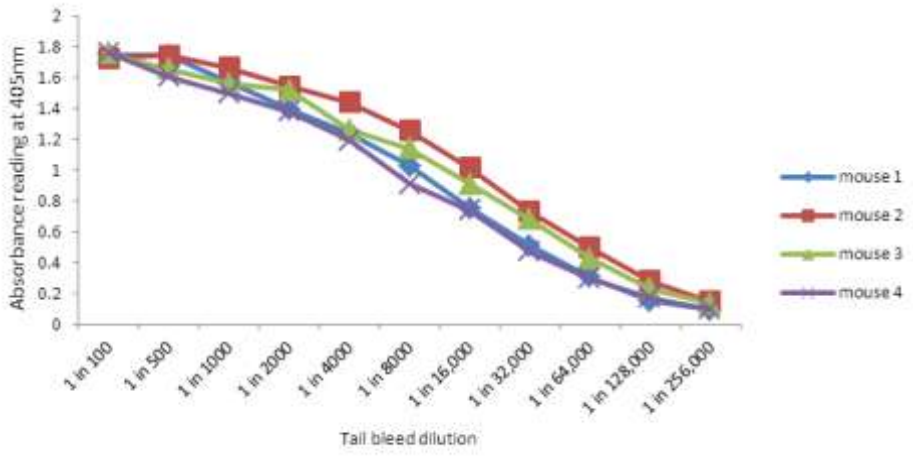

Figure 8: Mouse tail bleeds as tested by PTA-ELISA to *Pyrenopeziza Brassicae* conidial fractions.

REACTIVITY SCREENING OF SELECTED HYBRIDOMAS

Erysiphe cruciferarum **(Brassica powdery mildew**)

Mouse 6 was selected for the initial fusion. Thirteen hybridoma cell lines were then identified by PTA-ELISA and IF as producing antibodies which recognised components associated with *E. cruciferarum* conidial material (Fig. 9). In the initial reactivity screen only two of the hybridoma cell lines 2H10 (UW 254) and 1A10 (UW 255) showed limited or no reactivity to four of the five obligate fungal species when tested by PTA ELISA . Both cell lines did react however with the Tomato powdery mildew fungal plant pathogen (*Oidium neolycopersici* ; Order of Erysiphales and family *Erysiphacea*). By immunofluorescence no reactivity to the 'structural' component of *Oidium neolycopersici* was observed but both cell lines were observed to react with soluble material associated with *Oidium neolycopersici.* Expanded PTA ELISA reactivity tests with specific antibodies UW 254 and UW 255 suggest that they may both be genus specific with limited or no reactivity observed to fungal species tested outside the family Eryiphacea (Fig.10).

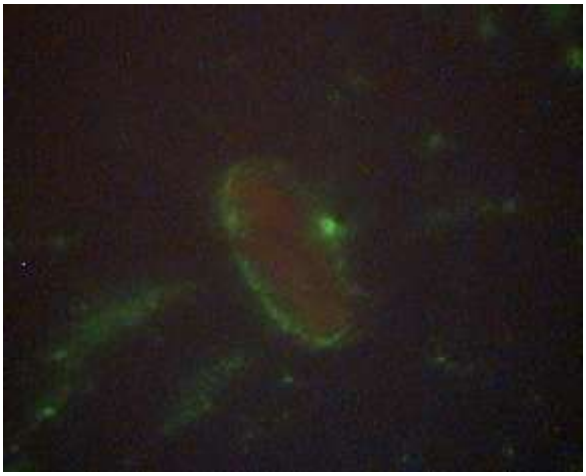

Figure 9. Conidial wall and soluble material of *Erysiphe cruciferarum* as labelled by the specific antibody UW 255 and visualised by immunofluorescence.

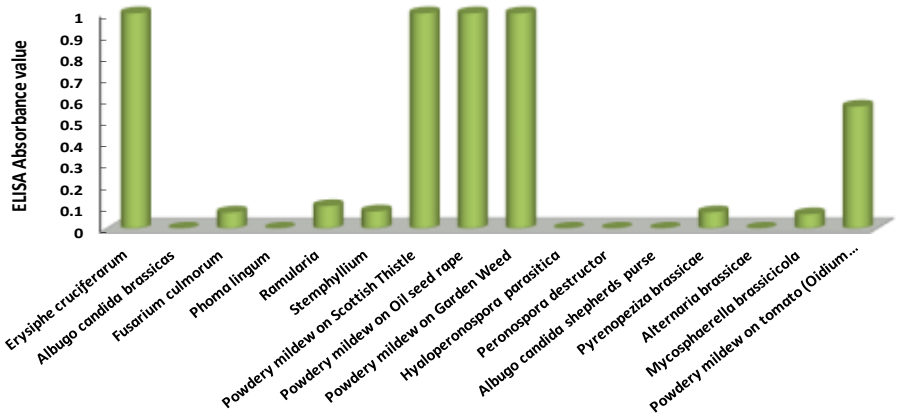

Figure 10. Reactivity of hybridoma antibody cell line 1A10 (twice cloned and coded UW 255) to a range of fungal spores types by PTA ELISA

Pyrenopeziza Brassicae **(Brassica light leaf spot**)

One of the mice died before fusions commenced. Fusions were carried out on all 3 remaining mice and a total of forty one cell lines were identified by PTA-ELISA and IF as producing antibodies which recognised components associated with *P. Brassicae* material. The 41 antibody cell lines were twice cloned to monoclonal and coded UW 268 – UW 309. One of the monoclonal antibodies, UW 277, exhibited high levels of activity to *P. Brassicae* disease inoculum but showed no reactivity to the other fungal species when tested by PTA-ELISA (Fig.11).

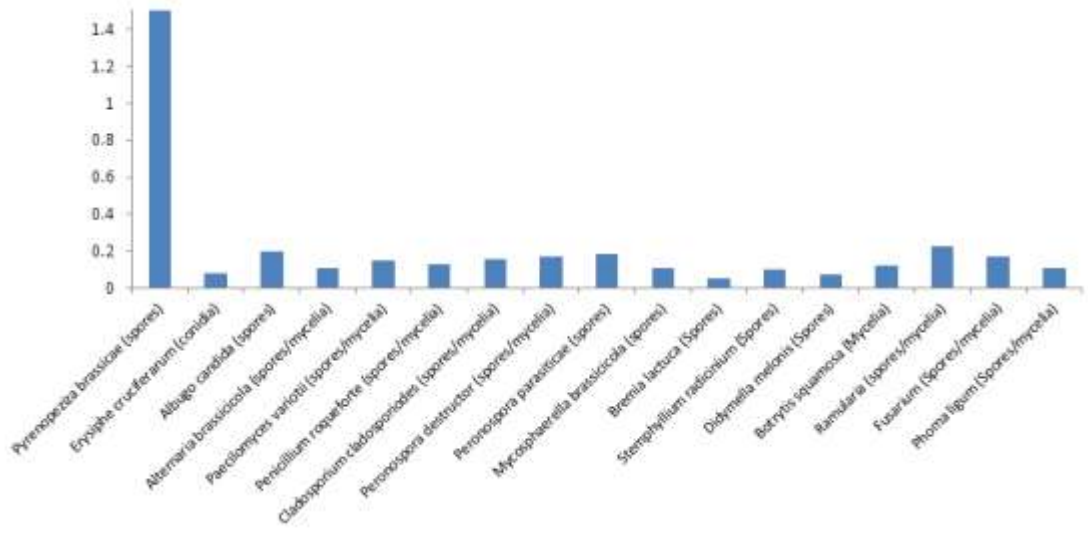

Figure 11. Reactivity of monoclonal cell line 8F6 G2 E5 (UW 277) to a range of fungal spores types by PTA ELISA.

The monoclonal antibody cell line UW 277 was shown by immunofluorescence to bind to the ascosporic spore wall of *Pyrenopeziza Brassicae* (Fig.12).

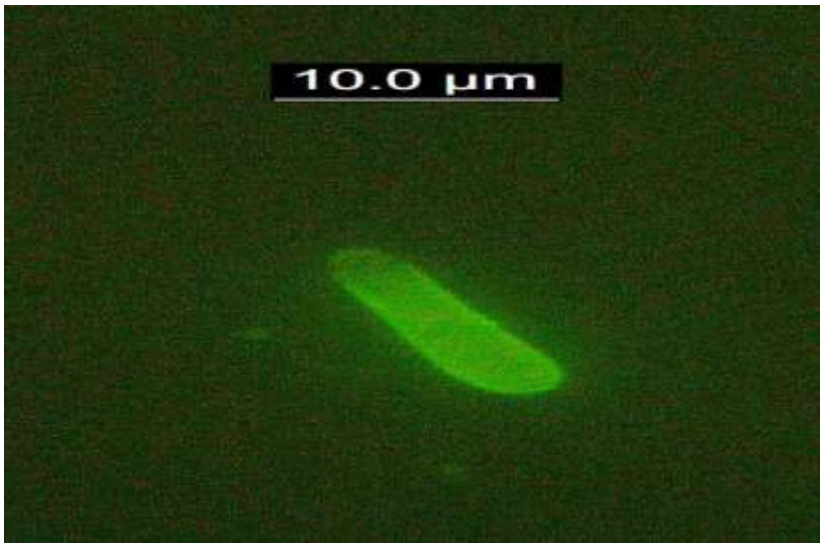

Figure 12: *Pyrenopeziza Brassicae* ascospore as labelled by the specific antibody UW 277 and visualised by immunofluorescence.

CONCLUSION

Preliminary studies have identified two antibody probes for use in the development of a system to monitor and quantify inoculum of Brassica powdery mildew and Light leaf spot. The selected hybridoma cell lines have been cloned to monoclonal and coded as UW 255 and UW 277. In Year 2 of the project the monoclonal antibody probes will be assessed for use in the 'in field' test system and in conjunction with the two air sampling procedures.

4. SUMMARY OF YEAR 2

YEAR TWO WORKPLAN

In Year two, 'in field tests' (competitive lateral flow devices) for the semi-quantitative measurement of disease inoculum of *Pyrenopeziza Brassicae* (light leaf spot) and *Erysiphe cruciferarum* (powdery mildew) were developed. The combined use of air sampling systems with these tests were assessed for the quantitative measurement of airborne disease inoculum of *Erysiphe cruciferarum* and *Pyrenopeziza Brassicae*. The collection and trapping efficiency of the air samplers for the target diseases was determined and where possible optimised to attempt a detection sensitivity that is appropriate to disease development in susceptible field crops. The field test prototypes were tested in a glasshouse cropping system for quantitative detection of *E. cruciferarium* disease inoculum and in the field for light leaf spot disease transmission events.

MATERIALS AND METHODS

OPTIMISATION OF TRAPPING ASSAYS FOR THE DETECTION AND QUANTIFICATION OF TRAPPED AEROSOLS OF BRASSICA POWDERY MILDEW AND LIGHT LEAF SPOT INOCULUM

Erysiphe cruciferarum (Brassica powdery mildew)

Production of Brassica powdery mildew. Sporulating *E. cruciferarum* leaf material, as identified on field grown Brassica crops at Wellesbourne, was collected. Healthy 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 12-14ºC 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 Brassica powdery mildew. An MTIST air sampling device (Burkard Manufacturing Co., Rickmansworth, UK) was used to assess the production and airborne transport of powdery mildew conidia. Airflow through the sampler is channelled through 32 trumpet-shaped nozzles and each directed at the base of a microtitre well (Kennedy *et al*.,

2000). 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}. A range of microtitre well coatings were assessed for their ability to trap and retain conidia of *E. cruciferarum* (Wakeham *et al*., 2004).

Enumeration of the collected powdery mildew spores. The numbers of *E. cruciferarum* spores (powdery mildew) impacted on the base of each microtitre well were 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. Using a monoclonal antiserum (UW 255), the impacted conidia were then immunoquantified by PTA ELISA (Wakeham *et al*., 2004).

***Pyrenopeziza Brassicae* (Light leaf spot)**

Production of Pyrenopeziza Brassicae ascospores. A collected *P. Brassicae* ascospore suspension was adjusted to $1x10^4$ ml^{-1} and using an aerosol sprayer, was applied to leaves of oilseed rape plants *cv.* Bristol. The inoculated plants were incubated according to Gilles *et al.,* (2001) and observations made to confirm production of mature apothecia. After the leaves had senesced, they were collected and dried at 20^{o}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^{o}C in darkness, petioles with mature apothecia containing ascospores were observed. Ascosporic release from the leaf material was facilitated by the use of an environmental chamber using conditions ascribed by Gilles *et al*., (2001b). An MTIST air sampler was cited within the chamber and operated at an air flow of 57 L per minute.

Enumeration of the collected light leaf spores. The collected microtitre wells of the air sampler were examined by bright field microscopy for the presence / absence of ascosporic inoculum of *P. Brassicae*. Using monoclonal antiserum (UW 277), the impacted ascospores were then immunoquantified by PTA ELISA (Wakeham *et al.,* 2004).

DEVELOPMENT OF LATERAL FLOW DEVICES FOR 'IN FIELD' DETECTION OF BRASSICA POWDERY MILDEW AND LIGHT LEAF SPOT

Competitive lateral flow device.

Lateral flows comprised of a Millipore 180 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) (Fig. 13). Using a flat bed air jet dispenser (Biodot Ltd, The Kingley Centre, West Sussex, UK), control lines of an anti-mouse serum were sprayed directly on to the membrane surface of each lateral flow. The lateral flows were then divided in two groups: Group A received a test line application of collected *P. Brassicae* soluble mycelial suspension, adjusted to a protein concentration of 500µg ml^{-1} in PBS and applied at a rate of 10mm $sec.^{-1}$. Group B, received a test line application of *E. cruciferarum* soluble conidial antigen, adjusted to a protein concentration of 500µg ml^{-1} in PBS and applied at a rate of 10mm sec^{-1}. The lateral flows were air dried at 35ºC for a period of 4 hours and cut in to 5 mm strips.

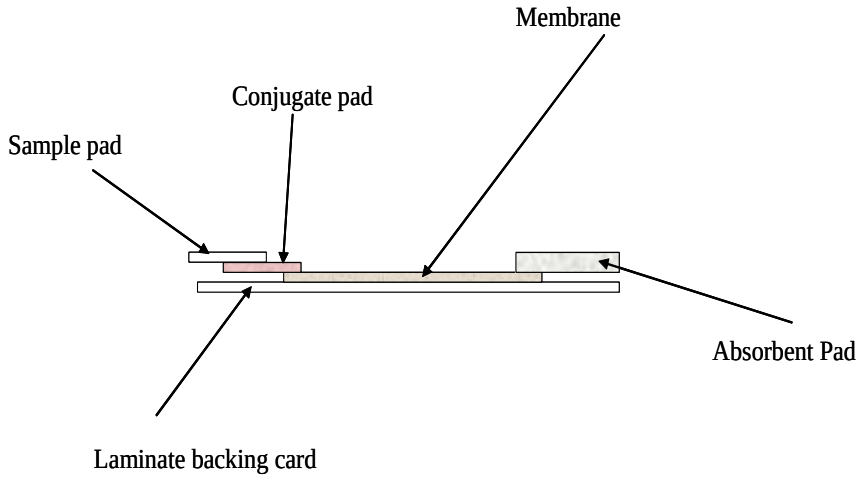

Figure 13: Components of a lateral flow device

A volume of 500 µl purified IgM monoclonal antibody which had been raised to to *P. Brassicae* (coded UW 277) 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). The solution was incubated at room temperature on a roller 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). Sample pads, taken from each *P.Brassicae* lateral flow device, each received 60µl of the antibody gold conjugate solution before air drying at 27ºC. After which the lateral flows devices were re-assembled with the UW 277 gold conjugate and mounted within a plastic housing device (European Veterinary Laboratory, Netherlands).

The lateral flow gold conjugation construction process was repeated using UW 254 (Brassica powdery mildew antiserum). The lateral flow devices which had received a test line application of soluble *E. cruciferarum* conidial material were re-assembled and mounted within a plastic housing device

ESTABLISH LATERAL FLOW SENSITIVITY FOR BRASSICA POWDERY MILDEW AND LIGHT LEAF SPOT DISEASE INOCULUM.

Detection sensitivity of the Brassica Powdery Mildew Lateral Flow.

Conidia of *E. cruciferarum* (Brassica powdery mildew) were produced as described in Section 3.2.1 , and a doubling dilution series (4800 to 38 conidia) was prepared in NPARU extraction buffer. Sample pads of prepared Brassica powdery mildew lateral flow devices, had 100µl aliquots of the *E. cruciferarum* spore dilutions applied to them. The lateral flows were read 10 minutes after sample application for test line development using a laboratory based Quadscan device.

Detection of *E. cruciferarum* in a glasshouse cropping system.

Daily air samples were collected using a Burkard 8-day cyclone multi-tube sampler in a controlled glass-house environment and, over a three week period. The air sampler was loaded weekly with eight 1.5ml microfuge tubes (Sarstedt 2013/4). By an integrated automated mechanism each tube was exposed once for a 24 H period for collection of air particulates After each eight day period the exposed tubes were collected and stored at -20^0 C. Disease free and infected powdery mildew Brussels sprout plants were introduced in to the glasshouse on day four. To determine trapped spore numbers in the collected air samples, 120µl of NPARU extraction buffer was aliquoted in to each of the glasshouse exposed multi-tubes and agitated using a Gallenkamp Spinmix, for 3 minutes at high speed. A 20µl sample was then viewed by bright field microscopy for *E. cruciferarum* presence. The remaining sample of each microtube was then applied to a sample pad of a Brassica powdery mildew lateral flow device. Determination of test line development was made by visual assessment and, using a laboratory ‘non portable’ Quadscan lateral flow reader.

Sensitivity of the Light leaf spot lateral flow.

An 'in vitro' *P. Brassicae* ten-fold serial dilution series, ranging from $1x10^7$ to10 ascospores ml^{-1} was prepared in NPARU extraction buffer. For each, a 100µl ascospore dilution was transferred to a microfuge tube. From a light leaf spot lateral flow device, an antibody (UW 277) gold conjugate sample pad was removed and inserted into the extraction buffer of each microfuge tube. This process was repeated for each ascospore tube dilution series. The pads were gently agitated to release the gold conjugate in to solution and allow binding to the ascosporic material present. After a 3 min. incubation period the antibody gold conjugate/ extraction buffer was removed and transferred to a light leaf spot lateral flow device. Determination of lateral flow test line development was then 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.

Field detection of light leaf spot ascospores in field aerosols.

A commercial crop of Brussels sprouts (*c.v.* Petrus), was monitored continuously over a period of 3 months for the presence of light leaf spot ascospores in air samples at Fife, Scotland. Air samples were collected using a Burkard single tube cyclone sampler and a microtitre immunospore trap (MTIST). The air samplers were operated daily for 12 H periods (06:00H – 18:00H), as previous studies had shown that ascospores of light leaf spot were present in air samples during daylight hours. The collection vessels in each of the traps were replaced at seven day intervals (by the agronomist). After field exposure the tubes of the cyclone sampler and the microstrips of the MTIST air sampler were sent to the NPARU, Worcester for processing.

Lateral Flow assay. 100µl of NPARU extraction buffer was added to each field exposed microfuge tube. For each test, an antibody (UW 277) gold conjugate sample pad of a lateral flow device was removed and inserted into the extraction buffer of a field exposed microfuge tube. The pad was gently agitated to release the gold conjugate in to solution and allow binding to trapped air particulates. After a 3 min. incubation period the antibody gold conjugate/ extraction buffer was removed and transferred to a lateral flow device developed for field assessment risk of the light leaf spot pathogen. Determination of test line development was made by visual assessment and using a Quadscan instrument. Each device was scanned on two occasions using the ESE QUANT hand held reader.

*MTIST PTA ELISA***.** Field exposed microtitre strips were washed 1 x 200µl /well with PBSTwc (0.05% Tween 20 and 0.1% Casein) and blocked with 200µl of 1% Casein buffer (1% (w/v) casein PBS) at 37ºC for 30 min. Residual blocking buffer was removed and wells were

washed four times for one min each with 200µl PBSTw C. Wells 1-4 of each strip then received 100 µl UW 277 and with the remaining wells of 5-8 each receiving 100µl of PBSTwC. Following incubation in a Wellwarm shaker incubater (37ºC) for a period of 30 mins, as above, wells were washed three times for one min each with 200µl PBSTwc. A DAKO duet amplification system was used according to manufacturer's instructions (DAKO Ltd, Angel Drive, Ely, Cambridge, UK; Cat no. K0492) to amplify the signal generated by bound antibodies of UW 277. The microtitre wells were then 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 H2S04 solution to each well. Absorbance at 450nm was determined with a Biohit BP800 ELISA plate reader (Alpha Laboratories, 40 Parham Drive, Eastleigh, Hampshire, UK).

RESULTS

OPTIMISATION OF TRAPPING ASSAYS FOR THE DETECTION AND QUANTIFICATION OF TRAPPED AEROSOLS OF BRASSICA POWDERY MILDEW AND LIGHT LEAF SPOT INOCULUM

Erysiphe cruciferarum (Brassica powdery mildew).

E. *cruciferarum* spore concentrations measured by the MTIST spore trap were significantly higher ($p > 0.001$) for microtiter wells pre-coated with a paraffin wax and petroleum jelly mixture when compared with the other well coatings (Table 1). The paraffin wax and petroleum jelly coatings trapped about 10 times as many powdery mildew spores than that of other well coatings. With the exception of well coatings Poly-L-lysine and Bovine serum albumin (BSA) most conidia were retained following the initial ELISA wash (Fig. 14). Wells pre-coated with a petroleum jelly and paraffin wax mixture proved most effective in retaining spores post ELISA. Air sampling caused the coating solution of 2% Casein to fracture making enumeration problematical.

Table 1. Mean numbers of spores trapped per cubic metre air sampled (Log_{10} spore concentration+1)

Well Coating	*Erysiphe cruciferarum*
2% Gelatin	203 (2.07)
2 % BSA	135 (1.92)
0.1mg ml $^{-1}$ Poly-L-lysine	213 1.75
PP/H (1:8)	1963 (3.21)
PP/H (1:16)	1535 (2.92)
PP/H (1:32)	1788 (2.82)
2 % Casein	101 (1.06)
Distilled water	121 (1.53)
d.f	97 97 97
s.e.d. (between coatings only)	0.0449 0.0673 0.141

BSA Bovine Serum Albumin
PP/H Petroleum jelly and paraffin wax dissolved in a hexane solution

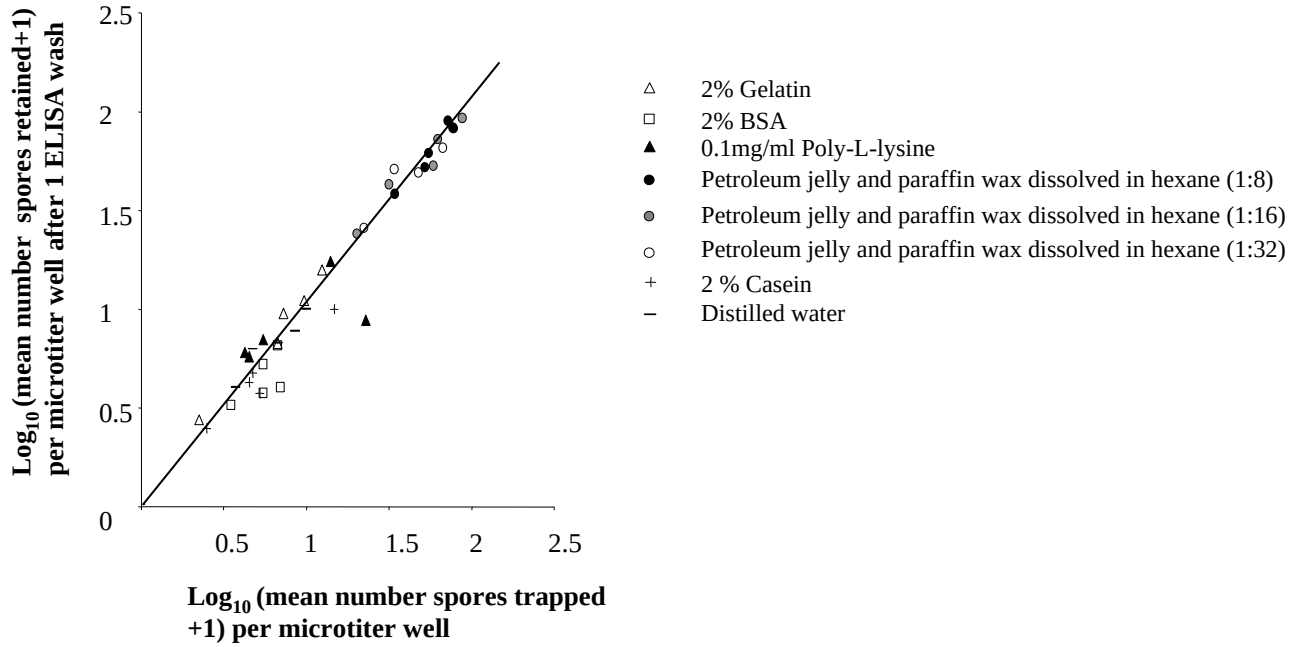

Figure 14. Retention of MTIST trapped *Erysiphe* conidia following an initial ELISA wash stage

***Pyrenopeziza Brassicae* (Light leaf spot).**

The MTIST proved efficient in the collection and retention of ascosporic inoculum of *P. Brassicae* airborne disease inoculum. Mature ascospores have a mucilage coating (Cullington, 1995) and this is likely to assist attachment to impacted surfaces. Pre-coating of the microtitre wells did not prove beneficial in enhancing the attachment and retention process. The pre-treatment of microtitire wells with sodium azide may prove useful to inhibit germination of the trapped ascospores during periods of high humidity (Wakeham & Kennedy, 2010).

DEVELOPMENT OF LATERAL FLOW DEVICES FOR 'IN FIELD' DETECTION OF BRASSICA POWDERY MILDEW AND LIGHT LEAF SPOT

Detection sensitivity of the Brassica Powdery Mildew Lateral flow.

A competitive lateral flow prototype (*clfd*), produced using monoclonal antiserum UW 254, proved the most sensitive for estimation of *Erysiphe cruciferarum* conidial number. A semi-quantitative *clfd* gave a visible test line formation when powdery mildew conidial numbers were at or below 300 (Fig.15). In glass house tests, where 24hr collected aerosols were monitored for *E. cruciferarum* conidial presence, the *clfd* provided a correlation of r^2 =0.8821 for observed conidial numbers by microscopic examination and test line *clfd* ESE optical density readings. The control standard of known *E. cruciferarum* conidial numbers, of between 4800 and 38 conidia in a doubling dilution series, resulted in a correlation of r^2= 0.9421 (Fig.16).

Figure 15. A semi-quantitative competitive lateral flow device with powdery mildew conidial numbers tested

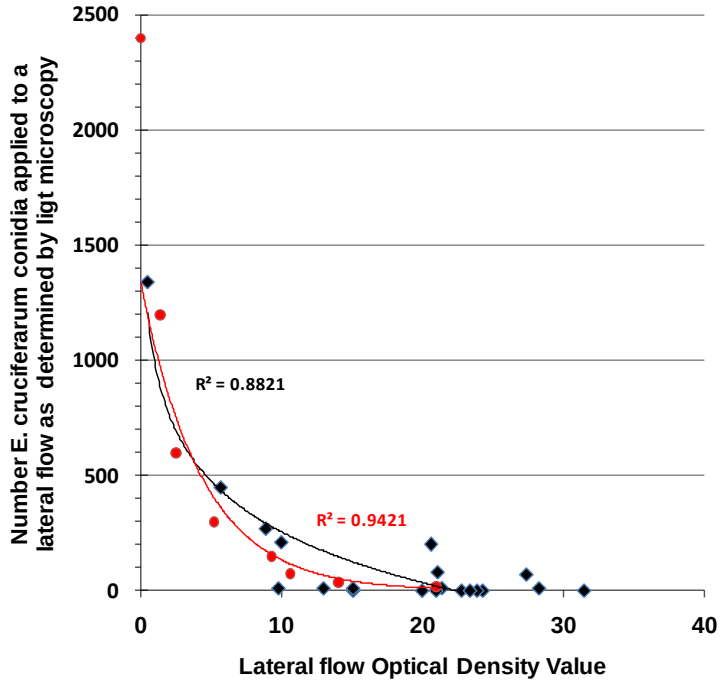

Figure16. Glass house tests using the powdery mildew competitive lateral flow (*clfd*) prototype for estimation of *E. cruciferarum* conidial numbers.

Detection sensitivity of the Light leaf spot Lateral flow.

A competitive lateral flow prototype (*clfd) was* produced using monoclonal antiserum UW 277 for estimation of ascosporic disease inoculum of *P. Brassicae*. A semi-quantitative competitive lateral flow device gave a visible test line formation when ascosporic numbers were at or below 1000 ascospores ml^{-1}.

Monitoring field aerosols for light leaf spot disease inoculum.

A UK commercial Brussels sprout crop was monitored for weekly airborne light leaf spot disease transmission events using two air samplers: a Microtitre Immunospore Trap (MTIST) and a single cyclone air sampler. The collected field exposed microtitre wells (4 x 8 wells) of the MTIST air sampler were processed by PTA ELISA for weekly concentration of light leaf spot ascospores (Fig. 17). Samples which recorded an ELISA value of greater than 0.2 were considered as risk periods on moderately susceptible commercial crops. At Fife, light leaf spot risk was considered extreme (>0.5) between the 8 – 14 September 2010.

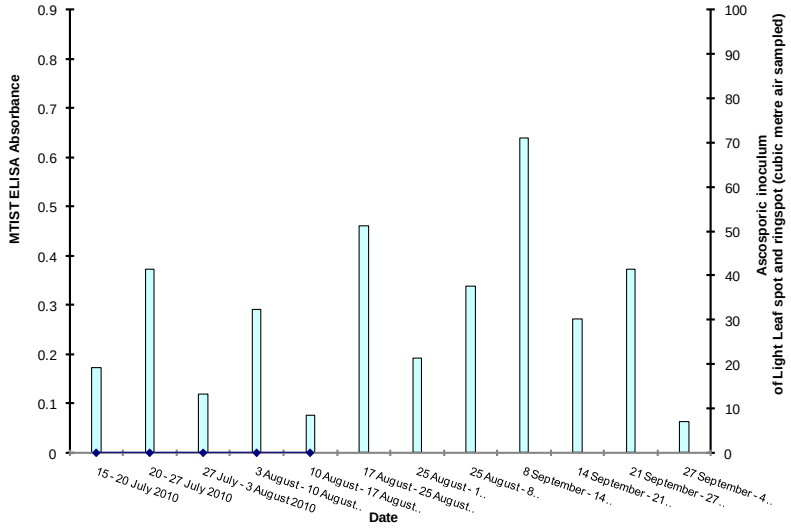

Figure 17. Light leaf spot ascospores in air samples at Fife (Crail) (July 2010 – October 2010

Collected aerosols of the single tube cyclone air sampler were processed by competitive lateral flow assay *(clfd* – the 'infield' test*)* and results agreed with those observed using the MTIST ELISA (Fig. 18). The lateral flow assay however detected an increased crop risk to light leaf spot a week in advance of the MTIST ELISA and was at significant risk to light leaf spot disease pressure for the period 25th August to 23rd September, 2010.

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.

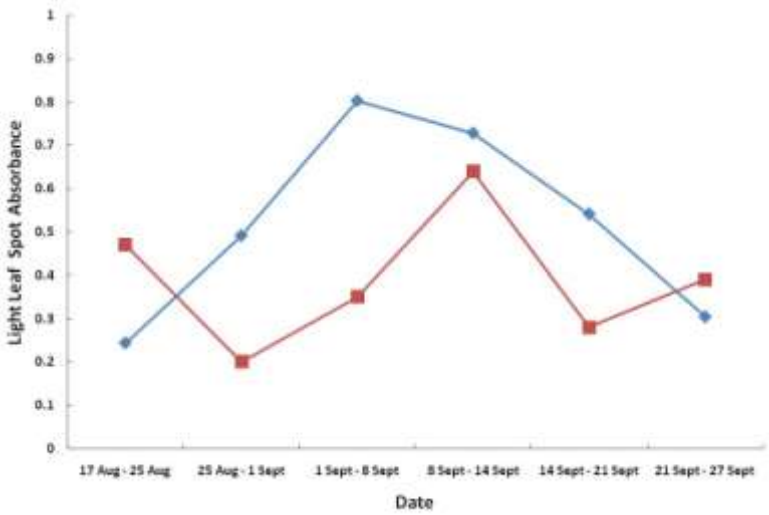

Figure 18. Comparison of detection of light leaf spot in air samples collected in Fife using lateral flow (♦) and ELISA (■).

CONCLUSION

Air sampling

A microtiter immunospore air sampler (MTIST) showed potential for the collection of airborne disease inoculum of *E.cruciferarum* and *P. Brassicae*. Pre-coating the microtiter wells with paraffin wax and petroleum jelly would be a prerequisite for monitoring aerosols of powdery mildew disease inoculum, with 10x more airborne conidia trapped and maintained in the wells post sampling. The pre-treatment of microtitre wells with sodium azide (0.5mg ml^{-1}) would be advised for light leaf spot sampling to inhibit germination of trapped ascospores (Wakeham& Kennedy, 2010). At sites around the UK, particularly in coastal regions of Brassica production, where the high summer temperatures (>18^0C) and sea fogs give rise to high humidity, would provide conditions optimal for spore germination. This could give rise to an overestimation in disease inoculum when ascospore numbers are quantified by enzyme-linked immunosorbent assay.

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

Field tests which can semi-quantify aerosols of light leaf spot and powdery mildew disease inoculum have been developed. Developing these tests has formed the major part of the year two work on this project. Both tests exhibit a detection test sensitivity which should prove useful in a field setting for monitoring disease aerosols of Brassica powdery mildew and light leaf spot. The air sampling systems (MTIST PTA ELISA and cyclone tube sampler) used to collect the field aerosols proved efficient in the collection of disease inoculum and when used in conjunction with the antibody probes enabled semi-quantitative measurements to be made. Both air sampling systems identified similar disease risk periods for each plant pathogen and will provide the end user with the option of an 'in field test' or a laboratory processed system.

5. SUMMARY OF YEAR 3

WORKPLAN

A new batch of lateral flow devices were prepared for the detection and quantification of field airborne transmitted Brassica powdery mildew and light leaf spot disease inoculum. Test sensitivity and stability of the lateral flow devices was examined and the tests then deployed for field usage. Two air sampling systems, as used previously in Year 2 of the study, were operated in commercial Brassica field crops. An MTIST air sampler was operated weekly as a reference trap to validate the semi-quantitative daily and weekly readings of the lateral flow system.

MATERIALS AND METHODS

OPTIMISATION OF LATERAL FLOW DEVICES FOR IN-FIELD DETECTION OF BRASSICA POWDERY MILDEW AND LIGHT LEAF SPOT

Repeat Batch sensitivity

Competitive lateral flow devices (*clfds*) comprising of a Millipore 180 HiFlowTM cellulose ester membrane were produced as described in Section 4.2.2 for the semi-quantitative analysis of *E. cruiferarum* and *P. Brassicae* disease inoculum. Serial dilutions of *E. cruciferarum* and *P. Brassicae* were individually prepared and applied to the lateral flows as described previously in Section 4.2.3. After a 10 minute development time the *clfds* test lines were measured electronically using an ESEQuant reader.

MODIFICATION TO LATERAL FLOW DEVICES ALLOWING TESTING OF WEEKLY AIR SAMPLES FOR BOTH POWDERY MILDEW AND LIGHT LEAF SPOT

Background

For use in routine field tests at Growers' holdings the lateral flow batches require a minimum shelf life of three months. Studies were carried out to determine the stability of the developed lateral flow tests and where possible modifications were made to the lateral flow process to aid test line or conjugate pad stability.

Lateral flow test line study

Competitive lateral flow devices (*clfds*) comprising of a Millipore 180 HiFlowTM cellulose ester membrane were produced as described in Section 4.2.2 for the semi-quantitative analysis of *E. cruiferarum* and *P. Brassicae* disease inoculum. However variable test line components were tested:

Group 1, received a test line application of collected *P. Brassicae* soluble mycelial suspension or *E. cruciferarum*, adjusted to a protein concentration of 500µg ml^{-1} in PBS and applied to the membrane at a rate of 10mm sec^{-1}

Group 2, received a test line application of collected *P. Brassicae* soluble mycelial suspension or *E. cruciferarum* adjusted to a protein concentration of 500µg ml^{-1} in 1/4 strength PBS, 0.05% Trehalose, 1% Isoproponol and applied to the membrane at a rate of 10mm sec^{-1}

Group 3, received a test line application of collected *E. cruciferarum* soluble mycelial suspension, adjusted to a protein concentration of 500µg ml^{-1} in ¼ strength PBS, 0.05% Trehalose, 1% Isoproponol and 2% sucrose and applied to the membrane at a rate of 10mm sec^{-1}

The lateral flows were then assembled and to three lateral flows, taken from each group / fungal pathogen test line type, 100µl of NPARU extraction buffer was applied to the sample pad of each. The sample pad (antibody gold conjugate pad) was prepared 'fresh' at the time of usage. 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. This process was repeated on a monthly basis for three months.

Lateral flow Antibody Sample Pad stability study

Competitive lateral flow devices (*clfds*) comprising of a Millipore 180 HiFlowTM cellulose ester membrane were produced as described in Section 4.2.2 for the semi-quantitative analysis of *E. cruiferarum* and *P. Brassicae* disease inoculum. For each study the lateral flow devices were made up as new whilst the antibody gold conjugate pads were prepared at the start of the study and then assessed for activity over time.

To three lateral flows of each fungal pathogen test line type, 100µl of NPARU extraction buffer was applied to a sample pad. 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. This process was repeated during a month period.

UK FIELD VALIDATION OF LATERAL FLOW DEVICES

Lancashire.

An MTIST air sampler and an eight day multivial cyclone air sampler were positioned within the crop canopy of a commercial Brussels sprout crop at Croppers Farm, Bickerstaffe, Lancashire in August 2011. Held within the base plate of the MTIST machine were four coated eight well microtitre strips. Two strips received a microtitre well coating solution of 100µl / well of petroleum jelly and paraffin wax dissolved in hexane (Sec. 4.2.1). The remaining two strips were coated with 0.05mg ml^{-1} sodium azide at 100µl well. The microtitre strips were changed at seven day intervals and prior to PTA-ELISA analysis were stored at -20^{o}C. The multi-cyclone

air sampler was placed 2 m from and adjacent to the MTIST spore trap. The air sampler was loaded weekly with eight 1.5ml microfuge tubes. By an integrated automated mechanism each tube was exposed once for a 12 H period for collection of field air particulates. Both air samplers were operated by an automated timer to provide a sample exposure period of 06:00 to 18:00 H daily.

A data logger (Smartlog, Aardware Design, Walton on Thames, UK) recorded the air temperature, leaf wetness, relative humidity and rainfall at 30 min intervals. This information was downloaded daily for use in a mathematical model to ascribe infection disease risk periods for Brassica powdery mildew and light leaf spot disease.

Fife, Scotland.

An MTIST and an eight day multi-vial cyclone air sampler were positioned within the crop canopy of a commercial Brussels sprout crop at Inch Farm, Pittenweem, East Fife in July 2011. In this cropping environment, only measurements of light leaf spot inoculum were considered. Air samples were taken daily for 12H periods from 06:00 H to 18:00 daily. All wells of the MTST air sampler were coated with 0.05 mg ml^{-1} sodium azide in distilled water. As in the previous field study, the environmental parameters were recorded daily and a mathematical model ascribed infection risk periods for light leaf spot.

During September to November 2011 the eight day multi-vial cyclone air sampler was removed from the cropping system and replaced with a single tube cyclone air sampler. The air sampler was operated as previous but with the exception that weekly aerosols were collected into a single microfuge tube. All tubes were stored at-20ºC prior to analysis.

Semi-quantitation of disease potential for the collected field air samples

MTIST PTA ELISA. Visual examination of the base of the field exposed microtitre wells (MTIST air sampler) determined that a high concentration of air particulates would prevent enumeration of target inoculum by bright field microscopy. Using the developed antibody probes (Sec. 3.4 : Year 1, Conclusion), trapped aerosols of *E. cruciferarum* and *P. Brassicae* were semi-quantified by PTA-ELISA (Sec 4.2.3: MTIST ELISA).

Lateral Flow. To each field exposed microtitre tube 200µl of NPARU B2 buffer was added and agitated using a Gallenkamp Spin Mix for 5 seconds at high speed. A lateral flow device developed for the field assessment risk of the Brassica powdery mildew pathogen (Sec.5.2.1) was used to semi-quantify trapped airborne disease inoculum of *E. cruciferarum* at the Lancashire site. A 100ul aliquot of the spore suspension (Lancashire field site only) was

applied to the sample pad of the lateral flow device and test line development was assessed 15 min. later using an ESE Quant reader.

The remaining NPARU B2 buffer (approx. 100µl) from each of the field exposed tubes had an antibody (UW277) gold conjugate sample pad of a light leaf spot lateral flow device (Sec. 5.2.1) inserted into it. The pad was gently agitated to release the gold conjugate in to solution and allow, if present, binding to trapped disease inoculum of *P. Brassicae*. After a 3 min. incubation period the antibody gold conjugate/ extraction buffer was removed and transferred to a lateral flow device developed for field assessment risk of the light leaf spot pathogen. Determination of test line development was made using an ESE QUANT hand held reader 10 min after application start.

RESULTS

OPTIMISATION OF LATERAL FLOW DEVICES FOR IN-FIELD DETECTION OF BRASSICA POWDERY MILDEW AND LIGHT LEAF SPOT

Repeat Batch sensitivity

A good correlation (r^2= 0.997 (*E. cruciferarum* conidida) and r^2=0.9176 (*P. Brassicae* ascospore)) between disease inoculum and the corresponding lateral flow ESE Quant portable scanner reading was observed for each test device (Figs. 19 and 20). Detection sensitivity was similar to that observed in Sec. 4.3.2

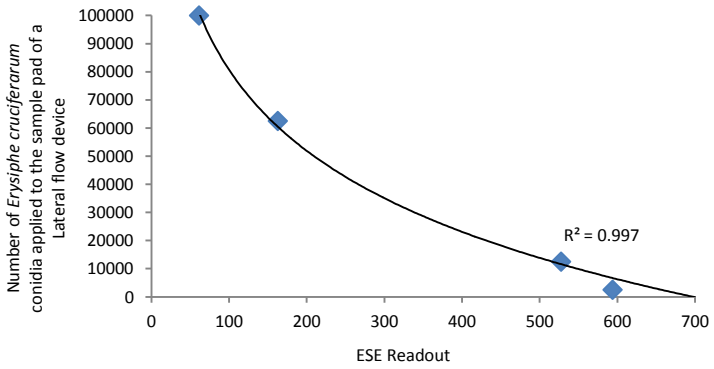

Fig.19 ESE Quant Lateral flow readings as recorded for a conidial dilution series of *Erysiphe cruciferarum*.

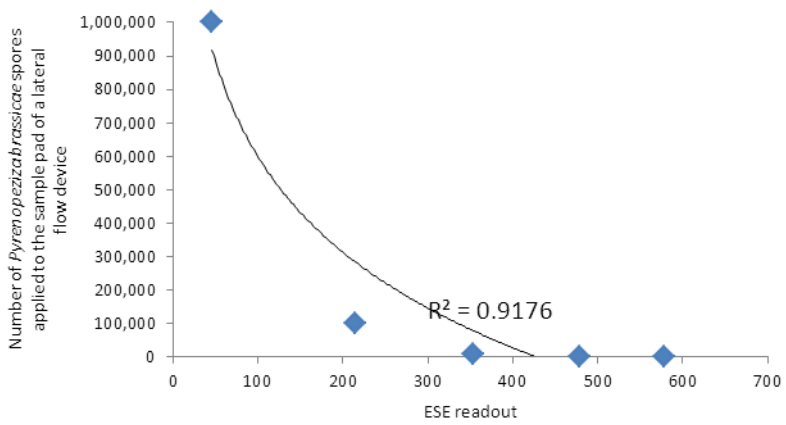

Fig.20 ESE Quant Lateral flow readings as recorded for an ascosporic dilution series of *Pyrenpeziza Brassicae*

MODIFICATION TO LATERAL FLOW DEVICES ALLOWING TESTING OF WEEKLY AIR SAMPLES FOR BOTH POWDERY MILDEW AND LIGHT LEAF SPOT

Lateral flow test line study

The test line activity of both lateral flow types was seen to decrease over time when applied in a ¼ strength PBS (Figs. 21 and 22). The addition of 0.5% Trehalose and 1% Isopropanol provided little effect over the time periods tested. The addition of 2% Sucrose enabled test line stability to be retained at three months compared to the readings made directly after manufacture (Figs 21 and 22).

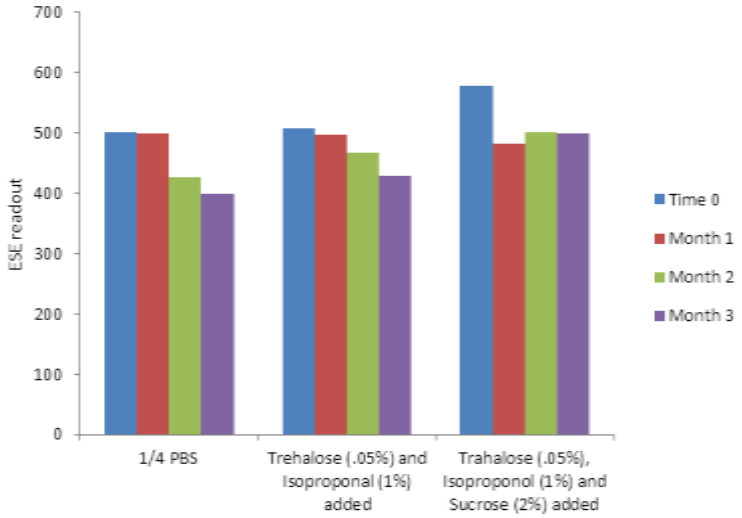

Fig. 21. The effect of additives to the test line stability of the light leaf spot lateral flow as monitored over time.

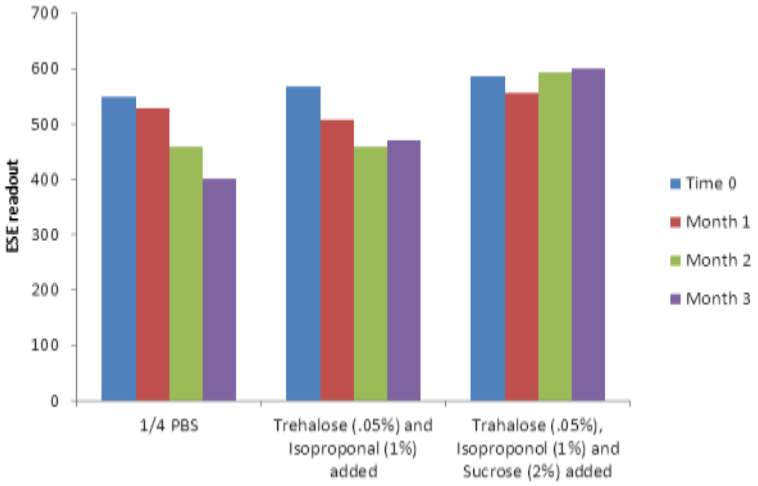

Fig. 22. The effect of additives to the test line stability of the Brassica powdery mildew lateral flow as monitored over time.

Lateral flow Antibody Sample Pad stability study

The addition of sucrose at concentrations >5% to the antibody gold conjugate solution enabled the activity of the light leaf spot lateral flow to be preserved in relation to the control lateral flow (no sucrose added) one month after production (Fig. 23). This was not observed for the Brassica powdery mildew lateral flow where antibody activity was affected within a 24hr storage period (Fig. 24).

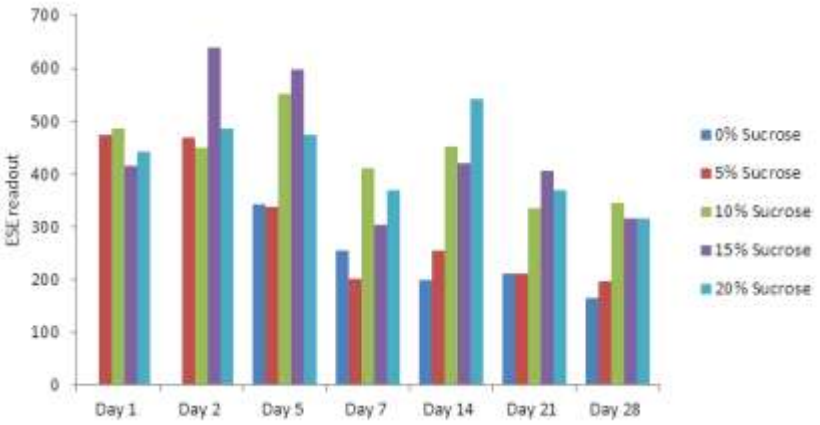

Fig. 23 The effect of sucrose on the stability of the dried antibody gold conjugate over time of the Light leaf spot lateral flow.

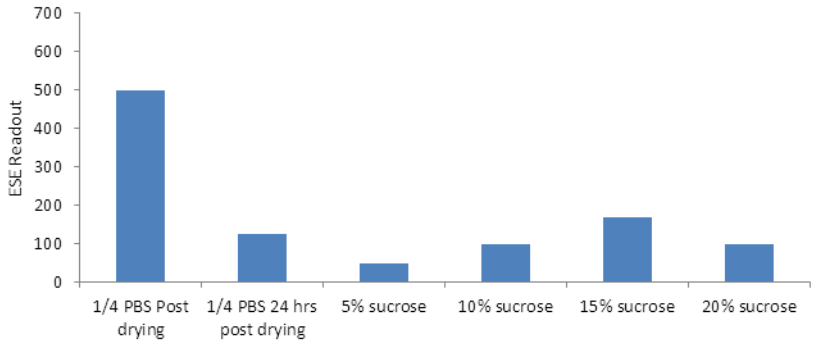

Fig. 24 The effect of sucrose on the stability of the dried antibody gold conjugate over time of the Brassica powdery mildew lateral flow.

UK FIELD VALIDATION OF LATERAL FLOW DEVICES

Lancashire

At Croppers Farm, Bickerstaffe, Lancashire, low / negligible Brassica powdery mildew disease transmission periods were recorded by the MTIST PTA ELISA and daily lateral flow test ('in field tests') for the period of August to October, 2011 (Figs. 25 & 26). No alerts were issued for Powdery Mildew disease risk. Crop walking determined that the crop was free of powdery mildew for the period monitored.

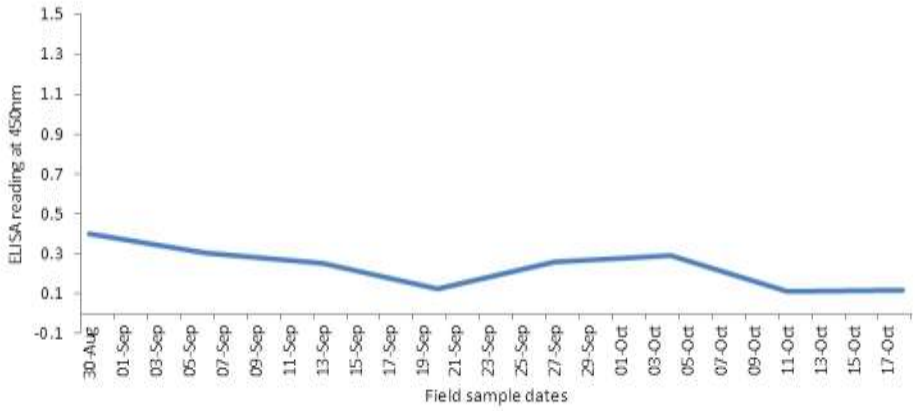

Figure 25 Immunoquantification of MTIST trapped airborne disease inoculum of *E. cruciferarum* in a UK Brussels sprout commercial crop during August to October, 2011.

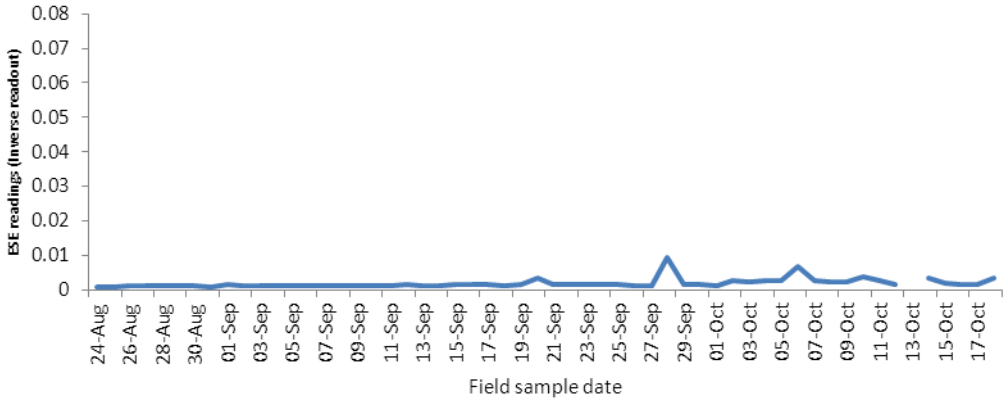

Figure 26. Monitoring *E. cruciferarum* disease inoculum in field collected aerosols during August to October, 2011 in a UK commercial Brussels sprout crop using lateral flow devices.

Conversely light leaf spot disease transmission was recorded by both test systems and a relationship observed between the MTIST PTA ELISA and the developed lateral flow tests when weekly readings were compared (Fig. 27). Crop walking indicated little or no disease development.

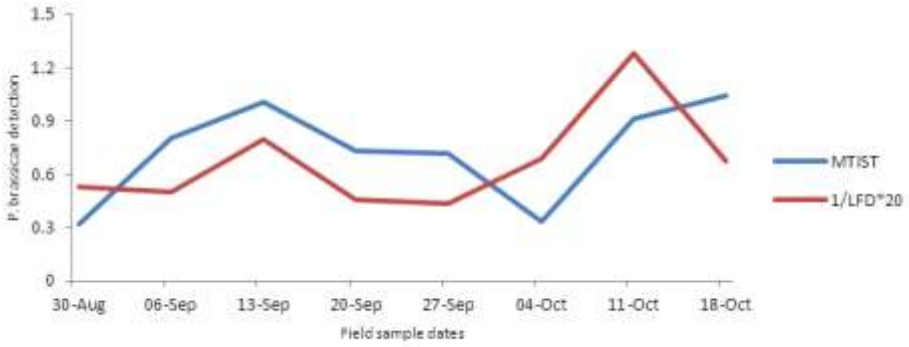

Fig. 27. Monitoring *P. Brassicae* disease inoculum in field collected weekly aerosols during August to October, 2011 in a UK commercial Brussels sprout crop using lateral flow devices and MTIST ELISA.

Fife, Scotland.

During July 2011, both the MTIST PTA-ELISA (weekly sampling) and the daily lateral flow field tests determined little or no risk of light leaf spot disease inoculum in the collected field air samples (Fig. 28).

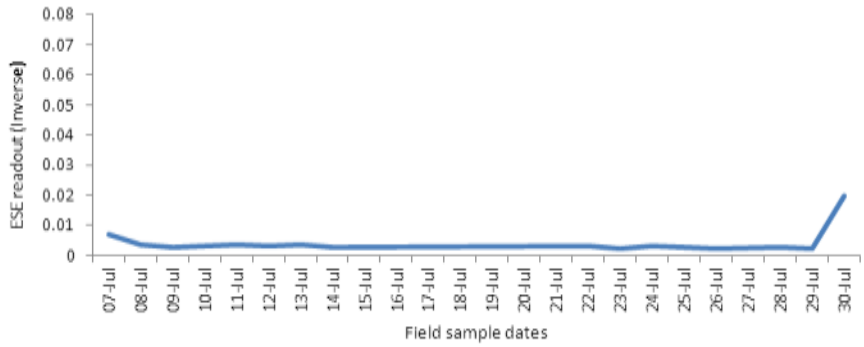

Fig. 28. Monitoring *Pyrenopeziza Brassicae* disease inoculums in field collected aerosols during July 2011 in a Scottish commercial Brussels sprout crop using daily lateral flow devices.

Throughout the September to November 2011 period, when air samples were collected weekly, multiple peaks of *P. Brassicae* disease inoculum was observed in the field aerosols when tested by MTIST PTA ELISA and the lateral flow (Fig. 29). A moderate risk was determined when signal readings were in excess of 0.2 and a high disease risk pressure when in excess of 0.4 reading.

Results for November are excluded as the air sampling equipment was reported to have failed due to battery problems.

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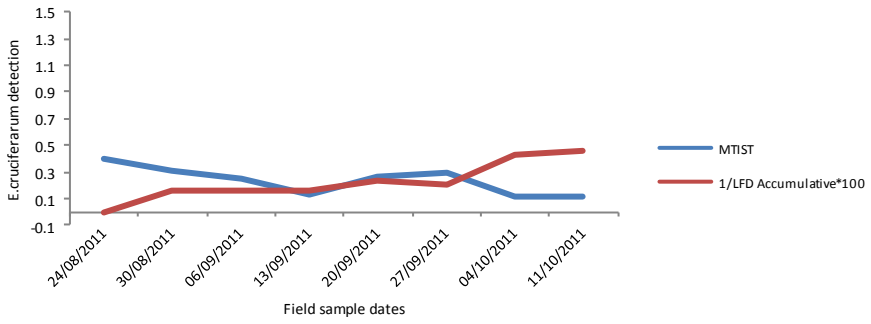

Fig. 29. Quantification of *Pyrenopeziza Brassicae* ascospores (disease inoculum) in weekly collected field aerosols of a Scottish commercial Brussels sprout crop during September-November 2011 .

CONCLUSION

The lateral flow tests developed for the in field testing of Brassica powdery mildew and light leaf spot disease aerosols correlate with the results derived from the Microtitre Immunospore test. Daily readings taken by the lateral flow device determined that the commercial Brussels sprout crops grown in Lancashire was at no disease risk. Subsequent crop walking confirmed this. For light leaf spot both tests determined that the crop was at high risk due to the presence of inoculum. Crop walking recorded a small number of plants expressing light leaf spot disease symptoms following application of disease control measures.

In Scotland both the MTIST PTA-ELISA and the in field tests proved negative for *Pyrenopeziza Brassicae* during the month of July. No disease was observed on the exposed field plants during this period. From September through to November both tests showed a relationship in the quantification of trapped light leaf spot disease inoculum and crop protection warnings were raised on several occasions. These latter tests were based on weekly air samples. Further work should now investigate whether daily or weekly sampling for airborne inoculum is required.

The use of weekly estimates of pathogenic inoculum in air samples has been reported for other diseases of field crops (Wakeham and Kennedy, 2010). Nevertheless, in providing a robust field disease monitoring system, it is necessary to determine the inoculum concentration required for infection and symptom development and the effect of environmental parameters on this process. This could provide useful information in determining whether daily or cumulative (weekly) field readings could then be made.

Further work is also required to assess the shelf life of the developed lateral flow devices and develop processes to enable stable devices over a prolonged period *i.e* a full growing season. The stability of the *Erysiphe cruciferarum* probe was disappointing and studies are underway to determine how this can be improved. Alternatively other monoclonal antibody cell lines produced earlier in the study may prove useful and show improved stability.

6. DISCUSSION

Control of Light Leaf Spot in Vegetable Brassica Crops

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 Brussels 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. 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. 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. Additionally the fungicide Rudis (which contains triazolinthione) has also given good control of light leaf spot. 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 partially resistant cultivars such as cv. Petrus has been successful in producing disease free crops late in the growing season until final harvest in the following year. Consideration should be given to separating cultivars in the field into different areas as this should reduce the risk.

Control of 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, 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.

Improved control using 'in field' detection systems to identify periods of airborne disease transmission.

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. 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 airborne diseases (Kennedy *et.al.* 2006, Wakeham & Kennedy, 2010). This study details the development of 'in field' tests for Brassica powdery mildew conidia and light leaf spot ascospores. If the technique is to be of value practically *i.e.* targeted and effective usage of

crop protectants, the method of detecting spores in the field should be combined with information derived from forecasts based on environmental risk of infection (Brassica^{TM} Spot).

The system described in this report 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 inoculum before it is established within the crop. 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. In addition, using crop protectant measures in a timely, efficient and effective way should assist in the prevention of the breakdown of plant resistant varieties and the potential for increase of virulent pathotypes. A reduction of chemical usage with an informed targeted approach towards disease control will prove popular not only with the producer user on a cost / benefit case but also with with the supermarkets and the end user *i.e* the consumer. A win win situation.

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