Project title:	Brassicas: Development of a rapid field based immunomonitoring assay and its use in controlling disease establishment in transplanted crops.
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Project leader:	Dr Roy Kennedy
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The results and conclusions in this report are based on an investigation conducted over one year. The conditions under which the experiment was carried out and the results obtained have been reported with detail and accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results especially if they are used as the basis for commercial product recommendations.

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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CONTENTS PAGE

1. GROWER SUMMARY

1.1 The Problem

Ringspot (*Mycosphaerella brassicicola*) and dark leaf spot (*Alternaria brassicae*) are two of the most serious problems with the most widespread geographic distribution in vegetable brassica production in the UK. Environmental conditions can be used to predict infection but often these over estimate the real risk of disease establishment in crops. Systems which can be used to determine risk of infection /disease establishment are required if fungicide efficiency is to be improved. Many air-borne pathogens (ringspot) of vegetable brassicas have long latent / incubation periods (time between infection and appearance of disease). This means that success or failure of control is only apparent in some cases weeks after fungicide applications. Often this leads to diseases becoming well established in crops before the disease is really visible. Additionally many of these diseases are difficult to diagnose correctly, as they all first appear as small black spots. A major problem exists in differentiating ringspot from dark leaf spot in its early stages of development.

1.2 Usage of lateral flow devices for inoculum detection

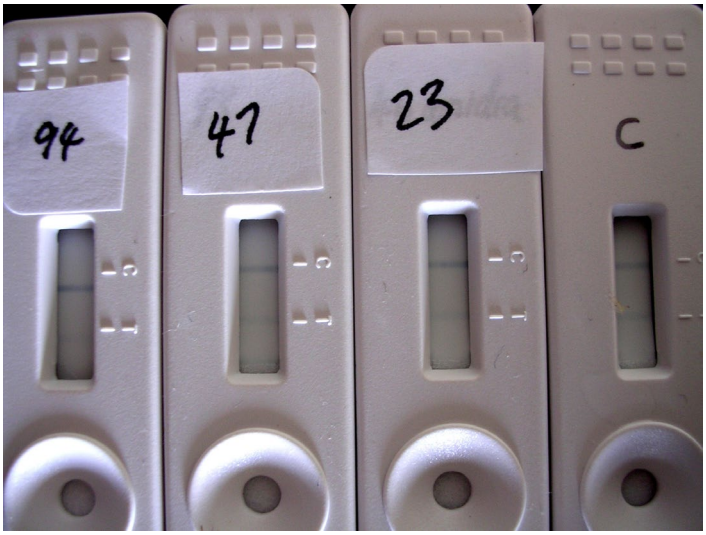
This research report outlines the development of 'in field' test kits which can be used used to monitor the presence or absence of diseases in the air. This is a new and revolutionary approach to crop protection, which will help growers and producers reduce their reliance on pesticides especially if they use the information within existing disease forecasting systems (such as Brassica *spot*). The format that the test will take is described as a "lateral flow test". This type of device is based on the recognition of target spores by antibodies, which react with them specifically. The more specific the antibody the more accurate the results are likely to be.

1.3 Optimisation of lateral flow device for dark leaf spot conidia

A lateral flow format for detection of dark leaf spot conidia has been produced. The competitive lateral flow device is described in the first year annual report for FV233 (Kennedy, 2003). In this type of lateral flow test the absence of a test line represents a positive result. A second control line indicates that the test has functioned normally. The competitive lateral flow assay for dark leaf spot proved very sensitive in its reaction to low numbers of dark leaf spot conidia in test samples. These tests were carried out on laboratory grown cultures of dark leaf spot. The lateral flow device when used on these samples could detect between 47 and 23 dark leaf spot conidia per sample. These levels of dark leaf spot conidia in samples were extremely low in epidemiological terms. Additionally the competitive lateral flow tests did not react to the presence of other fungal contaminants in the

sample even when these were present in high levels. These cross-reactivity tests did not include other species of *Alternaria*. However, previous results using the EMA 212 antibody (which was used in the final lateral flow test format) has shown a low cross reactivity to other species of *Alternaria* when used in other immunoassay formats (ELISA).

Lateral flow tests for dark leaf spot conidia (94 - 23 conidia per sample + control)



1.4 Specificity of the dark leaf spot test

The information would be important in determining potential errors, which might occur under field conditions. For example, if the tests were used to determine the presence and predicted development of dark leaf spot in brassicas, but the location of the test site was adjacent to a crop of carrots heavily infected by *A. dauci* there would be potential for some error in the results. Additionally if the device was used in older crops where there was the presence of dead leaves with the presence of *Alternaria* sp. again the results maybe prone to some errors. However in both these occasions the test would give a false positive result which could trigger fungicide spray applications. In reality careful choice of trapping location and usage of disease forecast software which would indicate environmental risk would eradicate these types of errors. Additionally the lateral flow test is sensitive enough to detect epidemiologically small numbers of conidia which, might be below the threshold for disease establishment although this point will require further tests carried out during year three of the project. The tests will also be used in conjunction with disease forecasts, which will provide the background risk at each location.

1.5 Lateral flow test formats for detecting ascospores of ringspot

Several lateral flow formats have been tested for detection of ringspot ascospores. The most successful format to date has been the competitive lateral flow format using antibody EMA 187. No new antibodies could be raised to ringspot using immunisations at CSL. However, a range of antibodies which are specific to soluble components of ringspot ascospores are available. These will be used within the competitive lateral flow format to designate which antibody will be optimal for ringspot ascospore detection. These tests are ongoing at present.

1.6 Practical usage of the dark leaf spot lateral flow test under field conditions

Usage of the test in the field would require environmental data from a weather station and an additional spore trap which, would be integrated with the weather station. At each location both the environmental data and the air-borne spore risk could be assessed (the later using the lateral flow device). The spore sample would be collected within a vial in the trap. For each test period the vial would be replaced with a fresh one and the sample tested by adding buffer from a dropper. The contents with vial with the added buffer would be shaken and a specific amount of buffer removed and placed on the sample pad of the dark leaf spot lateral flow device. The results of the test would be visible as lines on the lateral flow device. This would indicate the presence of dark leaf spot in the sample. By using the existing sensitivity the system has the advantage of detecting the very earliest possibility of disease transmission. The end user would always be in this situation because fungicides could be used to prevent disease establishment. It is hoped that the system will enhance the activity of protectant fungicides or even biological control agents.

1.7 Action points for growers

There are currently no action points arising from this project. By project completion growers should have access to validated spore detection kits.

1.8 Anticipated practical and financial benefit

The spore trapping system will be used in conjunction with disease forecasts. Growers must therefore have access to disease forecasting systems. The Brassica *spot* system and other disease forecasting systems are available and can be used by growers directly to meet these requirements. This means that there will be few extra costs to the grower in keeping the system running once established. The additional traps needed to determine inoculum in the air will be add-on equipment to this system. By using the system it is possible that growers will be able to reduce spray numbers and place more reliance on cheaper protective fungicides.

By using the test costing approximately £2-4 the grower can confirm the need or otherwise of applying sprays. These sprays can cost between £20-30 per hectare. The cost saving will depend on the area of brassicas that the test will be applied to.

With the development of the dark leaf spot test kit it will possible for the grower to obtain this data on the risk to crops due to pathogenic inoculum in a rapid and inexpensive way. By using traps in conjunction with forecasts the grower will be able to assess the risks precisely from dark leaf spot to his crops. Using this information the grower will then be able to determine precisely which crops require fungicides and which do not. This offers the possibility of producing crops using very little or no fungicides by breaking the cycle of disease establishment and transmission. By using this approach the grower will be able to cope with reduced numbers of actives available in brassica production and any subsequent decline.

SCIENCE SECTION

2. INTRODUCTION

2.1 Air borne disease problems affecting vegetable brassica crops

There are many air-borne fungal pathogens of vegetable brassicas which are difficult to control despite the usage of fungicidal sprays. Two of the most difficult pathogens to control in Brussels sprouts are ringspot caused by *Mycosphaerella brassicicola* and dark leaf spot (*Alternaria brassicae* and *A. brassicicola*). Other fungal pathogens such as white blister (*Albugo candida*), powdery mildew (*Erysiphe cruciferarum*) and light leaf spot (*Pyrenopeziza brassicae*) can be difficult to control in some years and are endemic in some vegetable growing areas. Complex interactions between the environment, plant and air-borne fungal pathogens determine the rate of plant disease development within agricultural ecosystems. Brussels sprout crops in many areas would normally receive 4 - 6 fungicide applications to control these diseases and maintain the high quality of produce demanded by the market. However these diseases are still problematical on cauliflower and broccoli crops. Fungicides are thus often used to prevent disease establishment within the crop. The long period between disease infection and symptom appearance which is a characteristic of many of these diseases often leads to diseases becoming well established in crops before the disease is really visible. Additionally many of these diseases are difficult to diagnose correctly and at low levels in crops are difficult to detect and observe.

2.2 Methods for determining the risk of air-borne diseases in vegetable brassica crops

Existing methods can be used to determine the likelihood of infection and development by airborne vegetable brassica pathogens. This can be achieved by monitoring environmental conditions necessary for infection by different pathogens. Dark leaf spot requires free water for spore germination and infection. At optimal temperatures of 20 ºC, infection by dark leaf spot spores may occur within 6 h but for substantial disease development at least 10 h of wetness is required. Both fungi require at least 12 – 14 h with a relative humidity of greater than 90 % for sporulation to occur. However, ringspot infection requires only short periods of leaf wetness at optimal temperatures. Ringspot requires prolonged periods of temperature and wetness to complete spore production within fungal structures on the lesion (Cullington, 1995). These requirements have been programmed into computer based models (Brassica *spot* and DACOM disease forecasting systems). These systems can be used in conjunction with in field weather data collected by data loggers to determine the risk of infection by different pathogens. However these systems can in many circumstances over estimate the risk of disease occurrence at an early stage in the season. Often favourable environmental

conditions occur in the absence of disease inoculum. Although the environmental risk is high the actual disease risk under these circumstances would be low or zero. Additionally inoculum can be imported into disease free crops from other localities/areas but assessments based on environmental risk alone do not take this factor into account. In order to avoid these problems new and rapid methods of detecting and quantifying pathogenic inoculum are required. These estimates can be used in conjunction with environmentally based risk forecasts to determine the actual disease risk. With this more precision approach there will be reductions in the amounts of fungicide required to control disease by eliminating unnecessary fungicide applications, which are based on weather information alone.

2.3 Using air-borne spore numbers within disease forecasting systems

By assessing airborne spore numbers disease can be predicted accurately before it is visible in the crop. Peaks of airborne spores are always detected prior to crops becoming infected. This, results from the requirement for a threshold of inoculum to initiate disease establishment in crops and this must coincide with favourable weather conditions. Peaks in airborne spore number have been reported to precede the first observed symptoms of the potato blight pathogen *Phytophthora infestans*. Fungicide applications were initiated when the daytime airborne sporangial concentration reached 30 sporangia/m^3 (disease was not yet visible when this threshold was reached). By using this, criteria in combination with disease forecasts based on weather information the number of fungicide applications could be reduced with no impact on disease development.

Similar results were obtained using *Botrytis* blight (*Botrytis squamosa*) on onion crops where thresholds of 15 - 20 conidia/m^3 could be used to reduce fungicide application by up to 100%. Thresholds of inoculum required for disease establishment have also been reported for *M. brassicicola* which is the fungal pathogen responsible for ringspot on vegetable brassicas (Kennedy *et al.,* 2000). The use of air-borne spore numbers, as criteria, within forecasting systems is a new and exciting development in disease forecasting. This work suggests that big reductions in fungicide applications could be made for control of a range of pathogens on a range of common vegetable crop types.

In these studies (with the exception of those on ringspot) the information on spore number had to be collected manually using a microscope which was slow and time consuming. Tests which, can be conducted in the field are necessary if information on air-borne inoculum concentration is to be of more practical value.

2.4 New methods for detecting disease transmission in vegetable brassica crops

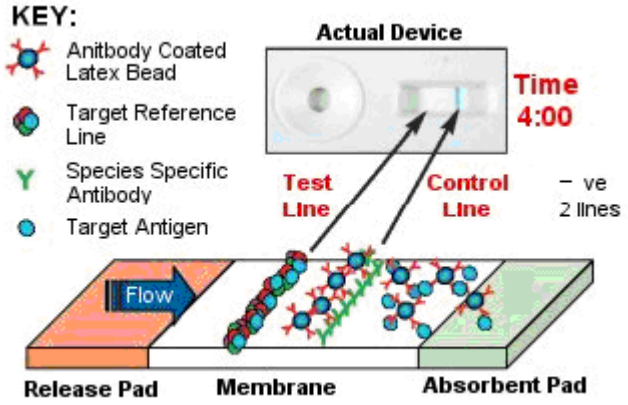
Previous studies using spore traps in the field have demonstrated that epidemiologically significant levels of *M. brassicicola* inoculum in the air could be detected both reliably and rapidly. Large numbers of ringspot ascospores (the spore type responsible for disease transmission) could be detected at the ringspot source and up to 1.6 km away. The distance (and amounts) that ascospores will travel depended on the amount of disease at source. For example there were enough ascospores produced by an infected plot (of dimensions of 5x5m) to give high ascospore numbers in the air 1.6 km from source. The results have great epidemiological significance for the control of ringspot and other diseases in the field. Overwintered unsprayed cauliflower plots with heavy levels of infection are common in field vegetable production areas. These can on average be approximately 10 to 20 hectares in size and must represent considerable sources of inoculum for plantings carried out during the new season (May onwards). It is likely that these crops have the potential to spread significant amounts of disease over entire production areas. In addition it is also clear that summer-grown cauliflower and broccoli which have reduced spray numbers act as considerable sources of disease for over-wintered cauliflower and late season Brussels sprouts crops. To be useful these tests must not be laboratory based. Information on airborne inoculum is required immediately and needs to be ascertained directly in the field.

2.5 Lateral Flow Device (Competitive Assay only)

Lateral flow assays rely upon the competition for binding sites by sensitised latex particles. Antibodies (polyclonal or monoclonal) raised to a specific target spore, are bound by passive or covalent means to dyed latex particles. These sensitised particles 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. Two lines of reagents are immobilised on to the membrane using a sophisticated reagent dispenser. The target reference or test line comprises an antigen or conjugate of the target to be identified 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 (Diagram 1). Two to three drops of sample extract obtained from an air sampler used to collect the spores) are added to the well, releasing the specific antibody bound latex particles, which then begin to flow across the membrane. If the target antigen is present in the sample extract, antibody binding will occur to produce a latex/antibody -antigen complex. Any antibody conjugated latex particles that fail to bind to an antigen will attach to the immobilised test line as they traverse the membrane; thus producing a visible line of deposited latex. The anti-species antibody then captures excess sensitised latex particles to produce an internal control line, providing a visible confirmation of latex flow. Sufficient target presence induces complete inhibition of

latex attachment to the test line, a result that is indicated by a single line of latex deposition. Two lines of equal colour intensity indicate a negative result.

Diagram 1. The Lateral Flow Device



The competitive lateral flow device assay is a semi-quantifiable test. Use of reader technology allows the line intensity, and therefore level of latex accumulation to be calculated using reflectance photometry. However this is not yet available for use under field conditions. By introducing internal control latex particles to the assay, a standard control line can be produced for use as a reference against the test line intensity. Variations in line intensity can be distinguished using a reader, facilitating the differentiation between target concentrations.

2.6 Developing 'in field' tests for detecting the presence or absence of dark leaf spot and ringspot spores (Year one results)

Accurate 'in field' tests for inoculum,based on lateral flow devices, require specific antibodies that can differentiate between different types of pathogenic spores. In year one of this project much of the work centred on the production of specific antibodies which could recognise dark leaf spot spores. A range of antibodies have been raised against dark leaf spot and tested for their cross-reactivity to related organisms found on carrot, wallflower and decaying brassica tissues. These antibodies did not recognise other spores (notably *Botrytis* sp) but reacted weakly to *Alternaria alternata* spores. The antibodies raised to spores of *A. brassicae* (dark leaf spot of brassicas) have been tested for their ability to quantify numbers of dark leaf spot conidia. The results showed that some antibody test lines reacted strongly with increasing number of dark leaf spot spores. Of the monoclonal cell lines tested Mab 220/1.D1.C2.E11 gave the best results with a linear relationship between the signal (absorbance) and the number of dark leaf spot conidia per microtitre well. Antibody EMA

HRI 212.C6 was included in further tests, exhibiting a relatively high level of specificity to dark leaf spot spores. Immunoflourescence studies showed however that this antibody bound to an epitope that was associated with spore germination.

Tests were carried out to optimise the collection and deposition of dark leaf spot spores in sample tubes from the air. The optimised system with the most specific monoclonal antibodies which, selectively detect conidia of dark leaf spot, were used in controlled environment tests where a source of dark leaf spot spores were present. Tests were conducted in the presence of 20 plants of Brussels sprouts, cv. Golfer with sporulating lesions of powdery mildew. These tests showed that antibodies Mab 220/1.D1.C2.E11 and Mab 220/2.G2.A7.D5 were highly specific to dark leaf spot spores (*A. brassicae* only). Antibody 212.C6 was also selective for dark leaf spot conidia. These antibodies were suitable for inclusion in studies to determine applicability within lateral flow tests for dark leaf spot. A good relationship between ELISA estimates of *A. brassicae* spore number and dark spot lesion numbers on exposed Brussels sprout plants given, infection conditions after exposure, was observed using Mab 220/1.D1.C2.E11 and Mab 220/2.G2.A7.D5.

All available antibodies raised and existing from other studies were used in year two for incorporation into lateral flow test formats for detecting conidia of dark leaf spot in the field. Development of antibodies specific to acospores of the ringspot pathogen continued in year two.

3. PRODUCTION OF ANTISERA

3.1 Production of antisera to *Mycosphaerella brassicicola*

3.1.1 Materials and Methods

3.1.1.1 Production of ascosporic inoculum of *M. brassicicola*

The pathogen was isolated on a sprout leaf decoction agar (SLD: agar-amended filtrate from 100g fresh-weight senescent leaves homogenised in 140ml distilled water) from a single ringspot lesion on leaves of a diseased Broccoli crop at Freiston Shore, Lincolnshire. Leaves were surface sterilised by dipping in a 70 % ethanol solution followed immediately by placing in an aqueous sodium hypochlorite solution (4% w/v available chlorine) for 30 sec. Isolations were excised after 21-28 days growth, excess agar was removed and, a mycelial suspension produced in 10ml sterile distilled water (SDW) using an Ultra Turrax homogenizer (Janke Kunkel C., Stausen in Breisgau, Germany). Aliquots (0.5ml) of mycelial suspension were pipetted onto SLD agar Petri plates. The suspension was spread evenly over the entire surface of the agar, using a sterile plate spreader. The inoculated plates were partially air dried under sterile conditions and then stored at 17 ±2°C for 14 days in a growth room under warm white fluorescent / black light (Osram F7/AD/Phillips TLD 18 W/08 tubes). Cultures were examined under x 100 magnification at intervals of 4 – 5 days until pseudothecia were observed on the culture surface. Selected cultures were misted with sterile distilled water and, incubated as described above. Discharged ascopores of *M. brassicicola*, identified on the surface of the Petri dish lid, were then removed by agitation with a sterile plate spreader in 5ml of sterile distilled water.

3.1.1.2 Production of monoclonal antibodies to *M. brassicicola*

The collected ascosporic suspension was concentrated by first freeze-drying (Modulyo 4k, Edwards) and then rehydrating to a final volume of 5ml in phosphate buffered saline solution (PBS). Three female Balb C mice were immunised (by intraperitoneal injection) each with 50µl of the ascospore preparation mixed with an equal volume of Titermax adjuvant (Sigma T-2684). The mice were immunised twice more at 4 weekly intervals. Following tail bleeds and, employing a plate trapped antigen ELISA (PTA-ELISA Plate-Trapped Antigen Enzyme-Linked ImmunoSorbent Assay), a mouse was identified which exhibited a level of sensitivity to *M. brassicicola*. The selected mouse received a final pre-fusion boost (ascospore preparation mixed with adjuvant) and the spleen was removed four days later. A fusion was carried out according to standard CSL protocol and cell hybrids were fed on days 3, 6, and 10. Cell culture supernatants were screened by PTA ELISA 14 days after cell fusion for the presence of antibodies, which recognised ascosporic epitopes of *M.brassicicola*.

3.1.2 Results

The initial screen was unsuccessful with false positives recorded. A plate-trapped antigen ELISA which had been optimised at HRI employing a streptavidin / biotin amplification system, was used to repeat the screen but no hybridoma cell lines were identified positive to *M. brassicicola*. With poor hybridoma fusion (16 % wells displaying hybridomas) the fusion was aborted on tissue culture instruction

A second fusion was carried out (Mouse 231.1) with a post fusion hybridoma rate of 30% recorded. Twenty four positive hybrids were selected by PTA ELISA however negative results were this time observed at the retest stage. Examination of culture wells showed an absence of hybrid cells in twelve of the 24 selected wells. Selection of cell lines was based on culture well observation rather than *Mycosphaerella brassicicola* positive immunoassay results. At the 1st clone retest examination by immunoflourescence identified six cell lines positive to asoosporic inoculum of *M. brassicicola* as follows: 251/4.F3.E1, 251/4.E6.C8, 251/1.D9.G9, 251/1.B8.E10, 251/4.E2.A5, 251/3.C8.H12. These cell lines were cloned and sent to HRI Wellesbourne for further testing by PTA ELISA. All proved negative to pseudothecial material of the ringspot pathogen, when tested by PTA ELISA (Figure 1). A postive control of HRI EMA 187 and HRI 98/4/p (*M. brassicicola* positive monoclonal and polyclonal) were included in the test.

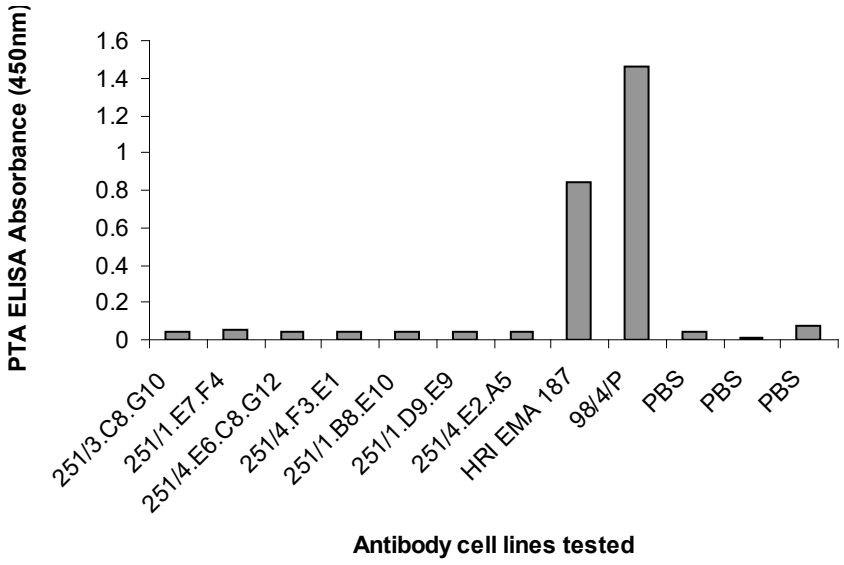


Figure 1. Reactivity of selected clones to soluble material associated with the fungal plant pathogen *Mycosphaerella brassicicola* (pseudothecial stage).

3.1.3 Conclusions

Production of antibodies for ringspot proved difficult and all cell lines produced were shown to be non reactive to ringspot culture material in comparison to existing monoclonal antibodies raised in earlier research projects (MAFF project HH1759SFV). Antibody EMA HRI 187 and HRI 98/4/p, (ringspot positive monoclonal and polyclonals) were used in subsequent lateral flow production for the ringspot pathogen. Although these antibodies have been shown to react to non-soluble components of ringspot ascospores it is possible by careful choice and lateral flow optimisation that these antibodies would be highly selective to ringspot ascospores in lateral flow tests.

3.2 Production of monoclonal antiserum to airborne stage of *Alternaria brassica*: Fusion 3

3.2.1 Materials and Methods

3.2.1.1 Condial production of *A. brassicae*

Isolates of *A. brassicae* (Table 1), taken from the HRI culture collection, were grown on a vegetable juice agar (V8) for one week. A 5cm cube of mycelium was then removed, homogenised in 5 ml of sterile distilled water and transferred in 500µl aliquots to 10 x 5ml sterile clarified V8 juice medium. This process was repeated for each isolate. To induce conidial production the V8 mycelial suspensions were agitated prior to incubation at 25º C. At a magnification of x 100 conidial production was noted seven days later. Conidia of *A. brassicae* were harvested and, collected in a 0.1 % glucose solution collected ($3.5x10^3$ conidia per ml). The conidial suspension was shaken for 1 hour on a wrist action shaker prior to being directly sprayed on to disease free Brussels sprout seedlings (*Brassica oleracea* var. *gemmifera*) c.v. Golfer, each with three true leaves. To provide optimal disease conditions the inoculated plants were exposed to a relative humidity of 100% for 48 hours and thereafter retained in a glass house operating at a constant temperature of 18 ºC. Approximately two weeks after inoculation conidia of *A. brassicae* were identified and collected by agitating segments of *A. brassicae* sporulating leaf material in sterile distilled water for a period of 30 minutes. The plant debris was then removed by filtering the collected suspension through a membrane of 97µm pore size. To remove bacteria, plant cell components and other leaf contaminants the conidial suspension received a final filtration through a membrane of 37µm pore size. The retained conidia of *A. brassicae* were resuspended in 5ml PBS ($1x10^5$ conidia per ml), and completely disrupted (broken open) using a Fast Prep (Qbiogine, UK) and then dispatched to Central Science Laboratory, York for immunization.

Table 1. Isolates of *A. brassicae* for use in immunization study

Code No.	Host	Area	Date isolated
AA3	Brussels sprout	Wellesbourne, HRI	1994
AA3-1	Brussels sprout	Wellesbourne, HRI	1994
AA3-2	Brussels sprout	Wellesbourne, HRI	1994
AA4	Brussels sprout	Wellesbourne, HRI	1992
AA5	Brussels sprout	Lincolnshire	1993
AA10-1	Kohl rabi	Lincolnshire	1994
AA10-2	Kohl rabi	Lincolnshire	1994
AA10-5	Kohl rabi	Lincolnshire	1994
AA11-4	Kohl rabi	Lincolnshire	1994

3.2.1.2 Production of monoclonal antibodies to *A. brassicae*

The remaining Balb C mouse was immunized with 50µl of the prepared conidial immunogen preparation mixed with an equal volume of Titermax adjuvant. The mouse was immunised twice more at 14 day intervals but this time without the adjuvant. Following a tail bled and the collected serum was screened for affinity to its homologous antigen using a plate trapped antigen ELISA (PTA-ELISA). After which and, following a final pre-fusion boost, the spleen was removed four days later. The fusion was carried out according to standard CSL protocol. Cell hybrids were fed on days 3, 6, and 10. Cell culture supernatants were screened by PTA ELISA 14 days after cell fusion for the presence of antibodies, which recognised conidial epitopes of *A. brassicae*. To determine specificity selected *A. brassicae* positive cell lines were screened, by PTA-ELISA against a range of fungal species.

3.2.2 Results

The hybridoma fusion yielded a number of *A. brassicae* positive results. However subsequent cloning steps yielded only two cell lines which were determined as positive to soluble conidial components of *Alternaria brassicae*. Reactivity tests, as carried out by PTA ELISA, determined that each of the cell lines did not exhibit the level of specificity required for inclusion within a lateral flow format. An *A. brassicae* postive control (HRI EMA 212 monoclonal antiserum) was included within the test format (Figure 2a -c).

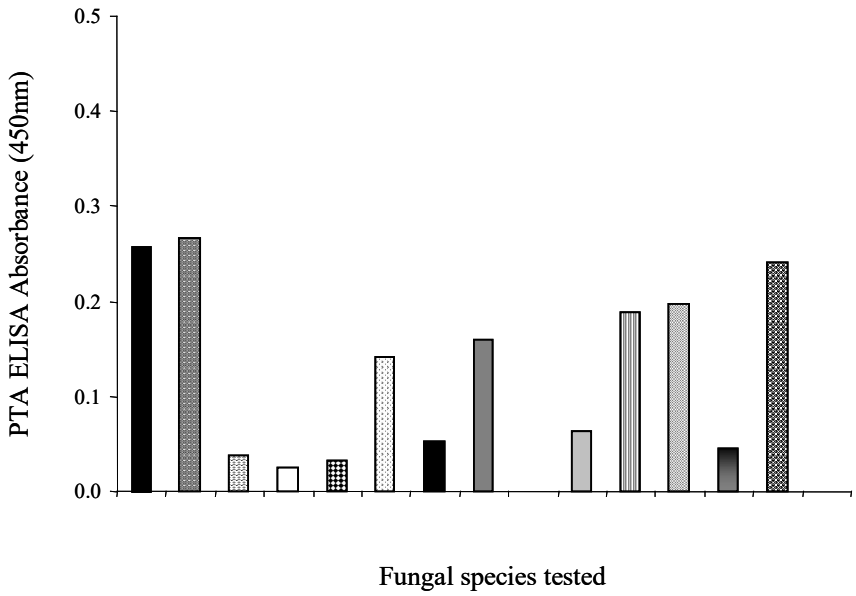


Figure 2a. Reactivity screening of cell line 253/2.A10.C4.A9

- *Alternaria brassicae* (1x10^{3}conidia ml^{-1})
- *Alternaria dauci* (1x10^7 conidia ml^{-1})
- *Alternaria cheiranthi* (1x10^3 conidia ml^{-1})
- *Alternaria brassicae* (mycelial suspension / low conidial numbers present)
- *Penicillium* sp. mycelial / conidial suspension
- *Phoma* sp. mycelial / conidial suspension
- *Stemphyllium* sp. mycelial / conidial suspension
- *Pyrenophora* sp. mycelial / conidial suspension
- *Paecilomyces* sp. mycelial / conidial suspension
- *Botrytis cinerea* mycelial / conidial suspension
- *Ascochyta* sp. mycelial / conidial suspension
- *Aureobasidium* sp. mycelial / conidial suspension
- *Fusarium* sp. mycelial / conidial suspension
- *Botrytis allii* mycelial / conidial suspension
- Negative control

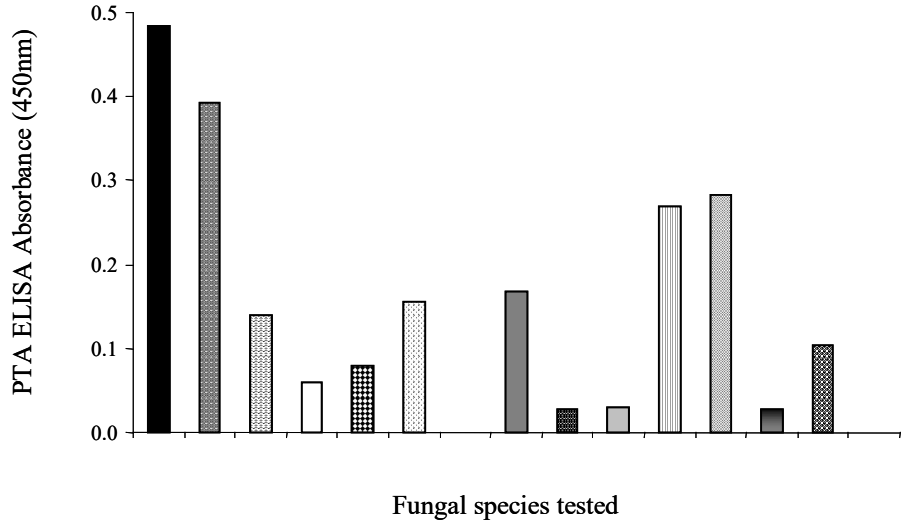


Figure 2b. Reactivity screening of cell line 253/1.D6.G10

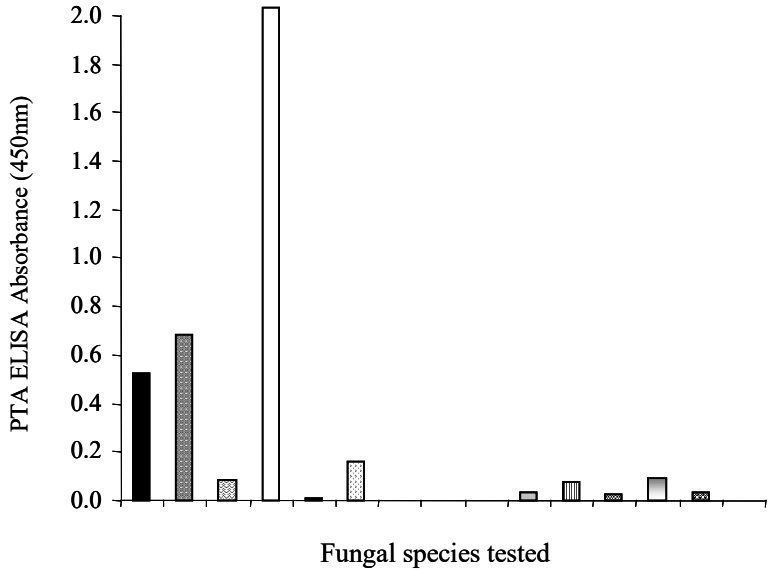


Figure 2c. Reactivity screening of HRI EMA 212 (positive control)

3.2.3 Conclusions

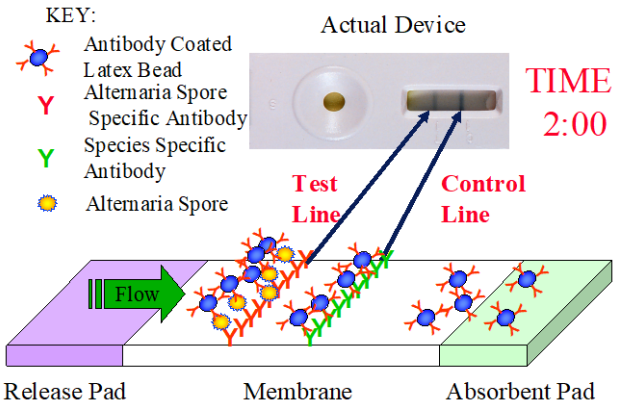
Previous work (Year 1 report Fv 233) indicated that there was a close relationship between, the numbers of dark leaf spot lesions on plants exposed to dark leaf spot spores, and the ELISA values obtained when using antibodies Mab 220/1.D1.C2.E11 and Mab 220/2.G2.A7.D5. Results of year two work yielded no further antibodies selective enough to conidia of *A. brassicae*, which could be used in lateral flow development. Cell line 253/2.A10.C4.A9 and cell line 253/1.D6.G10 were cross-reactive to a range of other common air-borne fungi.

4. DEVELOPMENT AND OPTIMISATION OF LATERAL FLOW FOR DETECTION OF *ALTERNARIA BRASSICAE* (DARK LEAF SPOT) AND *MYCOSPHAERELLA BRASSICICOLA* (RINGSPOT)

4.1 Development and optimisation of lateral flow device for detection of conidia of dark leaf spot

4.1.1 Double Antibody Sandwich (DAS) test format

Two sources of antibody are required within the double antibody sandwich test format, which can comprise either a pair of the same or two different antibody types. One antibody type is bound to the nitrocellulose membrane (test line) the other is labelled with a visual marker (in all tests listed below blue latex spheres are used) and held within a release pad (Figure 3). To ascertain successful test operation a control line was prepared on the same membrane as the test line to capture additional latex particles



- Negative sample = Observation of the control line.
- Positive sample = Observation of the test line and the control line

Figure 3. Schematic drawing of a Double Antibody Sandwich (DAS) lateral flow device *(Positive result shown)*

4.1.1.1 DAS lateral flow test procedure

When a few drops of the test sample containing the target spore are placed on the lateral flow release pad the latex conjugated antibodies are released in to solution and flow with the sample laterally towards the antibody test line. If the target antigen (conidia of *Alternaria brassicae*) are present within the sample the specific antibody conjugated latex spheres bind to the target

antigen (*Alternaria*) and, as this complex flows over the test line, it is captured by the immobilised test line antibody. This reaction is visualised by the formation of a blue line (Figure 3). If no target antigen (no *A. brassicae* conidia) is present within the sample the antibody conjugated latex spheres are not captured on the test line and no line is visible. In either situation, excess antibody conjugated latex spheres will become immobilised at the control line and a clearly visible blue line will form showing that the test has operated satisfactorily.

4.1.1.2 Capture and detector antibodies for inclusion within a DAS lateral flow device

To determine the applicability of the DAS lateral flow format, for the detection of conidial material of *Alternaria brassicae* selected antibody combinations were examined (Table 2). A species antibody was included in each format on the control line. For each combination positive test samples of both mycelial and conidial preparations of *Alternaria brassicae* were used.

Table 2. Antibody combinations used in the DAS lateral flow assessment

Latex conjugated antibody		Test line antibody
Alternaria polyclonal antiserum (PAb) MAb Y80 MAb Y81 MAb Y94		MAb 191
Monoclonal Antiserum (MAb) MAb Y80 MAb Y81 MAb Y94	Y80	MAb 191
Monoclonal Antiserum (MAb) MAb Y80 MAb Y81 MAb Y94	Y81	MAb191
Monoclonal Antiserum (MAb) MAb Y80 MAb Y81 MAb Y94	Y94	MAb 191
Monoclonal Antiserum (MAb) MAb Y80 MAb Y81 MAb Y94	191	MAb 191
Monoclonal Antiserum (MAb) MAb Y80 MAb Y81 MAb Y94	212	MAb191

MAbs recoded from 1st year report

- MAb Y80 recoded from 220/1.D1.C2.E11
- MAb Y81 recoded from 220/2.G2.B7.D5
- MAb Y94 recoded from 220/3.F10

4.1.1.3 Membranes and buffers used

4.1.1.4 Results

Clear control lines were observed when the dark leaf spot polyclonal antiserum (PAb) was conjugated to the latex spheres. However for each of the monoclonal antibody (MAb) combinations poor control lines and / or no control lines were observed. For each antibody

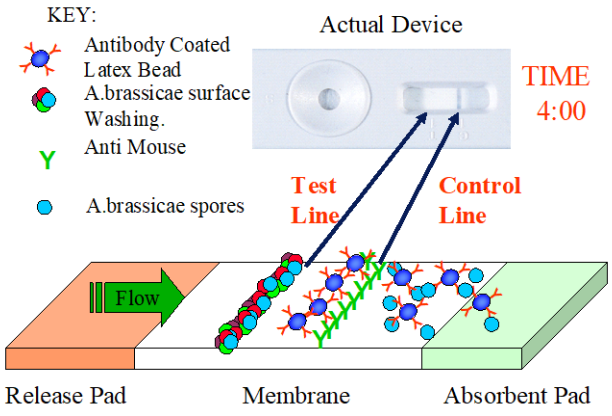
combination when mixed with an *A. brassicae* (dark leaf spot) positive sample no test line was observed.

4.1.1.5 Conclusions

The double antibody sandwich lateral flow test format did not work using the range of specific antibodies available. With the range of antibodies tested it was unclear why the format did not work. However all monoclonal antibodies tested in this format were of IgM class type. IgM's are generally of low affinity and would require longer contact times with the target sample (dark leaf spot conidia) for successful binding to occur during travel over the test line. This may contribute to the absence of test line development. Some weak control lines were observed however it is likely that an IgG antibody class type was applied at the control line and, with enhanced affinity for its target, the formation of control line may be more readily visible. Clear control lines were observed when polyclonal antibodies (raised in rabbit) were employed within the assay format. However, as a result of the meticulous optimisation processes involved the use of latex conjugated polyclonal antibodies, a DAS format is generally avoided, particularly where the donor species is rabbit.

4.1.2 Competitive assay test format and procedure

Interpretation of results using a competitive lateral flow test format is the opposite to that expressed using the DAS lateral flow format. The absence of a test line represents a positive result (Figure 4). As in the DAS format a control line is observed to demonstrate successful test operation.



- Negative sample = Observation of the test line and the control line
- Positive sample = Observation of the control line. No test line formation

Figure 4. Schematic drawing of a competitive lateral flow device: Positive result shown.

Test operation is as described for the DAS lateral flow format. The sample extract is applied to the release pad, facilitating the 'immobilised' specific antibody bound latex particles to flow across into the nitrocellulose membrane. As the sample extract and the specific antibody bound latex particles flow laterally though the membrane there is potential for binding between target antigen (if present within the test sample) and specific latex labelled antibody. The formation of a target antigen / latex conjugated antibody complex this time inhibits capture at the test line. With sufficient target antigen present (*Alternaria* conidia) complete inhibition occurs and no test line is observed. In a negative sample (target *Alternaria* antigen absent) the antibody conjugated latex particles remain unbound and are captured at the test line to produce a visible line of deposited latex. In either situation, excess antibody conjugated latex spheres will become immobilised at the control line. The control line is composed of an anti-species antibody which will react with the latex/antibody complex flowing from the sample pad. A clear visible blue control band will form showing that the test has been completed satisfactorily.

4.1.2.1 Membranes and buffers used

4.1.2.2 Assessment of *A. brassicae* specific antibodies for inclusion within a competitive lateral flow device

To determine the applicability of the competitive lateral flow format for the detection of conidial material of *Alternaria brassicae* selected antisera were tested within the competitive assay format using a range of membrane types (Table 3). *Alternaria brassicae* specific antibodies were conjugated with spherical latex beads. Where a monoclonal antiserum (raised in mice) was used to detect the conidial material of *A. brassicae* anti-mouse capture antibodies were sprayed on to the control line of the lateral flow device. Lateral flow devices employing polyclonal antibodies to detect the conidial material of *A. brassicae* received an anti-rabbit serum as the control line. For all tests mycelial soluble washings of *A. brassicae* were applied to the test line of each lateral flow device.

Table 3. Assessment of antibodies for inclusion within a competitive lateral flow test format: test line development

Latex labelled detector antiserum	Membrane type 1	2	3	4	5	6
Y80N	-	-	tl+	tl+	-	tl+
Y80N	-	-	-	-	-	-
Y81N	tl+	tl+	tl+	tl+	tl+	tl+
Y81N	-	-	-	-	-	-
Y94N	-	-	-	-	-	-
Y94N	-	-	-	-	-	-
EMA 212 (12.16mg ml^{-1})	TL+	TL+	TL+	TL+	TL+	TL+
EMA 212 (1g ml^{-1})	-	-	-	-	-	-
Polyclonal Ab	-	-	-	-	-	-

-	No test line development
tl+	Weak test line development
TL+	Clear test line development denoting *Alternaria* negative sample

4.1.2.3 Results

Employing a range of membrane types only the HRI EMA 212 antibody worked satisfactorily within this competitive lateral flow format producing a blue test line (negative result) when probed with *Alternaria brassicae* negative samples (Table 3). Antiserum MAb Y80 (recoded from 220/1.D1.C2.E11) and antibody MAb Y81 (recoded from 220/2.G2.B7.D5) demonstrated some potential for use with weak test line development using a number of membrane types.

With clear test line development an optimised lateral flow test was produced employing HRI EMA 212. Employing test samples of conidial material of *Alternaria brassicae* single control bands were readily observed, denoting dark leaf spot spore presence (positive test samples).

4.1.2.4 Conclusions

The results demonstrated that the competitive lateral flow format using a range of specific antibodies conjugated to latex was specific enough to detect dark leaf spot positive samples. The competitive lateral flow format using EMA 212 conjugated to latex will be tested further for its sensitivity and specificity.

4.1.3 Competitive Lateral Flow Assay – *Alternaria brassicae* detection threshold

4.1.3.1 Method

Isolates of *A. brassicae* (Table 1), taken from the HRI culture collection, were grown on a vegetable juice agar (V8) for one week. A 5cm cube of mycelium was removed, homogenised in 5 ml of sterile distilled water and transferred in 500µl aliquots to 10 x 5ml sterile clarified V8 juice medium. This process was repeated for each isolate. To induce conidial production the V8 mycelial suspensions were agitated prior to incubation at 25º C. At a magnification of x 100 conidial production was noted seven days later. Conidial washings (includes mycelial fragments) of *A. brassicae* were harvested, collected and mixed in a lateral flow Type C extraction buffer ($1x10^5$ *A. brassicae* conidia per ml). A serial doubling dilution series was made to provide 60µl aliquots of conidial/mycelial material of *A. brassicae* ranging from 6000 to 12 dark leaf spot conidia present. Each 60µl conidial suspension was applied to a separate lateral flow device (prototype BO2) and, following the 4 minute assay processing time the development of Test and Control lines were recorded using a Biodot Quadscan device. A negative control of extraction buffer alone was included within the test format

4.1.3.2 Results

The results (Table 4) show that the competitive lateral flow device had the ability to detect dark leaf spot conidia to approximately 47 - 23 conidia per sample. Weak test lines were observed when samples containing 47 conidia per sample were applied to the device. A clear test line was observed in samples containing 23 conidia. A clear control line was observed in all tests showing that the device had operated successfully. Samples contained some mycelial fragments in addition to the number of whole dark leaf spot conidia (taken from *Alternaria* plate washings). The lateral flow devices tested with varying numbers of conidia per sample are shown in Plates 1 - 3. No test lines are observed in plates 1 and 2 which show tests with 6000 - 187 dark leaf spot conidia per sample. Clear test lines were observed in samples

containing 23 - 0 dark leaf spot conidia (Plate 3). Weak test lines were observed in lateral flow devices tested with samples containing 47 dark leaf spot conidia (Plate 3). Results were confirmed when a lateral flow reader (Biodot Quadscan) was used to detect and quantify the test lines in comparison to the control lines in each device (Figure 5). Only those samples containing 23 - 0 dark leaf spot conidia per sample gave significant test line measurements on the reader device.

Table 4. Detection threshold for *Alternaria brassicae* using a competitive lateral flow assay

Total no. *A.brassicae* Spores applied to lateral flow device *(+ mycelial fragments)*	**Test line**	**Control line**	***A.brassicae* detected**
6000	-	+	++
3000	-	+	++
1500	-	+	++
750	-	+	++
375	-	+	++
187	-	+	++
94	-	+	++
47	+	+	+
23	+	+	-
12	+	+	-
0	+	+	-

+	Clear line development
+	Weak test line development
-	No test line development

Plate 1 Lateral flow tests for dark leaf spot conidia (6000 - 1500 conidia per sample)

Plate 2 Lateral flow tests for dark leaf spot conidia (750 - 187 conidia per sample)

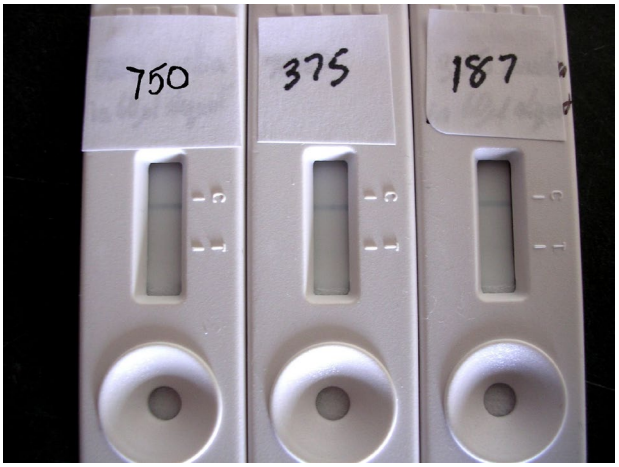
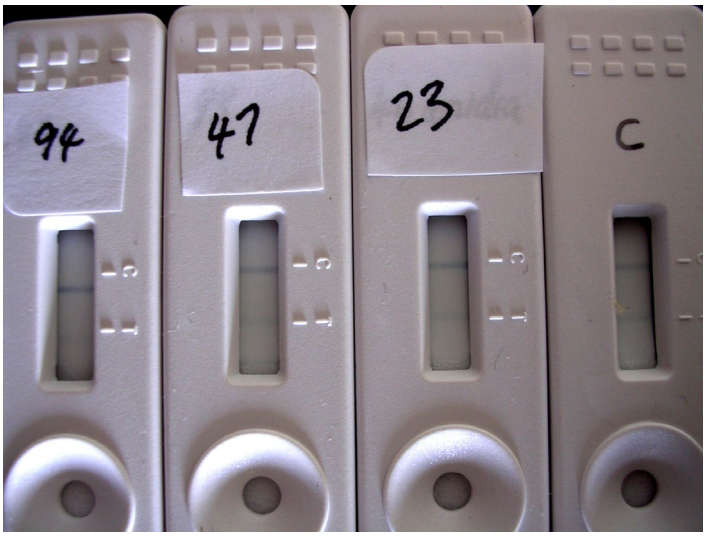


Plate 3 Lateral flow tests for dark leaf spot conidia (94 - 23 conidia per sample + control)



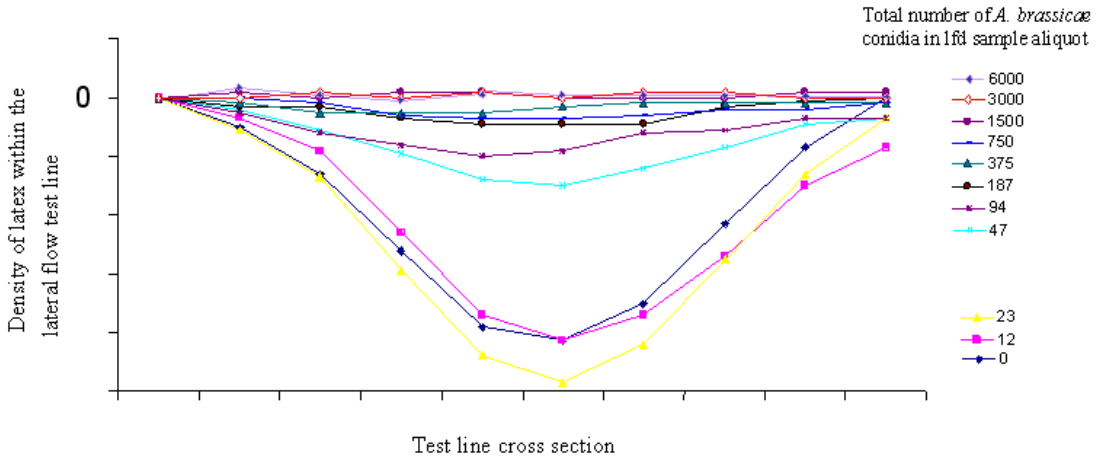


Figure 5. Detection threshold of *Alternaria brassicae* conidia employing lateral flow prototype

4.1.3.3 Conclusions

The competitive lateral flow assay was the best format for detecting and quantifying the presence of dark leaf spot conidia in samples. The devices were sensitive when low numbers of dark leaf spot conidia and mycelial fragments were present. The detection threshold of 47 - 23 conidia per sample may be below the threshold required for disease establishment within vegetable brassicae crops. Further field tests are required to investigate this possibility.

4.1.4 Assessment of reactivity of competitive lateral flow prototype B02 with other fungal spore types found in vegetable crops

4.1.4.1 Materials and Methods

A range of fungi, common in vegetable crops were grown on V8 media. Cultures were taken from the HRI fungal culture collection. Cultures were grown in a 20º C incubator in the dark and culture material was harvested after 3 weeks growth. Spore and mycelial fragments of a range of fungal species were collected from sterile culture in lateral flow Type C extraction buffer (provided by CSL) and, spore concentrations were determined (Table 5). Each of the fungal preparations were then agitated, using a Gallenkamp Spinmix, for a period of 3 minutes at high speed. A 60µl aliquot of the each spore suspension was then applied to the sample pad of individual competitive lateral flow devices (prototype BO2).

Table 5. Fungal species tested for reactivity within competitive lateral flow format (prototype BO2.)

Fungal species tested	No. conidia ml^{-1} extraction buffer
Phoma betae	2.7×10^6
Penicillium sp.	1.2×10^6
Fusarium culmorum	4.5×10^5
Ascochyta rabei	9.1×10^5
Aureobasidium pululans	1.3×10^5
Botrytis cinerea	3.8×10^5
Botrytis allii	2.2×10^5
Stemphyllium sp.	3.2×10^3
Alternaria brassicae	1.0×10^5
Paecilomyces variotii	1.3×10^6
Botrytis squamosa	1.5×10^5

4.1.4.2 Results

The competitive lateral flow format gave negative tests when samples containing different fungi (Table 5) were tested. Clear test lines were observed with all cultures tested. Clear control lines were observed indicating that the flow devices had functioned normally. Samples containing dark leaf spot conidia (*Alternaria brassicae*) gave positive readings (no test line observed). Results were confirmed when a lateral flow reader (Quadscan) was used to detect and quantify the test lines in comparison to the control lines in each device (Figure

6). Only those samples without dark leaf spot conidia gave significant test line measurements on the reader device (Figure 6).

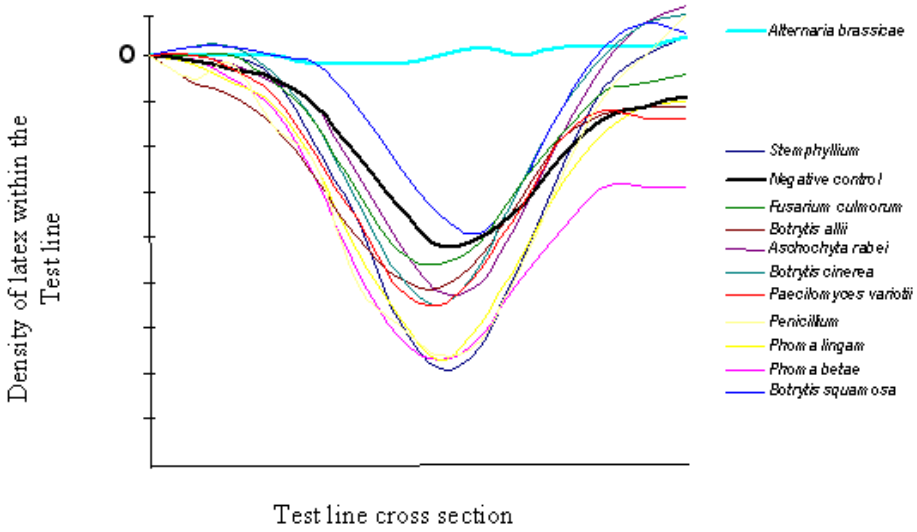


Figure 6. Reactivity of lateral flow prototype (BO2): Density of immobilised latex particles monitored on the test line of exposed lateral flow strips.

4.1.4.3 Conclusions

The results of cross-reactivity test although not fully comprehensive confirmed the specificity of the lateral flow device to dark leaf spot conidia. Further tests are required using *Alternaria alternata* (a common non-pathogenic field fungal contaminant). However previous tests using other immunoassay formats using the EMA 212 antibody (which is used in the competitive lateral flow device) indicated that these antibodies were only weakly cross reactive to *A. alternate* and *A. dauci*. Further evaluation of the dark leaf spot lateral flow device is required under field conditions in the presence of other fungal and non fungal contaminants (notably pollen).

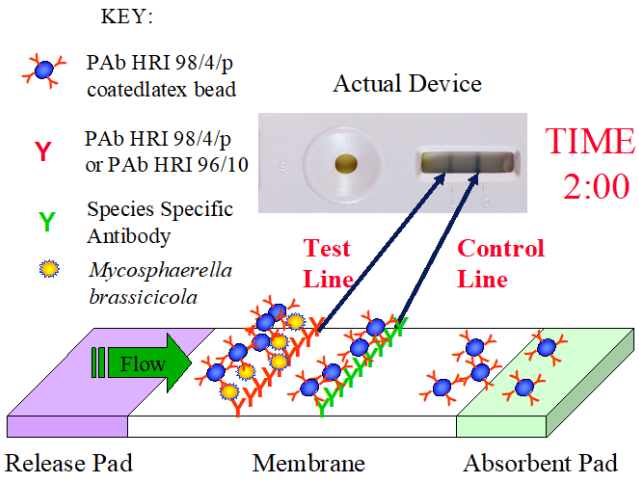
4.2 Development and optimisation of lateral flow device for detection of ascospores of ringspot

A number of lateral flow test formats were investigated for *Mycosphaerella brassicicola* detection. In year two the double antibody sandwich test format was investigated using available antibodies.

4.2.1 Double Antibody Sandwich (DAS) test format

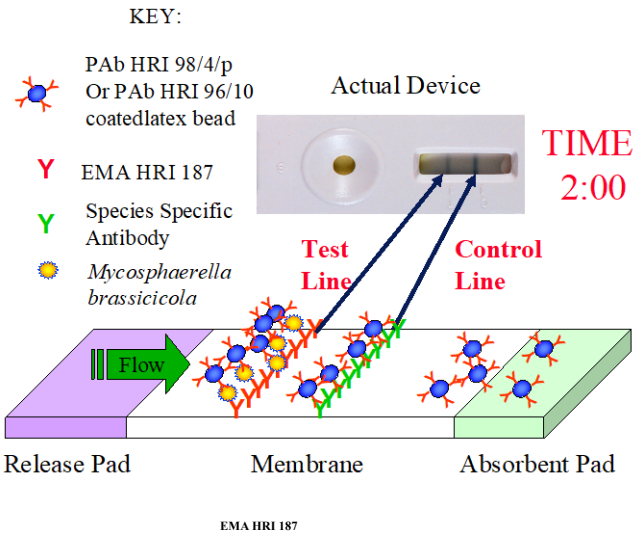
4.2.1.1 Capture and detector antibodies for inclusion within a DAS lateral flow device

To determine the applicability of the DAS lateral flow format, for the detection of ascosporic material of *Mycosphaerella brassicicola,* a range of antibody combinations (produced at Warwick HRI) were examined (Figures 7a,b). A species specific anti-antibody was used at the control line in each format. Two types of format were tested. In the first format type A, polyclonal antibodies (either Pab HRI 98/4 or Pab HRI 96/10) were placed at the test line (Figure 7a). In the second format type B monoclonal antibodies (EMA HRI 187) were placed on the test line (Figure 7 b). For each format type polyclonal antibodies of sera types Pab HRI 98/4 or Pab HRI 96/10 were conjugated to latex and held within the sample release pad to capture the ringspot material in the sample. As a positive control mycelial fragments of the ringspot pathogen (*M. brassicicola*) were suspended in Buffer C. As a negative control Buffer C alone was used.



- Negative sample = Observation of the test line
- Positive sample = Observation of the test and control line.

Figure 7a. DAS lateral flow prototype A



- Negative sample = Observation of the test line
- Positive sample = Observation of the test and control line.

Figure 7b. DAS lateral flow prototype B

4.2.1.2 Results

Clear control lines for each of the lateral flow formats were observed indicating that the test had functioned satisfactorily. However, for each double antibody sandwich lateral flow format tested no test lines were produced when a positive sample of *M. brassicicola* was used.

4.2.1.3 Conclusions

The double antibody sandwich lateral flow test (DAS lateral flow device) in the format used did not work employing the range and combination of specific antibodies tested. However as a result of the critical optimisation processes involved with the use of latex conjugated polyclonal antibodies within a DAS lateral flow device this assay format is generally avoided, particularly where the donor species is rabbit. However it would be useful to assess the DAS lateral flow device format employing EMA 187 conjugated to the latex spheres and, employing each of the PAbs at the test line. Studies employing a range of membrane types and buffers may warrant further investigation. The DAS lateral flow device test may however prove inappropriate for assay development for the detection of ringspot with the antibody combinations available. This may result from the characteristics of the target antigen (*M. brassicicola*) and epitope binding sites available for antibody binding.

4.2.2 Competitive assay test format and procedure

Visualisation of results using a competitive lateral flow test format is the opposite to that expressed using the DAS lateral flow format. The absence of a test line represents a positive result. As in the DAS lateral flow format a control line is observed to demonstrate successful test operation.

4.2.2.1 Assessment of *M. brassicicola* specific antibodies for inclusion within a competitive lateral flow device

To determine the applicability of the competitive lateral flow format for the detection of inoculum of *Mycophaerella brassicicola* selected polyclonal antisera (PAbs HRI 98/4/P or 96/10) were conjugated to latex spheres and tested within a competitive assay format (Fig 8). Mycelial fragments of a culture of *Mycosphaerella brassicicola* were applied to the test line. An anti-rabbit species specific antiserum was applied to the control line. As a positive control material of the ringspot pathogen (*M. brassicicola*) was suspended in Buffer C and applied to the sample pad. As a negative control, Buffer C alone was used.

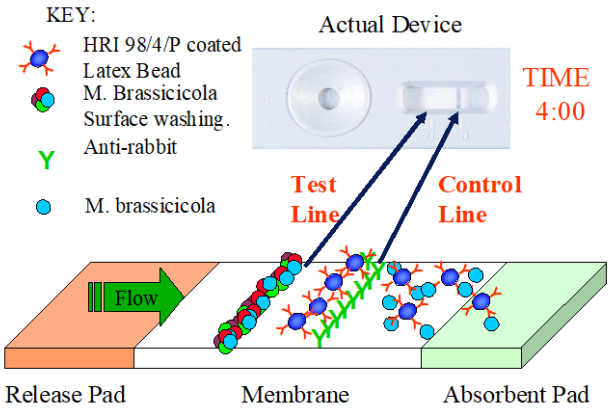


Figure 8. Competitive lateral flow format showing PAb HRI 98/4/p conjugated to latex beads and *M. brassicicola* mycelial washings sprayed at the test line.

4.2.2.2 Results

The competitive lateral flow test in the format used did not work employing the range and combination of antibodies tested. No test lines were formed when ringspot material was absent from the sample (negative result). Control lines were observed showing that the latex

conjugated antibodies were able to move freely through the membrane and conditions were appropriate for antibody binding.

4.2.2.3 Conclusions

A test line should have resulted when no ringspot material was present in the sample (ascospores of *M. brassicicola*). However the results show that the latex conjugated polyclonal antibodies are unable to bind to the components of the test line. This was also observed in the development of a competitive assay format for *Alternaria brassicae*. Reports have previously shown that the use of polyclonal antibodies within lateral flow formats can be problematical. Given the success of the dark leaf spot competitive prototype (employing a monoclonal antiserum conjugated to latex spheres) it would be appropriate to investigate the use of EMA 187 and other antibodies recently available within a competitive assay format. This work will be carried out in year three of the project.

5.0 EVALUATION OF LATERAL FLOW PROTOYPE (A01, BO2) FOR MONITORING AIRBORNE CONIDIA OF *ALTERNARIA BRASSICAE* WITHIN MIXED AIR SPORA SAMPLES

5.1 Materials and Methods

5.1.1 Monitoring airborne inoculum of *Alternaria brassice* (dark leaf spot) in a controlled environment

5.1.1.1 Collection of airborne spora

Decaying brassica leaf material, heavily contaminated with dark leaf spot (*Alternaria brassicae and A. brassicicola*), and other fungal organisms (ringspot, *Alternaria alternata*, *Phoma* sp. *Botrytis cinerea*, *Stemphyllium* sp. *Penicillium*), was collected from a field plot of Brussels sprouts (cv. Golfer) at Warwick HRI. The leaf material was placed in a misting hood for 48 hrs at 97% humidity. The material was dried off and examined using a binocular microscope for the presence of sporulating material of *Alternaria brassicae*.

After conidial production was observed the infected leaf material was placed in a controlled environment cabinet operating at 80% r.h. with continuous light. Over a 24 hr sampling period discharged air spora were collected by impaction into the eppindorf sample vessel of a Burkard cyclone sampler. After which the eppindorf was removed, sealed and stored at -20ºC. This process was repeated for a further 10 sampling periods.

5.1.1.2 Detection and quantification of collected spore samples

Approximately 200µl of Buffer C was added to each of the collected eppindorfs. The eppindorfs were agitated using a Gallenkamp Spinmix for 3 minutes at high speed. Removing a small volume of this material a range of spore counts were made (Table 6) by bright field microscopy (x 1600) using a haemocytometer. The numbers of *A*. *alternata* and *Stemphyllium* spores were also counted in each sample after which a 60µl aliquot of each spore suspension was applied to the sample pad of an individual competitive lateral flow device (prototype BO2). Determination of test line development was made by visual assessment and using a Biodot Quadscan.

5.1.1.3 Results

Significant quantities of dark leaf spot spores were collected within each sample tube as shown in Table 6. Both *Stemphyllium* and *Alternaria alternata* spores were also present in significant levels. There were three samples where dark leaf spot spores were found at higher levels. Dark leaf spot spores were accurately quantified in samples using a lateral flow device where they occurred at higher levels (greater than approximately 1000 spores per sample). In samples

where dark leaf spot spores were not present or occurred in low levels the lateral flow device gave a negative result.

Table 6.

No spores per 60µl aliquot Buffer C

Sample No.	*A. brassicae* / *A. brassicicola*	*A. alternata*	*Stemphyllium*	LFD Detection *A.brassicae*
1	-	-	-	✗
2	-	-	-	✗
3	-	-	-	✗
4	$3.8x10^3$	$1.4 x 10^7$	$3.8x10^3$	☑
5	$9.6x10^2$	$4.0 x10^3$	$4.2x 10^3$	✗
6	$1.9x10^3$	$8.4 x10^3$	$1.9x10^3$	✓
7	-	-	-	✗
8	$4.8x10^2$	-	-	✗
9	$4.8x10^2$	-	-	✗
10	$9.0x10^2$	$1.9x10^3$	$4.8x10^2$	✗
11	$6x10^3$	$8.4x10^3$	$1.9 x10^3$	✓

5.1.1.4 Conclusion

The lateral flow device did not detect very low levels of dark leaf spot. This may relate to the use of plate washings in the original sensitivity experiments with dilutions of dark leaf spot conidia (see section 4.3). In these studies, a mixture of mycelium and dark leaf spot conidia of *A. brassicae* were used. Where mycelial fragments are present the test is likely to become more sensitive. There was no germination of conidia in samples from controlled environments (samples frozen directly following exposure). The activity of the antibody (HRI EMA 212 reacts more sensitively with germinating spores) also confirms this likelihood. If appropriate, assay sensitivity may be improved by further test optimisation. A slower lateral flow run time may help achieve this. Using a different membrane type and / or buffer adjustment the speed of flow could be reduced. By lowering the amount of surfactant in the buffer (Tween 20) the flow rate of the lateral flow device would also be reduced potentially improving the sensitivity of the test. Determining the optimal conjugated antibody latex concentration in relation to test sample and test line development should be determined

5.2 Monitoring airborne inoculum of the dark leaf spot pathogens (*A. brassicae*/*A.brassicicola*) in inoculated overwintered Brassica crops

5.2.1 Materials and Methods

5.2.1.1 Monitoring dark leaf spot in air samples in relation to plant infection

An over-wintered, heavily infected (dark leaf spot, ringspot and white blister) field plot (20m x 10m) of Brussels sprouts (c.v. Golfer), was monitored continuously over a period of 3 weeks for the presence of dark leaf spot spores in the air using a Burkard cyclone samper and microtiter immuno-spore trap (MTIST). For each sampling period (one day or three days) the eppindorf sample collection vessel of the cyclone spore trap and, the microtiter strips (4x8 wells) of the MTIST spore trap were removed. The collection vessels of both spore traps were stored prior to assay development at -20ºC.

For each of the sampling periods six *B. oleracea* bait plants (Brussel sprouts c.v. Golfer, 10 true leaves), which had been grown in the absence of disease, were positioned adjacent to the spore traps. After each sampling period, the plants were removed from the field, and placed in an environment of 100% humidity for 48 hrs. This fulfilled the environmental requirements for infection by dark leaf spot. The plants were then removed, dried and retained in a glasshouse, at a temperature of 12 - 14ºC for 21 days. Plants were visually examined for expression of disease and confirmatory isolations (for dark leaf spot lesions) made on to sprout leaf decoction agar (Kennedy *et al*., 1999).

5.2.1.2 Detection and quantification of collected spore samples using lateral flow devices

Approximately 200µl of extraction buffer C was added to each of the collected eppindorf vessels (cyclone spore sampler) and, using a Gallenkamp Spinmix, agitated for a period of 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 (prototypes B01, BO2 used). Determination of test line development was made by visual assessment and, using a Biodot Quadscan. Each device was scanned on two occasions using the Quadscan reader.

5.2.1.3 Immunoassay process

Enumeration of trapped *Alternaria* spp on the base of the microtiter wells was determined using an inverted binocular microscope (x 200) by bright field illumination. Exposed MTIST microtiter wells were also assessed for dark leaf spot spores by using plate-trapped antigen ELISA employing antibody HRI EMA 212. The samples were incubated overnight in an enclosed chamber at 18ºC. After which unbound material was removed and the microtitre wells were washed once with 200 µl PBS Tinc (PBS mixed with 0.05% Tincture of Merthiolate 1 mg ml $^{-1}$ thimerosal, 1 mg ml $^{-1}$ pararosanoline in ethanol) per well. The

microtiter wells were blocked with 200 µl of 1 % casein buffer (1 % (w/v) casein PBS) and incubated at 37 º C for 45 min. Residual blocking buffer was removed and wells were washed four times for one min each with 200 µl PBS Tinc 0.05 % Tween 20 (PBSTincTw). Following this procedure wells received 100 µl per well of MAb HRI EMA 212 (diluted 1:20 PBST TincTw). Following incubation (as above) wells were washed three times for one min each with 200 µl PBSTincTw. After which a DAKO duet amplification system was used (catalogue no. K0492, DAKO Ltd, Angel Drive, Ely, Cambridge, UK) to amplify the signal generated by the bound antibodies of HRI EMA 212. Wells were washed as described above and to each well 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_2S0_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).

5.2.2 Results

5.2.2.1 Detection of dark leaf spot conidia in air samples using lateral flow devices

The results of using lateral flow device (prototype B01) are shown in Figure 9 (Quadscan reading for each sampling period) and Table 7 (visual assessment of the presence or absence of a test line). The Quadscan device gives an optical reading of the amount of captured latex spheres relative to the control line. Current results indicate that values of greater than 2.0 indicate the presence of a test line. The results show that dark leaf spot spores were detected in samples using the lateral flow device on only a few sampling periods in the field. Dark leaf spot conidia were detected using the lateral flow device in air samples collected on the 20 - 22 March 2004, 2- 3 April 2004, 4- 5 April 2004 and the 5 - 6 April 2004 (Figure 9). Low Quadscan values were obtained using lateral flow devices tested with material from vials containing particulates from air samples corresponding to these sampling period.

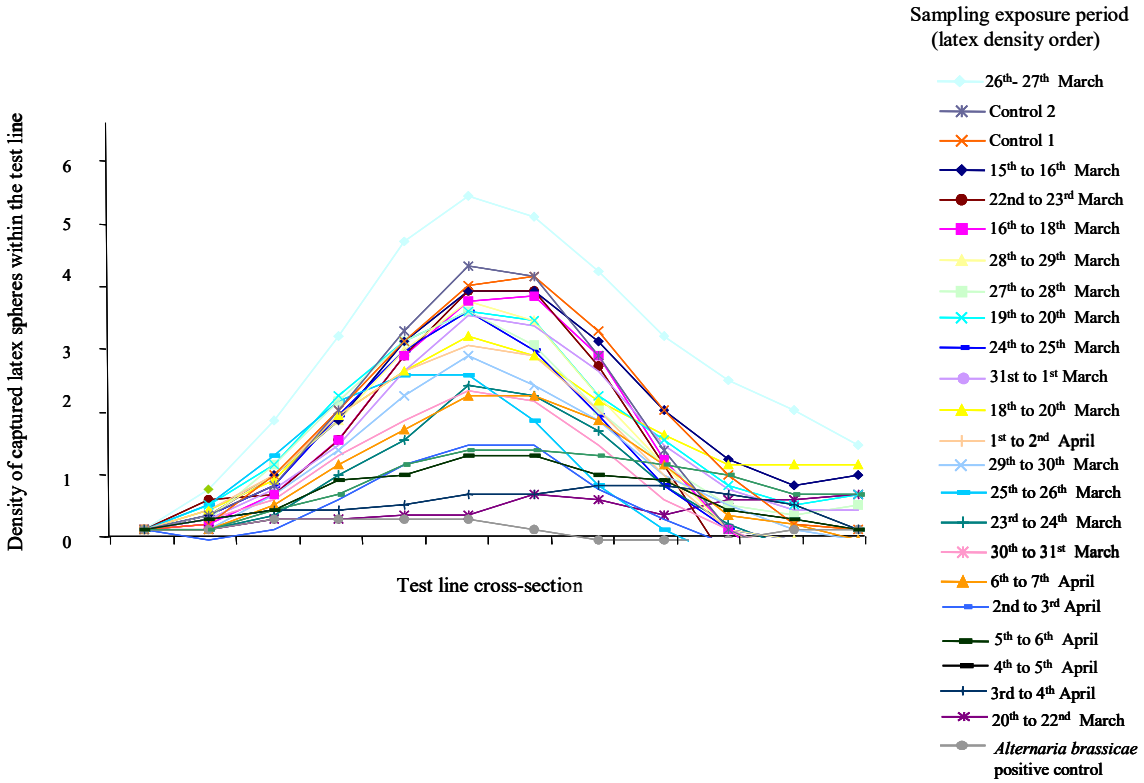


Figure 9. Quadscan readings of lateral flow test lines relative to control lines for dark leaf spot sampling periods

There was a relationship between the maximum value, obtained from each lateral flow device, (as measured by the Quadscan) and the number of dark leaf spot lesions observed on 6 trap plants (Figure 10). Trap plants were exposed at the same site over the same air sampling time period. Trap plants were given a 48 h wet period following exposure to ensure that all viable conidia of dark leaf spot or other fungal pathogens infected the trap plants. In the competitive lateral flow assay high values indicate negative results for dark leaf spot numbers.

Table 7. Visual assessment of test line for sampling periods

Field exposure period	Test line observation
15th to 16th March	Yes
16th to 18th March	Yes
18th to 20th March	Yes
20th to 22nd March	No
22nd to 23rd March	Yes
23rd to 24th March	Yes
24th to 25th March	Yes
25th to 26th March	Yes
26th to 27th March	Yes
28th to 29th March	Yes
29th to 30th March	Yes
31st to 1st April	Yes
1st to 2nd April	Yes
2nd to 3rd April	No
3rd to 4th April	No
5th to 6th April	No
6th to 7th April	Yes

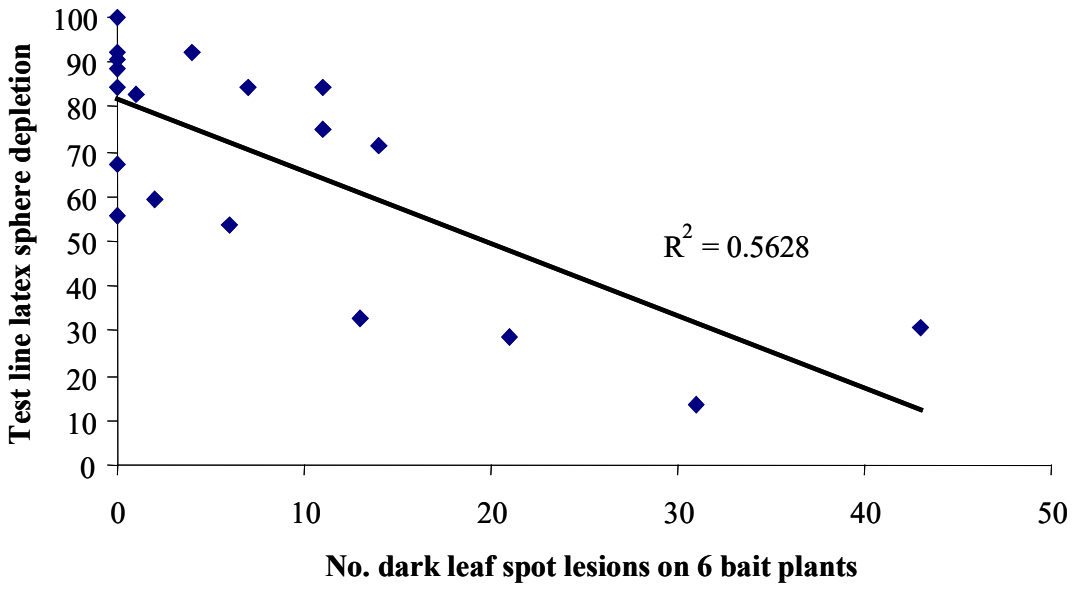


Figure 10. Maximum Quadscan readings for lateral flow test lines and corresponding number of dark leaf spot lesions on 6 trap plants.

5.2.2.2 Detection of dark leaf spot conidia in air samples using ELISA

Results of detecting of dark leaf spot conidia in air samples using ELISA is shown on Figure 11. The ELISA test indicated many days when dark leaf spot conidia were present in air samples. Significant numbers of dark leaf spot conidia were detected in air samples collected on the 15 - 18 March 2004 and continuously during the periods 20 March 2004 to the 27 March 2004, and the 1 April to the 7 April 2004 (Figure 11).

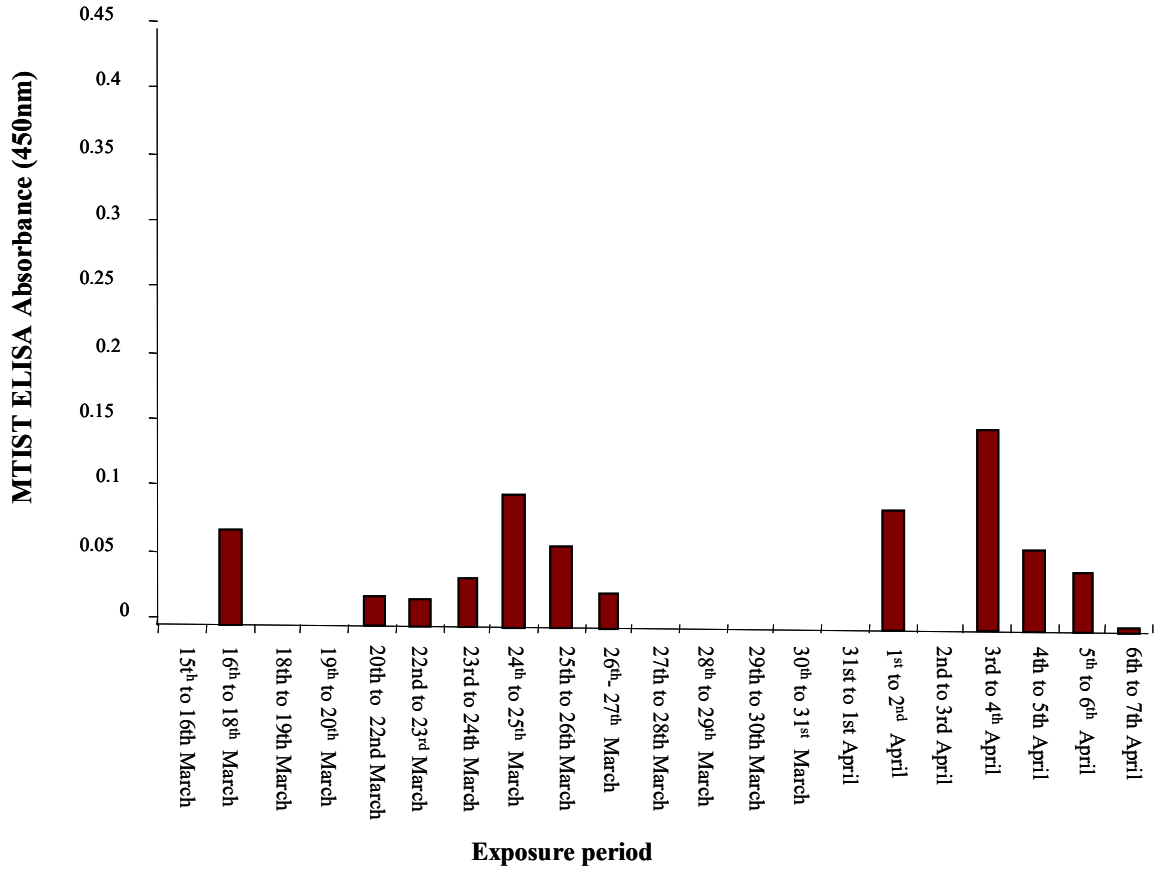


Figure 11. Detection of dark leaf spot conidia in air samples using ELISA

5.2.2.3 Number of dark leaf spot lesions on trap plants during air sampling periods

The numbers of dark leaf spot lesions on trap plants during each air sampling period is shown in Figure 12. Significant numbers of dark leaf spot lesions were observed on only 4 sampling periods. These were the 20 - 22 March 2004, 2-3 April 2004, 3-4 April 2004 and 5 - 6 April 2004 (Figure 12). Very small numbers of dark leaf spot lesions were observed on the 18 - 19 March 2004, 19 - 20 March 2004, 1 - 2 April 2004 and the 2 - 3 April 2004. It could not be confirmed if these were all dark leaf spot lesions.

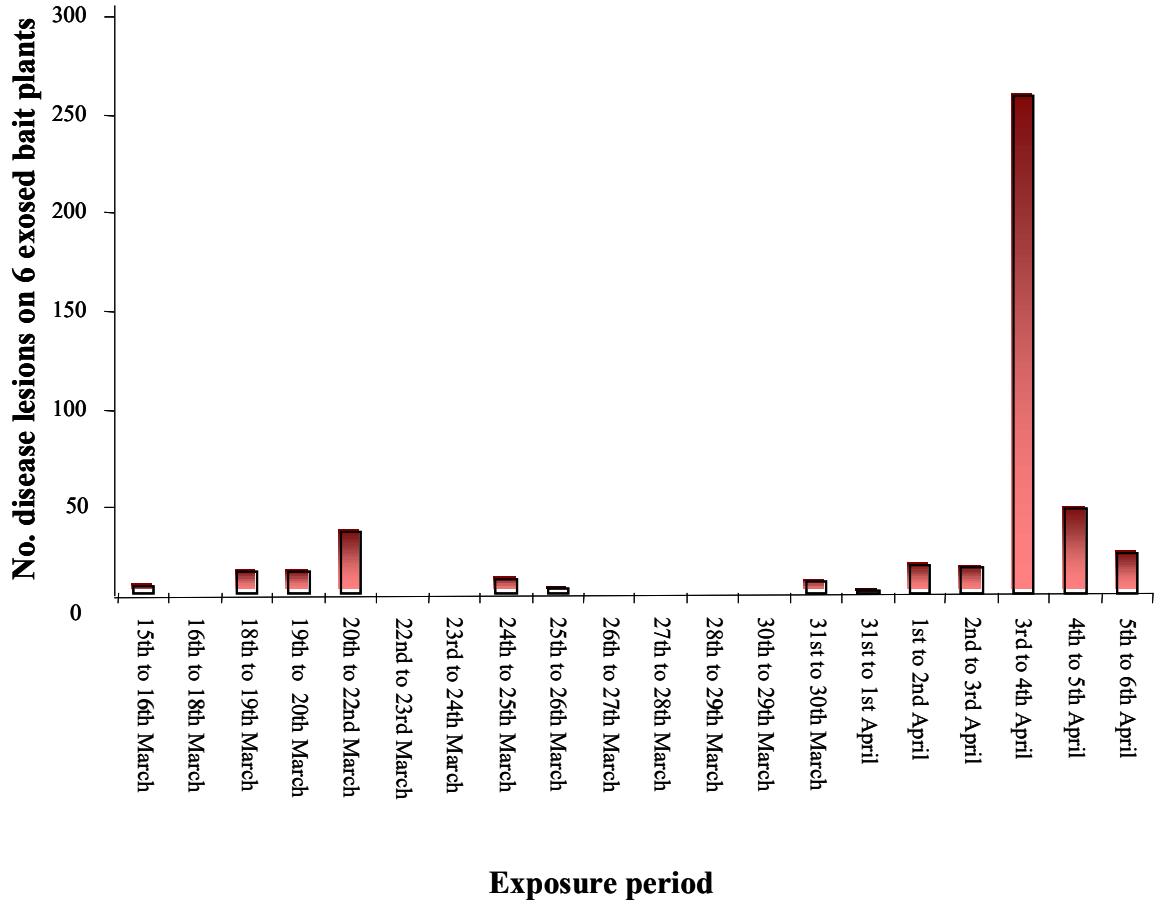


Figure 12. Number of dark leaf spot lesions on trap plants during air sampling periods (after 48 hrs of leaf wetness duration)

5.2.3 Conclusion

The results show that the dark leaf spot lateral flow test results for sampling periods corresponded more closely, to the numbers of dark leaf spot lesions on plants than results obtained using the conventional ELISA test. There were more false positive results observed using the ELISA test format than using the dark leaf spot lateral flow device. However it should be noted that the environmental conditions for infection by dark leaf spot were not fulfilled in the field during any air sampling periods tested.

6.0 DISCUSSION

6.1 Optimisation of lateral flow device for dark leaf spot conidia

The competitive format proved to be the most successful lateral flow format for detection of dark leaf spot conidia. Several formats were tested however lateral flow formats based on the movement of conidial material up the lateral flow membrane were not successful. This was because the pore size used in these tests was too small to allow the movement of relatively large conidial material. Dark leaf spot spores are particularly large however lateral flow formats for ringspot may not have this associated problem because the ascospores of ringspot are much smaller.

The competitive lateral flow assay for dark leaf spot proved very sensitive in its reaction to low numbers of dark leaf spot conidia in test samples. These tests were carried out on laboratory grown cultures of dark leaf spot. The lateral flow device when used on these samples could detect between 47 and 23 dark leaf spot conidia per sample. In this format the presence of a test line indicates the absence of dark leaf spot spores in a sample. These levels of dark leaf spot conidia in samples were extremely low in epidemiological terms.

6.2 Sensitivity and specificity of the competitive lateral flow device for dark leaf spot conidia under field conditions

The competitive lateral flow tests did not react to the presence of other fungal contaminants in the sample even when these were present in high levels. These cross-reactivity tests did not include other species of *Alternaria*. However, previous results using the EMA 212 antibody (which was used in the final lateral flow test format) has shown a low cross reactivity to other species of *Alternaria* when used in other immunoassay formats (ELISA). In this format it cross-reacts weakly with both *Alternaria dauci* (found on carrot) and *Alternaria alternata* (a common airborne fungal spore found mainly on dead tissues of plants). However it did not react with *Alternaria cheiranthi* (from wall flower) see section 3.2.2. Further tests are required on a range of *Alternaria sp*. to determine the full degree of cross-reactivity. The information would be important in determining potential errors which might occur under field conditions. For example, if the tests were used to determine the presence and predicted development of dark leaf spot in brassicas, but the location of the test site was adjacent to a crop of carrots heavily infected by *A. dauci* there would be potential for some error in the results. Additionally if the device was used in older crops where there was the presence of dead leaves with the presence of *Alternaria* sp. again the results maybe prone to some errors. However in both these occasions the test would give a false positive result which could trigger fungicide spray applications. In reality careful choice of trapping location and usage of disease forecast software which would indicate environmental risk

would eradicate these types of errors. False negative results (sprays not applied when a disease risk was present) are potentially a more problematical result for growers and end users. These result from errors in the reaction of the test itself. However, it is unlikely that these will cause problems when the test is used in the field because the presence of a control line on the test indicates when the test is faulty. Additionally the lateral flow test is sensitive enough to detect epidemiologically small numbers of conidia which, might be below the threshold for disease establishment although this point will require further tests carried out during year three of the project. The tests will also be used in conjunction with disease forecasts which will provide the background risk at each location.

6.3 Lateral flow test formats for detecting ascospores of ringspot

Several lateral flow formats have been tested for detection of ascospores of ringspot. The most successful format to date has been the competitive lateral flow format using antibody EMA 187. No new antibodies could be raised to ringspot (see section 3.1.1.2) using immunisations at CSL. A range of antibodies which are specific to soluble components of ringspot ascospores are however, available. These will be used within the competive lateral flow format to designate which antibody will be optimal for ringspot ascospore detection. These tests are ongoing at present, however it is unclear if a lateral flow detection device using these antibodies will be sensitive enough to detect low numbers of ringspot ascospores. During year three of the project it is hoped that these tests will be conducted on the finalised lateral flow format.

6.4 Practical usage of the dark leaf spot lateral flow test under field conditions

Usage of the test in the field would require environmental data from a weather station and an additional spore trap which, would be integrated with the weather station. At each location both the environmental data and the air-borne spore risk could be assessed (the latter using the lateral flow device). The spore sample would be collected within a vial in the trap. At each test period the vial would be replaced with a fresh one and the sample in the removed vial tested by adding from a dropper bottle the required amount of liquid buffer. The contents with vial with the added buffer would be shaken and a specific amount of buffer removed and placed on the sample pad of the dark leaf spot lateral flow device. The results of the test would be visible as lines on the lateral flow device. This would indicate the presence of dark leaf spot in the sample. By changing the format (run time) the sensitivity of the device can be adjusted however for initial field trials investigating the usage of the test the current sensitivity may be advantageous to establish a base line reaction. A reader device could be used to make the test semi-quantitative, however, this approach cannot be used in year three field trials but can be considered later. By using the existing sensitivity, the system has the advantage of detecting the very earliest possibility of disease transmission. The end

user would always be in this situation with regard to disease in his crop because of his usage of fungicides. It is hoped that the system will enhance the activity of protectant fungicides or even biological control agents.

6.5 Utilisation of disease forecasting criteria based on inoculum detection

Information on spore number could be used directly within crop protection programmes to rationalise fungicide application. Using these techniques the critical date for applying fungicide applications to the crop can be identified. With high sampling rates spore traps if positioned to reflect prevailing wind patterns could be used to designate to onset of disease risk in different areas and pinpoint specific transmission events affecting different crops and areas. This would be particularly useful early in the season as a method of preventing disease transfer between over wintered crops and freshly transplanted crops.

Other studies within the UK on *Pyrenopeziza brassicae* (the light leaf spot pathogen) have demonstrated that pathogenic inoculum builds up in the air before transmission is possible (unpublished data). By assessing air samples in Scotland where light leaf spot is problematical the real risk of light leaf spot transmission could be ascertained in Brussels sprouts crops. This was done using and ELISA format as no lateral flow device exists for detecting light leaf spot spores. Additionally spore trapping was able to indicate the likely source of pathogenic inoculum which will provide useful information for reducing disease occurrence within the system. This will help the brassica industry meet the current difficulties with declining numbers of active fungicides available for use on brassica crops and the costs of applying expensive eradicant fungicides.

7. REFERENCES

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