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Key workers:	Dr R Kennedy Ms A. Wakeham
Location of project:	Warwick HRI, University of Warwick, Wellesbourne, Warwick
Project co-ordinators:	Mr A. Richardson, Mr F Tyler
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1. GROWER SUMMARY

1.1 Headline

- Prototype detection systems (Lateral flow devices) for spores of dark leaf spot and ringspot been developed which can be used in the field to detect these diseases in air samples.
- Use of the dark leaf spot lateral flow device gave accurate predictions of disease development in crops before disease development was observed.
- This information will improve disease control (by informing spray decisions) in areas of intensive vegetable Brassica production where there are sprayed and unsprayed crops in the same locality.
- The test kits are going forward for wider scale use by industry during 2006.

1.2 Background and expected deliverables

A lateral flow format for detection of dark leaf spot conidia has been produced. The competitive lateral flow device is described in this report based on an immunogold antibody carrier system. 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 air 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. 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). The dark leaf spot lateral flow device has been produced at the appropriate sensitivity and mass produced by Bio-Diagnostics (www.bio-diagnostics.co.uk) for a series of trials conducted in commercial brassica crops 2005. A working lateral flow prototype has been produced in the laboratory which give a visualisation of the presence or absence of ascospores of the ringspot pathogen.

The expected deliverables from this project are:

- Detection and sampling systems for dark leaf spot and ringspot which will provide information on disease problems in crops before they are visible.
- Detection tests which can be used “in field” to determine the level of risk within localities where sprayed and unsprayed crops occur side by side.
- Less reliance on eradicant fungicide applications for dark leaf spot and ringspot control. More effective use of fungicides with protectant modes of activity which will improve the economics of production.

- Monoclonal antibodies which recognise conidia of dark leaf spot conidia and ringspot ascospores.

1.3 Summary of validation trials using the dark leaf spot lateral flow device in 2005

Tests were conducted using the dark leaf spot lateral flow device to monitor the number of dark leaf spot conidia in crops and compare these with dark leaf spot disease development in commercial crops of Brussels sprouts and cabbage. Trials were conducted in crops at two locations Skegness (T. A. Smith & Co, The Elms, Croft, Skegness, Lincs.) and Alphagrow (Hesketh Bank, Preston, Lancashire) and run in conjunction with grower/consultants. At Skegness a Brussels sprout crop was monitored and assessed for disease development. At Hesketh bank the trial was located in an over-wintered cabbage crop.

1.3.1 Results

Skegness 2005

The results of using dark leaf spot lateral flow device on air samples collected at Skegness (Biodiagnostics prototype) are shown in Table 1 and 2. The EVL one step reader device gives an optical reading of the amount of captured immunogold on the test and control line. However use of the reader enables the device to be used semi-quantitatively to determine the amount of dark leaf spot spores present in the sample. It was not possible to test all days where air samples were taken due to the restricted numbers of lateral flow devices available.

Table 1. Visual daily assessment of test line for sampling periods at Skegness in 2005

Field exposure period	Test line observation (Positive =No)
13th August	No
14th August	No
20th August	Yes
23th August	No
25th August	Yes
26th August	Yes
27th August	Yes
31st August	Yes
2nd September	Yes
6th September	Yes
7th September	Yes
10th September	Yes

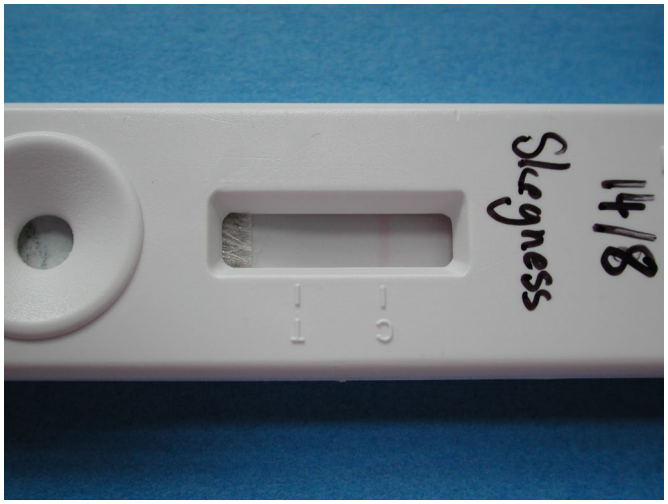
Table 2. Dark leaf spot conidial concentration per m^3 at Skegness in 2005

Field exposure period	Dark leaf spot conidia m^3	Immunogold Line Reading	
		Test	Control
13th August	31.9	0	0.7
14th August	37.0	0	1.0
20th August	18..5	1.1	1.8
23th August	32..8	0.6	1.3
25th August	6. 7	1.7	2.4
26th August	26.9	1.6	1.7
27th August	13.4	0.8	1.1
31st August	2.5	1.5	1.7
2nd September	2.5	1.1	1.2
6th September	NA	1.1	1.2
7th September	2.5	1.3	2.8
10th September	NA	1.1	2.2

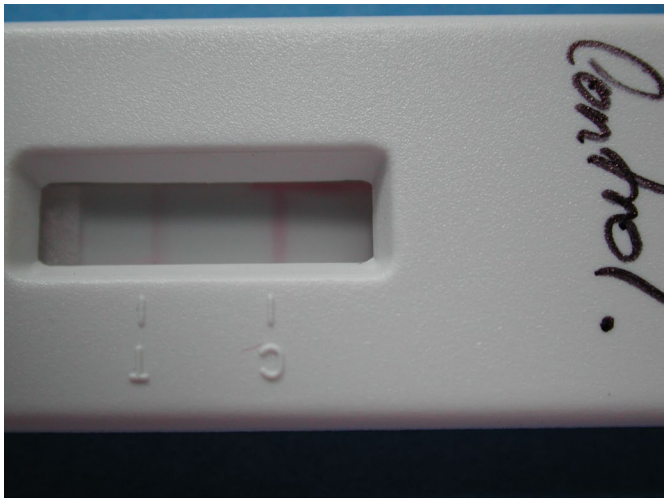
Using the dark leaf spot lateral flow prototype gave positive results using samples collected on the 13, 14, and 23 August 2005 (the absence of the test line). There was a close relationship between the amount of dark leaf spot conidia in each sample and the degree of accumulation of gold on the test line on each lateral flow test. The results for the 14 August 2005 are also shown in Plate 1 were there was no visualisation of the test line on the lateral flow device (a positive result).

Plate 1. Dark leaf spot lateral flow test Skegness 2005 (a) 14 August 2005 – positive (one line) (b) control test – negative (two lines)

(a)



(b)



The development of disease at the Skegness site is shown in Figure 2. Dark leaf spot lesions were already present in the Brussels sprouts crop at the beginning of the trial. There were two periods of disease development by dark leaf spot in the crop. These occurred after the 11 August 2005 and again after the 31 August 2005. There was one period of ringspot development in the crop which occurred after the 17 August 2005 (Figure 2). Both ringspot and dark leaf spot development on unsprayed plants at the Skegness site was high as approximately 90 - 100 lesions were recorded per plant by the beginning of September 2005.

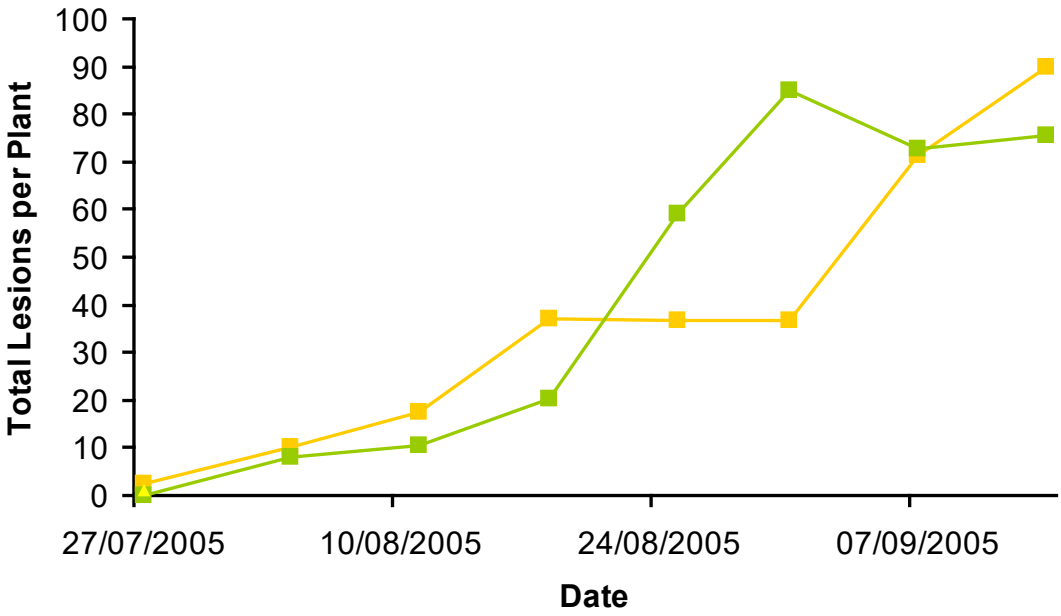


Figure 1. Ringspot (■) and dark leaf spot (■) disease development at Skegness in 2005.

Hesketh Bank 2005

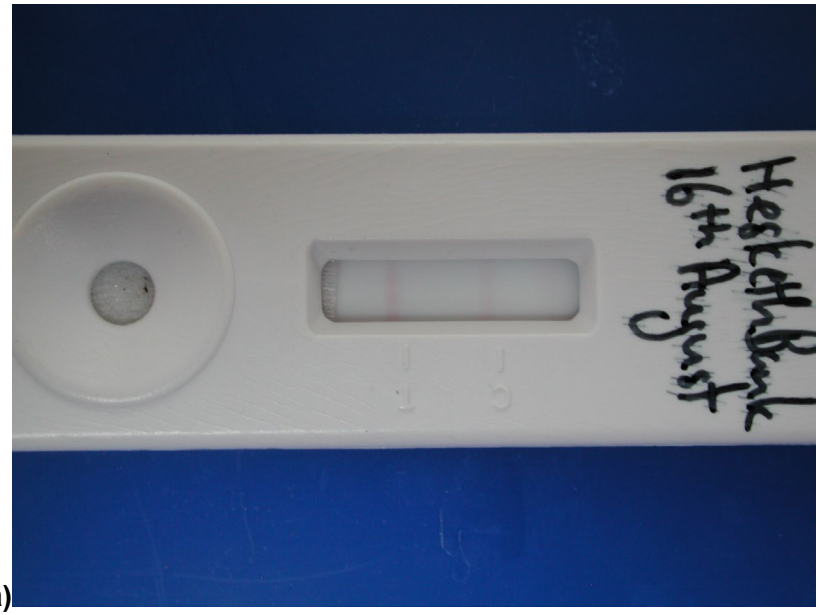
Results of testing air samples collected at Hesketh Bank (Biodiagnostics prototype) are shown in Table 3. The EVL one step reader device was used to give an optical reading of the amount of captured immunogold on the test and control line on the lateral flow device. The results from using the lateral flow reader matched the visualisations of the test line (see section 8.1.3.4). The dark leaf spot lateral flow prototype gave negative results using samples collected on all days tested (the presence of the test line, Table, 3). There was a close relationship between the amount of dark leaf spot conidia in each sample and the degree of accumulation of gold on the test line on each lateral flow test. The results for the 16 August 2005 and the 2 September 2005 are also shown in Plate 2. There was visualisation of the test line on the lateral flow device on both days indicating a negative result.

Table 3. Visual assessment of test line for sampling periods at Hesketh Bank in 2005 (daily samples)

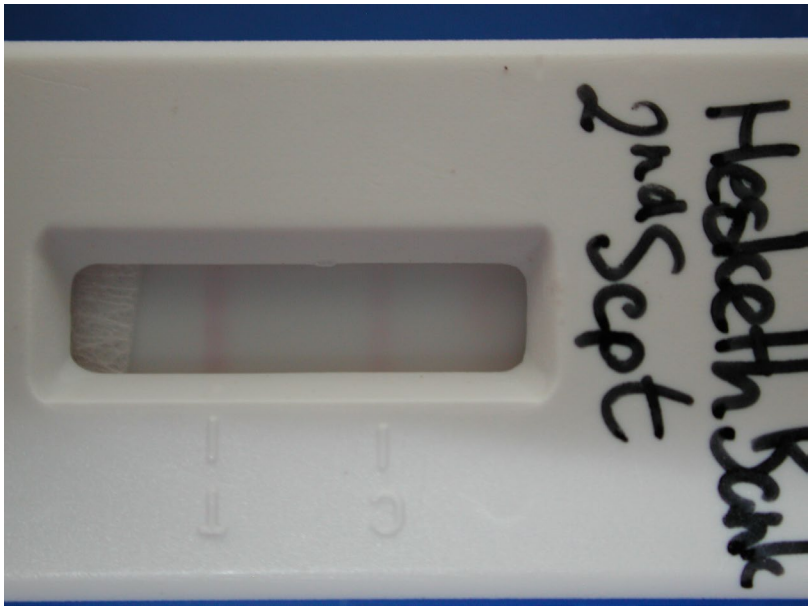
Field exposure period	Test line observation (Positive =No)
14th August	Yes
16th August	Yes
22td August	Yes
24th August	Yes
25th August	Yes
31st August	Yes
2nd September	Yes
9th September	Yes
10th September	Yes

Plate 2. Dark leaf spot lateral flow tests Hesketh Bank 2005 (a) 16 August 2005 – negative (b) 2 September 2005 – negative

(a)



(b)



Dark leaf spot lesions were already present in the cabbage crop at the beginning of the trial. There were low numbers of dark leaf spot lesions present in the trial site over the duration of the trial (approximately 1- 10 lesions per plant). Dark leaf spot and ringspot development occurred at a low level in the crop after the 8 September 2005. The results matched those obtained using the dark leaf spot lateral flow device.

1.3.2 Conclusions

Using the Biodiagnostics dark leaf spot lateral flow device positive results for dark leaf spot development were obtained at the Skegness trial site in 2005 before disease development was observed on the crop. The lateral flow device used at Hesketh Bank gave a negative result when tested on air samples collected from the crop. No disease development was recorded on the cabbage crop during the trial. Usage of the dark leaf spot lateral flow device gave accurate predictions of development of dark leaf spot at each site.

1.4 Lateral flow tests for detecting ascospores of ringspot

In year three of the project a working prototype lateral flow device for the ringspot pathogen has been developed using a competitive lateral flow format with antibody EMA 187. A working prototype which gave a visualisation in ascospore number at levels of between 50 and 100 was produced (which is almost the required level of sensitivity). With some further work this can be improved enabling the detection of ringspot ascospores in air samples at the required sensitivity for visualisation of reactions or over a concentration range using a lateral flow reader. The working ringspot prototype test requires further testing/development and production by Bio-Diagnostics.

1.5 Action points for growers

- Commercial quantities of test kits (for dark leaf spot) will be available for growers (from June 2006 onwards) to use in field tests to determine when disease development will occur in their crop.
- Prototype tests for ringspot will be available for use by growers from Sept 06.
- These tests and their accompanying instructions will be made available to growers. Growers should follow the communications on how to obtain these test kits.
- The system will be useable within localities to determine which areas are more disease prone at the start of the growing season.

1.6 Anticipated practical and financial benefit

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 rapidly obtain this data on the risk to crops due to pathogenic inoculum. 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.

- The usage of the “in field “ test for dark leaf spot and ringspot will improve the timing of the first application of fungicide for controlling these pathogens in vegetable brassica crops.
- There will be less need for and reliance on expensive eradicants which should reduce the costs associated with disease control in vegetable brassica crops.

SCIENCE SECTION

2. INTRODUCTION

2.1 Air borne disease problems affecting vegetable brassica crops

Many fungal pathogens occur on vegetable brassica crops and these can be 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 where the economics of spraying crops is variable. However there is ample opportunity for disease transmission from unsprayed cauliflower and broccoli crops on to long season crops such as Brussels sprouts. Forecasting disease outbreaks in vegetable brassica crops within intensive areas of production where unsprayed crops and sprayed crops are in the same vicinity is difficult. Additionally fungicides are often used to prevent disease establishment within crops. 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 ^{o}C, 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. Inoculum is in many instances 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, and early control of disease.

2.3 Using air-borne spore numbers within disease forecasting systems

It has been demonstrated that airborne inoculum plays a vital role in the development of epidemics caused by *Botrytis* leaf blight on onion crops (Carisse, 2005). Detection and quantification of airborne spore numbers can be used to predict disease 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. The importance of airborne inoculum has been recognised in the development of many diseases. Its use in practice has been limited because of the difficulties in quantifying it.

Detecting airborne spores of fungal plant pathogens is a useful tool in crop protection if this could be done rapidly and accurately. For example it has been reported that one or two peaks in sporangial concentration in the air of the potato blight pathogen *Phytophthora infestans* preceded the first observed symptoms of the disease in the field (Bugiani *et al*., 1998). These observations were validated in studies conducted by Philion (2003). In these studies the numbers of sprays applied to control potato blight could be successfully reduced without any impact on crop quality by monitoring the onset of thresholds of potato blight inoculum. 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. Given that potato blight is a difficult pathogen to control great scope exists in applying this approach in the control of other less aggressive pathogens and in different localities. This means that disease can be predicted accurately before it is visible in the crop. Similar results were obtained for *Botrytis* blight (*Botrytis squamosa*) on onion crops where thresholds of 15 - 20 conidia/m^3 could be used as a criteria for fungicide application which reduced applications by up to 100% (Carisse *et al*., 2003). 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). Similar results were obtained for the light leaf spot pathogen (*Pyrenopeziza brassicae*) on vegetable

brassicas in Scotland. In these studies (with the exception of 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. The use of air-borne spore numbers, as criteria, within forecasting systems is a new and exciting development in disease forecasting. One “in field test” which could be used in this respect is the lateral flow test for dark leaf spot and ringspot inoculum.

2.4 Immunological tests (Lateral Flow Devices)

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 by passive or covalent means 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.

The Competitive assay format

In a competitive assay format the test line comprises of homologous antigen (dark leaf spot and ringspot spore components) and the other, the control, is a line of anti-species antibodies. The release pad and membrane are assembled together with an absorbent pad into a plastic housing as illustrated below (Figure 2). 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 antigen (dark leaf spot and ringspot 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 an internal control line, providing a visible confirmation of antibody / particulate flow. Sufficient antigen target presence (dark leaf spot and ringspot spores), 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). Two lines of equal colour intensity indicate a negative result.

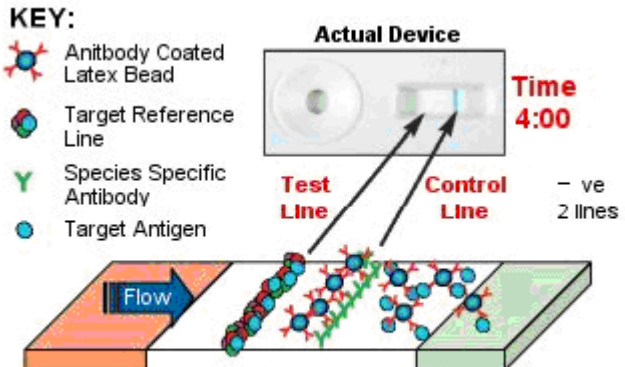


Figure 2. The Competitive lateral flow assay format.

The Non-competitive assay format

In a non-competitive assay format the test line generally comprises of an antibody complex which will bind, if present, to target antigen in the test sample. The control line will generally consist of an anti-species antibody, as in the competitive format, and bind material within the test flow to indicate successful test execution. The release pad and membrane are assembled as described above. The fluid test sample is added to the well, releasing the specific antibody bound coloured particles, which then begin to flow across the membrane. If the target antigen is present in the sample extract (dark leaf spot and ringspot), antibody binding will occur to produce a coloured particulate conjugated antibody-antigen complex. As this target complex passes over the test line capture of the antigen can occur, immobilising the antibody coated coloured particulates to produce a visible line of deposited coloured particulates at the test line. Excess coloured particulate material is captured at the control line, providing a visible confirmation of the success of the test. Two lines of equal intensity indicate a positive result.

Both assay formats 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. A number of readers are now available for use under field conditions. By introducing an internal control of coloured 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, making the test semi-quantifiable.

2.5 Epidemiological advantages of testing for inoculum in areas of intensive vegetable brassica production

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. The economics of production of many vegetable brassica crops vary as do the effect of disease on the marketability of those crops. There is ample opportunity for crop to crop spread of disease from unsprayed crops on to other crops where the presence of small amounts of disease adversely affects marketability (Brussels sprouts). 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 protectant fungicides.

2.6 Developing 'in field' tests for detecting the presence or absence of thresholds of dark leaf spot and ringspot inoculum

If accurate 'in field' tests for inoculum are to be constructed they will require specific antibodies that can differentiate between different types of pathogenic spores recognising only dark leaf spot and ringspot spores. Some specific antibodies have been raised and characterised for dark leaf spot in year one and two of this project. This report details the development of lateral flow prototypes which detect conidia of dark leaf spot and ascospores of ringspot. The use of developed lateral flow tests in the field is also reported. Cross-reaction of the test with spores of other pathogenic and non pathogenic species can be ascertained. Many of these pathogens survive on debris in the soil or are found on leaves of Brassica crops. Consequently tests which detect dark leaf spot and ringspot should not react with the conidia of other pathogens common in Brassica crops. The level of reactivity of the antibody to dark leaf spot conidia is also important as this will affect the sensitivity of the test and how it can be used to quantify the number of dark leaf spot conidia in samples. The third year of the work details the development of the “in field” tests for dark leaf spot and ringspot. The format of the lateral flow device required changes in year three of the project. Another important aspect of the system was the spore sampling regime. This was also investigated in year three of the project. The development of a working prototype lateral flow device for ringspot ascospores is also described.

3. SUMMARY OF YEAR ONE WORK (2002/3)

3.1.1 Production of antibodies to *Mycosphaerella brassicicola* and *Alternaria brassicae*

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. Fungal growth was 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 on to 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). After which the 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 rehydrated 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*.

3.1.1.3 Condial production of *A. brassicae*

Isolates of *A. brassicae* (Table 4), taken from the Warwick 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 collected into SDW ($3.5x10^3$ conidia per ml), disrupted using a Fast Prep (Qbiogine, UK) and then dispatched to Central Science Laboratory, York for immunization (Immunogen Type A).

Table 4. 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

This procedure was repeated (after 6 months), however, conidia were collected and suspended in a 0.1% glucose solution. Following agitation for 1 hour on a wrist action shaker the conidial suspension was then sprayed directly 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 18ºC. 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. After which the plant debris was 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 using a Fast Prep (Qbiogine, UK) and then dispatched to Central Science Laboratory, York for immunization (Immunogen Type B).

3.1.1.4 Production of monoclonal antibodies to *A. brassicae*

Three female Balb C mice each received 50µl of immunogen preparation A (*in vitro* produced conidia of *A. brassicae*) mixed with an equal volume of Titermax adjuvant. The mice were immunised twice more at 14 day intervals using preparation A without the adjuvant. The mice were then tail bled and the sera screened using a plate trapped antigen ELISA (PTA-ELISA). A mouse was identified which exhibited a level of sensitivity to *A. brassicae* 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 and immunofluorescence (IF), against a range of fungal species.

The immunization process was repeated with immunogen type B (which consisted of disrupted conidia of *A. brassicae* produced '*in planta*').

3.1.2 Results and Conclusions

No hybridoma cell lines were identified positive to *M. brassicicola* from the screen however previous work at Warwick HRI had established that polyclonal and monoclonal antisera could be raised to ascosporic inoculum of *M. brassicicola*. The remaining mice were re-boosted and, following tail bleeds, a further fusion was carried out to identify positive cell lines. Additionally existing antibodies which recognised the wall components of ascospores of *M*. *brassicicola* could be used in lateral flow development. In contrast four hybridoma cell lines were identified as positive to *Alternaria brassicae* from the first *Alternaria* fusion. The cell lines were twice cloned, purified and isotyped as IgM. In cross-reactivity studies each cell line was found to be highly non-specific reacting with a range of fungi tested.

Five hybridoma cell lines were identified as positive to *A. brassicae* in the second fusion. These were twice cloned and isotyped as IgM. In preliminary reactivity studies the selected clones exhibited a low level of recognition to fungal species outside the *Alternaria* genus. However with the exception of clone 190/191.C8 all clones exhibited a level of recognition to *Alternaria dauci* (Figure 3). It should be noted however that a later test revealed no reactivity with *A*. *dauci* when conidia remained ungerminated and intact (i.e. non-disrupted) when tested with monoclonals (MAbs) 220/1.D1.C2.E11 and 220/2.G2.C9.G11. The selected clones were tested against a greater range of air-borne fungi to determine specificity. The five cell lines were subsequently used in the development of a lateral flow device for the rapid detection and semi-quantification of trapped airborne inoculum of *A. brassicae.*

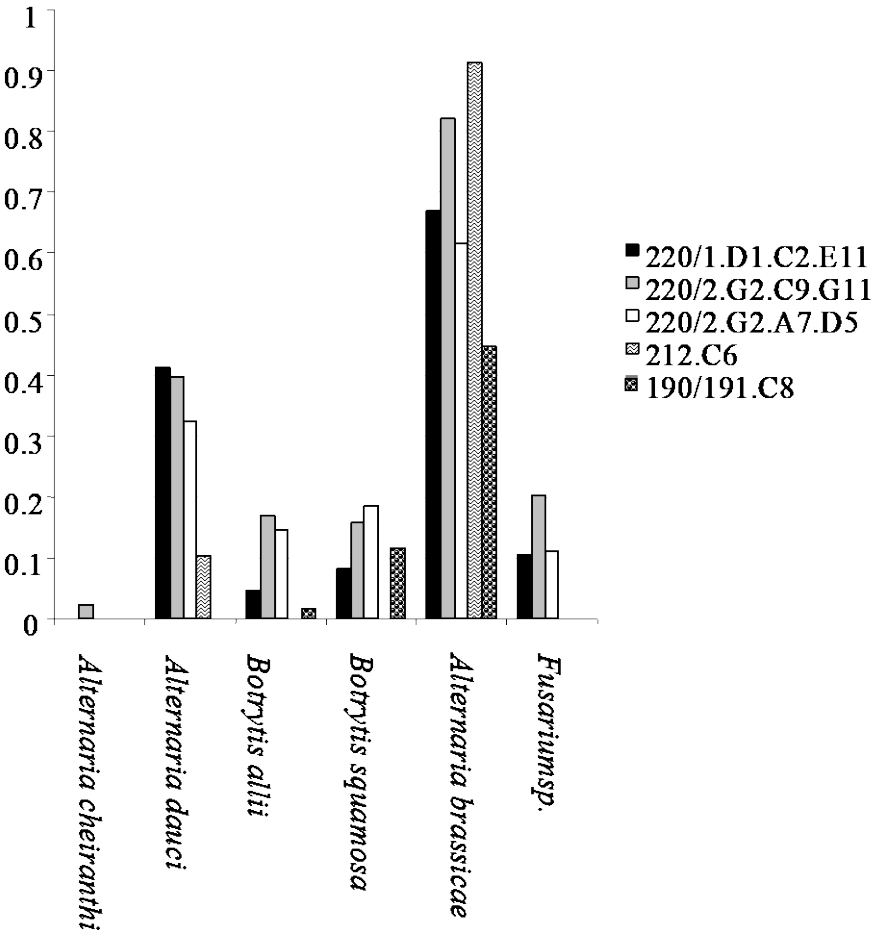


Figure 3. Reactivity screening of cloned cell lines (Fusion 2) by PTA ELISA

3.2 Development and optimisation of immunomonitoring assay formats

3.2.1 Collection of *Alternaria* spores

Brassica leaf material exhibiting dark leaf spot symptoms was collected from a commercial site in Lincolnshire. To promote spore production the leaf material was incubated in an enclosed chamber at a relative humidity (r.h.) of 97% for 48 hours. After which any fungal spores, which had been produced and released in to the airborne environment of the chamber, were monitored using a Burkard eppindorf cyclone sampler (Plate 3). Over a 76 hr period 9 sample collections were made. After each exposure period the eppindorf collection vessel was removed, sealed and stored at -20ºC.

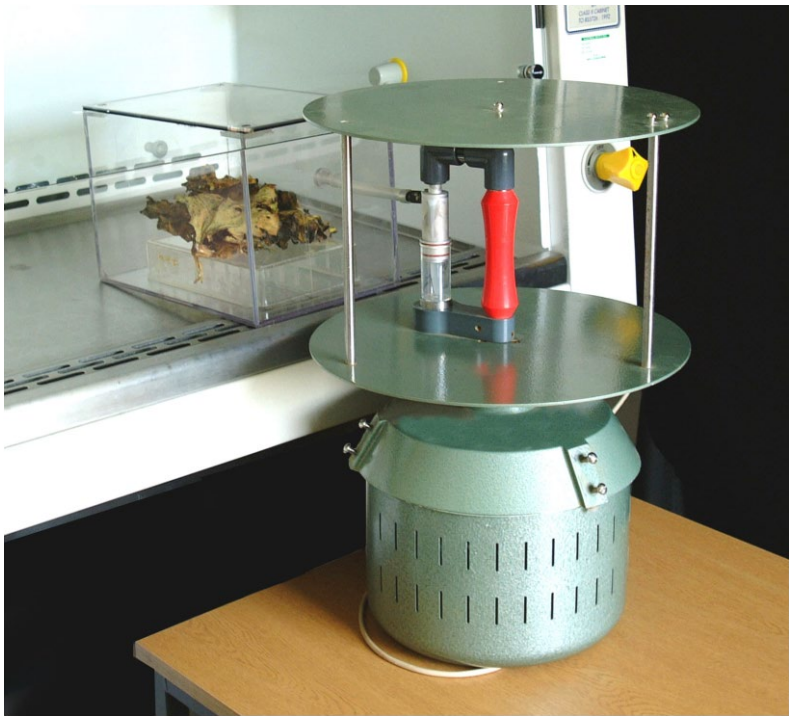


Plate 3. **Collection of airborne spora in to an eppindorf vessel using a Burkard cyclone sampler**

3.2.2 Preparation of spore samples for immunoassay

Each eppindorf sample had 500µl of distilled water added before the sample was agitated and the number of *A. brassicae* spores sampled determined (Table 5). To determine the optimal buffer for use in an immunoassay detection system 100µl of each sample was mixed with 1ml of sterile distilled water (pH 7), 1ml carbonate buffer (pH 9.6) and 1ml of Phosphate buffered saline solution (pH 7). To an ELISA microtitre well 80µl of each sample type was aliquoted and, for each sample type, three replicate wells were included. The conidial numbers of *A. brassicae* present in a range of wells, was determined using a Nikon TMS inverted microscope.

Table 5. Number of *Alternaria brassicae* conidia trapped into an eppindorf collection vessel

during each of the sampling periods

Sample	Total number of *A. brassicae* conidia trapped
1	225
2	5000
3	1620
4	330
5	7500
6	375
7	250
8	145
9	13.5

3.2.3 Immunoassay process

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 microtitre wells were blocked with 200 µl of 1 % casein buffer (1 % (w/v) casein PBS) and incubated at 37ºC for 45 min. Residual blocking buffer was removed and wells were washed four times for one min each with 200 µl PBS Tinc 0.05 % Tween 20 (PBSTincTw). Following this procedure wells received 100 µl per well of MAb cell line 220/1.D1.C2.E11 (diluted 1:2 PBST TincTw). Following incubation as above, wells were washed three times for one min each with 200 µl PBSTincTw. 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 cell line 220/1.D1.C2.E11. Wells were washed as described above and 100µl of 3,3', 5,5'- tetramethylbenzidene substrate (catalogue no. T-3405 and P-Sigma 4922 Sigma) was added to each well. The reaction was stopped by adding 25µl of a 20% 1M H_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). Conidial counts of *A. brassicae* in a selection of the microtitre wells was carried out before the ELISA procedure, following the Casein block stage, each of the antibody stages and post ELISA. The ELISA process was repeated with each of the selected *Alternaria* MAb cell lines (220/2.G2.C9.G11 and 212.C6, 190/191.C8) and as a negative control PBS alone was included at the primary antibody stage.

3.2.4 Results and Conclusions

Conidia in air samples are trapped in collection vessels from which estimates of numbers are taken. The cyclone spore trap when used in conjunction with the eppindorf capture vessel gave good sampling of airborne spores of *Alternaria brassicae*. The use of water to remove spores from the eppindorf collection vessel proved satisfactory as a method for determining dark leaf spot spore numbers. However, when used as a coating buffer water proved

inhibitory reducing antigen binding. Use of the MAb cell lines 220/2.G2.C9.G11 and 220/2.G2.A7.D5 gave a correlation between the numbers of *Alternaria* spores present and the absorbance values derived by ELISA (Figure 4a). Assay sensitivity was improved when a carbonate buffer was used but a phosphate buffered saline coating solution proved optimal in the development of a highly sensitive and rapid quantification assay for airborne inoculum of *Alternaria brassicae*. A similar result was observed when MAb cell lines 220/2.G2.A7.D5 or 190.191.C8 were used within the assay format (Figure 4b). Future development of a lateral flow device will incorporate, as the buffer component; a Phosphate buffered saline solution.

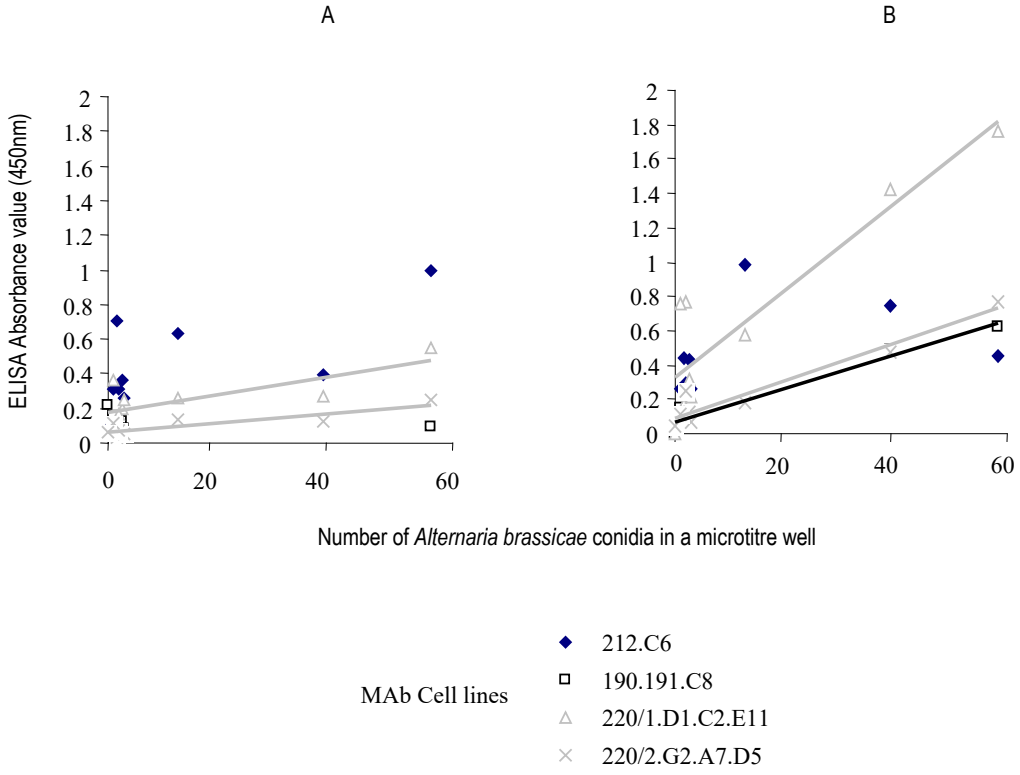


Figure 4. Relationship between the number of *Alternaria brassicae* spores pipetted into a microtitre well using (A) Water as a coating buffer and (B) PBS as the coating bugger and, the resulting ELISA absorbances.

3.3 Determination of biological interference with the developed assay test

3.3.1 Production of *Alternaria brassicae* (dark leaf spot) and *Erysiphe crucifearum* (powdery mildew)

Brassica leaf material heavily infected with *Alternaria brassicae* was collected from the field in Lincolnshire and Cornwall. Leaf material was placed in a misting hood for 48hr at 97% humidity in the glasshouse. Material was dried off after exposure and incubated under room temperature for a further 12-hr period. Leaves were examined for the presence of conidia of *A. brassicae* using a binocular microscope. Powdery mildew was maintained in the glasshouse on plant material of Brussels sprouts cv. Golfer. Plants were produced in Hassey trays before infection with powdery mildew at the 4 true leaf stage. After infection (which was achieved by rubbing infected leaf material on the surface of uninfected leaf material) plants were maintained under dry conditions in a glasshouse without overhead watering.

3.3.2 Sampling of *Alternaria brassicae* (dark leaf spot) and *Erysiphe cruciferarum* (powdery mildew)

After spore production was observed on leaves of infected leaf material (dark leaf spot) and infected plant material (powdery mildew), each type of material was placed in a controlled environment cabinet (Sanyo Gallenkamp, Loughborough, Leicestershire, U.K; Cat No. SGC970/C/RO HFL) operating at 80% r.h. with continuous light. Over a 9 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 eight sampling periods. For each sampling period 1ml of PBS Tinc (phosphate buffered saline solution, 0.05 %Tincture of Merthiolate added as an anti-bacterial agent) was added to the eppindorf collection vessels and the collected spores were resuspended in the solution. The total number of *Alternaria* (dark leaf spot) and *Erysiphe cruciferarum* (powdery mildew) was determined by bright field microscopy at a magnification of x 160. The remaining sample was aliquoted into ten microtitre wells (80µl per well) of a Polysorp microtitre strips (catologue no. 469957, Nunc Immunodiagnostics, Life Technologies Ltd, 3 Fountain Drive, Inchinnan Business Park, Paisley, Scotland) before incubation overnight at 18ºC. An ELISA was carried out, as described above, employing MAbs 220/1.D1.C2.E11, 220/2.G2.C9.G11 190/191.C8 and 212.C6. A negative control employing PBS alone was also included.The sampling efficiency of the cyclone spore trap was determined by positioning a Burkard 24-hr glass slide sampler adjacent to the cyclone spore trap. The Burkard 24-hr spore trap (similar characteristics to the 7-day sampler) has been used routinely for monitoring fungal air-spora in a variety of air sampling studies. Detection and quantification of impacted air spora on the glass slide was by bright field microscopy and at a magnification of x160.

For each sampling period 20 disease-free *B. oleracea* (Brussels sprouts) seedlings (cv. Golfer, 3 true leaves) were positioned in the controlled environment cabinet. Following each of these sampling periods the plants were removed, and placed into an environment of 100 % humidity for 48 hrs. The plants then were removed and retained in a glasshouse, at a temperature of 15 º C for 5 days. Plants were visually examined for expression of dark leaf spot lesions (*Alternaria*). To confirm dark leaf spot symptoms infected leaf tissue was removed; surface sterilised for 1 minute in aqueous sodium hypochlorite (4 % w/v available chlorine) and isolations made on to V8 juice agar. Comparisons between counts of conidial numbers (and

those of other fungi e.g. powdery mildew), ELISA absorbance, and lesion numbers were carried out to compare the sensitivity of the system using the antibodies raised as described in section 3.1.1.4.

3.3.3 Results

There was a close relationship ($r^2 = 0.9782$) between the numbers of conidia of *Alternaria* spp. (*A. brassicae* and *A. brassicicola*) trapped in a cyclone trap (the type of trap to be used in conjunction with the lateral flow test) with of the numbers trapped on a 24 h volumetric spore trap. Similar results were obtained for comparisons of conidial counts of *A. brassicae* alone where the relationship between the numbers in the two traps was very close ($r^2 = 0.966$). This data is presented in the 2003 annual report for FV233. The numbers of *Alternaria* sp. spores as estimated by ELISA and by microscope counts from the cyclone trap sample was close ($r^2 = 0.8544$) using monoclonal antibody (Mab) 190.191.G8 (Figure 5). This antibody recognised spores of both *A. brassicae* and *A. brassicicola* and did not cross react in the presence of high numbers of *Erysiphe cruciferarum* (powdery mildew) and other spores (*Botrytis* sp., *Cladiosporium* and *Phoma* sp.). There was no relationship between the ELISA absorbance values and the numbers of other spores present in the microtitre wells.

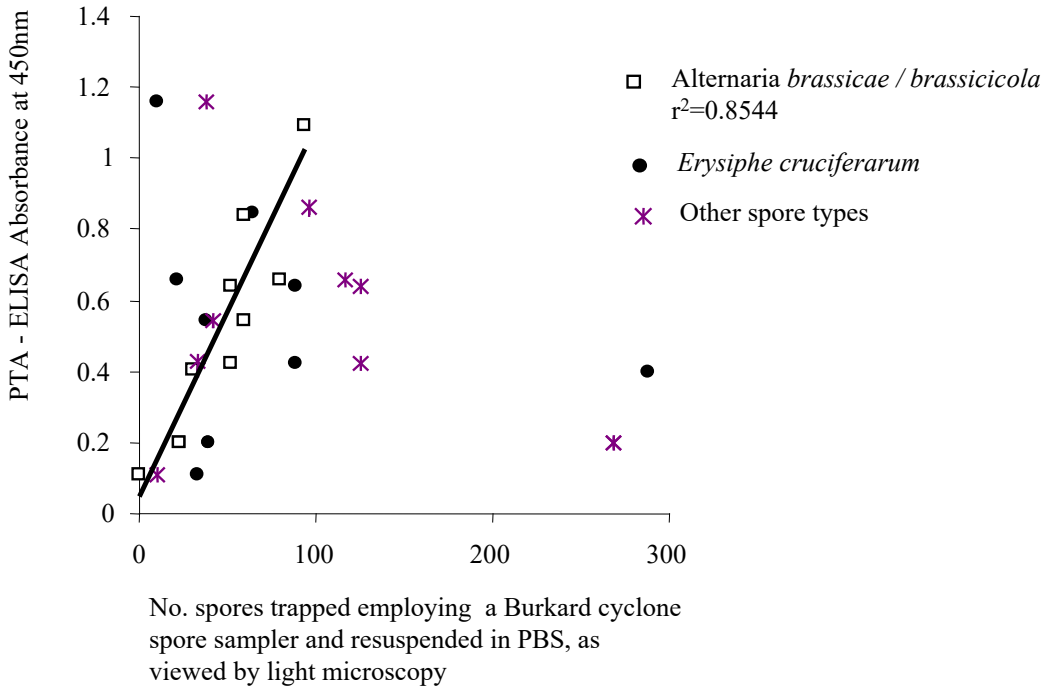


Figure 5. Selective detection and quantification of *A. brassicae* and *A. brassicicola* by PTA-ELISA employing Mab 190.191.G8 in a mixed spore sample.

The results of using antibodies Mab 220/1.D1.C2.E11 and Mab 220/2.G2.A7.D5, for the detection of dark leaf spot conidia (*A*. *brassicae* only), is shown in Figure 6. There was a very close relationship between the numbers of dark leaf spot spores in samples obtained from a cyclone trap and the ELISA value. Although only low spore numbers of *A*. *brassicae* were present there was a relationship between spore number and ELISA values of r^2 = 0.8131 and r^2 = 0.8049 using Mab antibody clones 220/1.D1.C2.E11 and 220/2.G2.A7.D5 respectively. Both antibodies did not recognise *A*. *brassicicola* which, was also present in these samples.

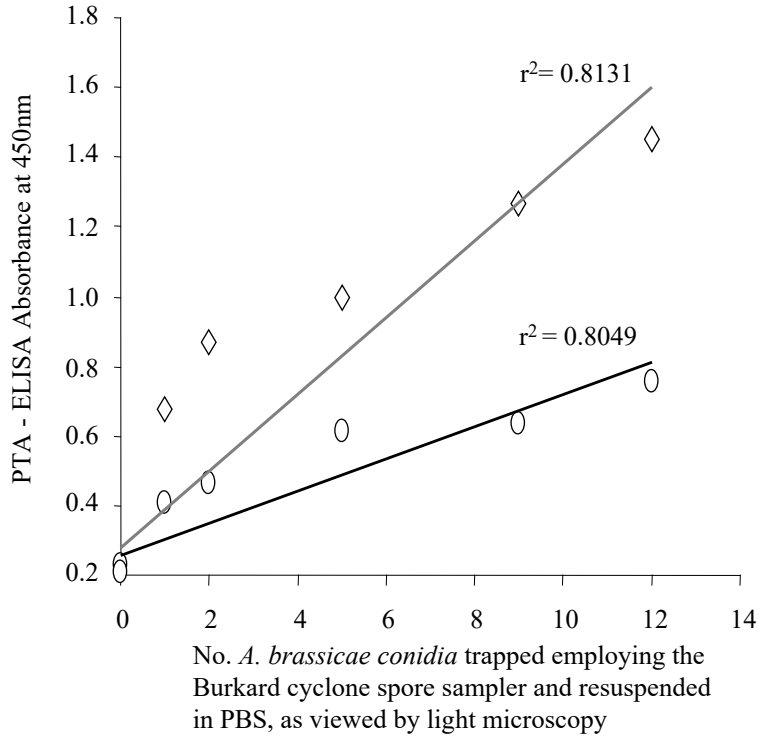


Figure 6. Selective detection and quantification of trapped airborne conidia of *A. brassica* by PTA-ELISA employing Mabs 220/1.D1.C2.E11(◊) and 220/2.G2.A7.D5 (○) in a mixed spore population.

There was a very 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 (Figure 6). After exposure to conidia plants were maintained under leaf wetting conditions for 48 H at temperatures which would result in infection by *A*. *brassicae*. Lesion numbers were counted 10 days after infection conditions had ceased. Isolations on to agar from leaf lesions confirmed that the dark leaf spot lesions resulted from infection by *A*. *brassicae* only despite the presence of *A*. *brassicicola* in the spore samples. A relationship between lesion number and ELISA value of r^2 = 0.6744 and r^2 =

0.6747 using antibodies Mab 220/1.D1.C2.E11 and Mab 220/2.G2.A7.D5 respectively was observed. There was no relationship between lesion number and ELISA value using antibody Mab 190.191.G8 (Figure 7).

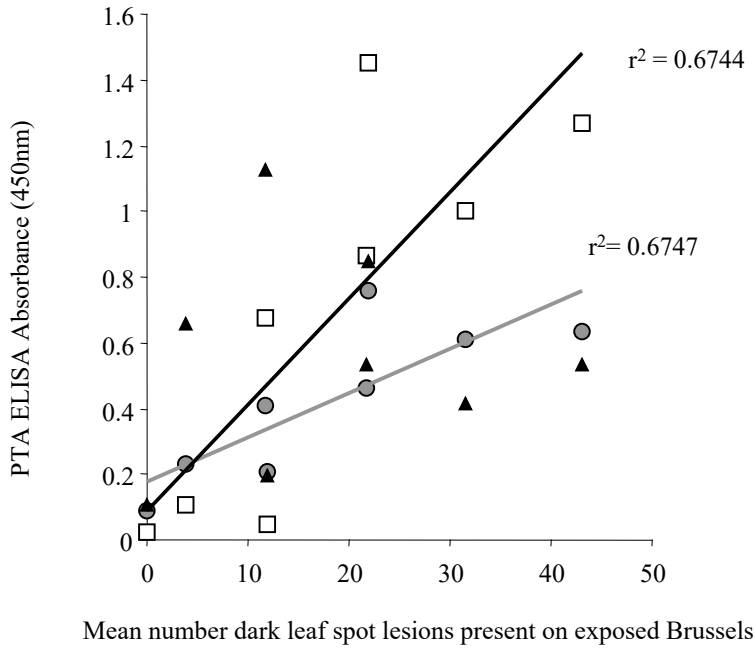


Figure 7. Relationship between predicted *A. brassicae* presence (PTA ELISA) and dark leaf spot disease development employing Mabs 190.191.G8 (▲) Mabs 220/1D1.C2.E11 (□) and 220/2.G2.A7.D5 (●) in a mixed spore population.

3.3.4 Conclusions

The results show that there was a very close relationship between the numbers of dark leaf spot spores trapped in the cyclone spore trap and the numbers trapped by a 24 h volumetric trap. The results show that antibody Mab 190.191.G8 recognises conidia of both *Alternaria brassicae* and *Alternaria brassicicola* which can both cause dark leaf spot. However *A. brassicae* is the most important cause of dark leaf spot in vegetable brassica crops. 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). This was further confirmed when ELISA values dervived from air samples using these two antibodies were compared with dark leaf spot lesion numbers on exposed plants. Despite the presence of *A. brassicicola* in the spore samples it appeared that only *A. brassicae* spores had resulted in infection on leaf material. No relationship was observed between the ELISA value and leaf lesion number when antibody Mab 190.191.G8

was used. These antibodies proved (with MAb cell line 212.C6) suitable for use in lateral flow development during year 2 of the project.

4. SUMMARY OF YEAR TWO WORK (2003/4)

4.1 Production of antibodies to *Mycosphaerella brassicicola* and *Alternaria brassicae*

4.1.1 Production of antibodies to *Mycosphaerella brassicicola*

In year 2 of the project further attempts were made to raise monoclonal antibodies to the ascosporic stage of *M. brassicicola* (as in year one).

4.1.1.2 Production of ascosporic inoculum of *M. brassicicola*

(See Section 3.1.1.1)

4.1.1.3 Production of monoclonal antibodies to *M. brassicicola*

(See Section 3.1.1.2)

4.1.1.4 Results

The initial screen was unsuccessful with false positives recorded. No hybridoma cell lines were identified as positive to *M. brassicicola* using a plate-trapped antigen ELISA which had been optimised at HRI employing a streptavidin / biotin amplification system. The initial fusion was aborted. A second fusion (using Mouse 231.1) gave twenty four positive hybrids (as selected by PTA ELISA). However negative results were this time observed at the retest stage. Selection of cell lines was based on culture well observation rather than *Mycosphaerella brassicicola* positive immunoassay results. Six cell lines (identified by immunoflourescence) were positive to ascosporic 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 Warwick HRI, Wellesbourne for further testing by PTA ELISA. All proved negative to pseudothecial material of the ringspot pathogen, when tested by PTA ELISA (Figure 8). A postive control of HRI EMA 187 and HRI 98/4/p (*M. brassicicola* positive monoclonal and polyclonal) were included in the test.

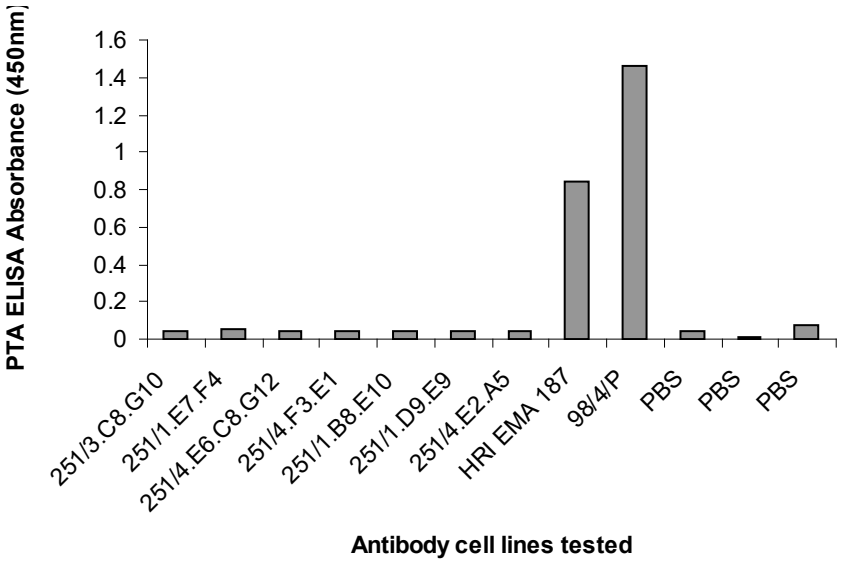


Figure 8. Reactivity of selected clones to soluble material associated with the fungal plant pathogen *Mycosphaerella brassicicola* (pseudothecial stage).

4.1.1.5 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.

4.1.2 Production of monoclonal antiserum to airborne stage of *Alternaria brassicae*

4.1.2.1 Condial production of *A. brassicae*

(see section 3.1.1.3)

4.1.2.2 Production of monoclonal antibodies to *A. brassicae*

(see section 3.1.1.4)

4.1.2.3 Results

A further *A. brassicae* 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 9a -c).

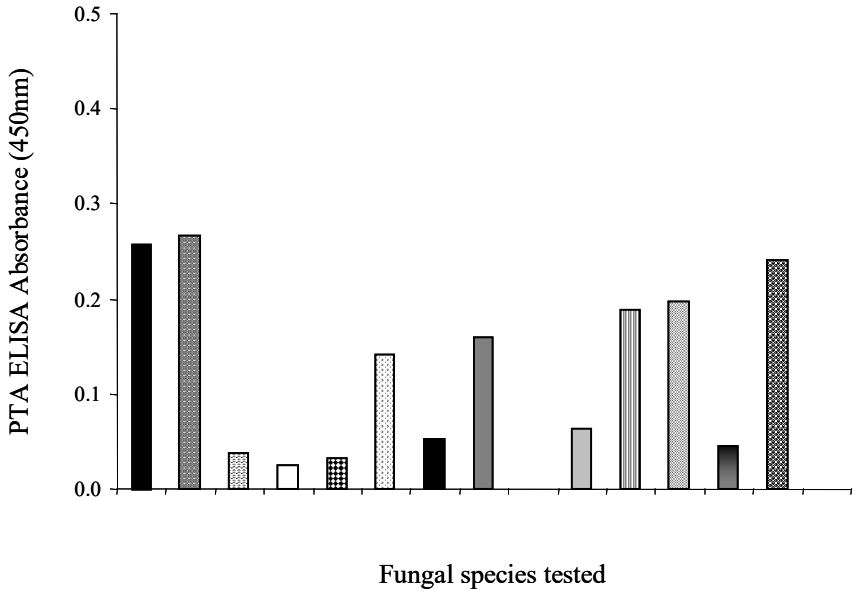


Figure 9a. Reactivity screening of cell line 253/2.A10.C4.A9

- *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

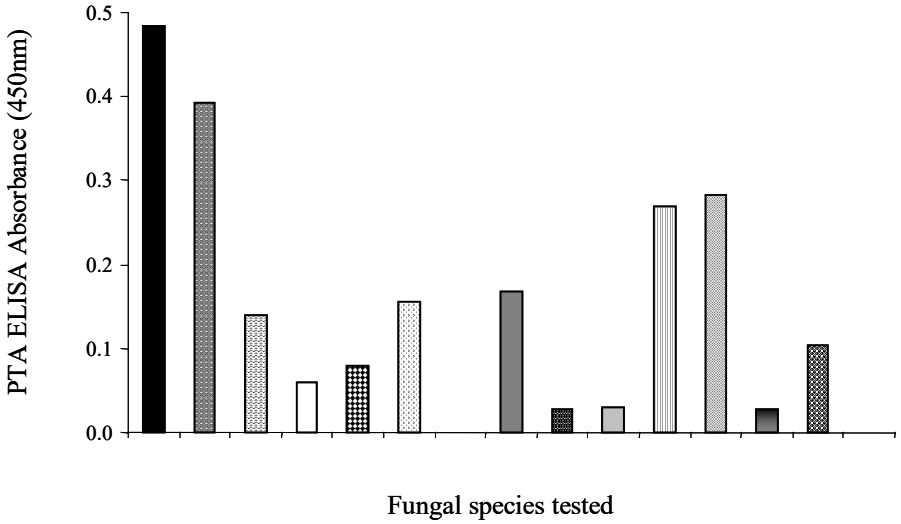


Figure 9b. Reactivity screening of cell line 253/1.D6.G10

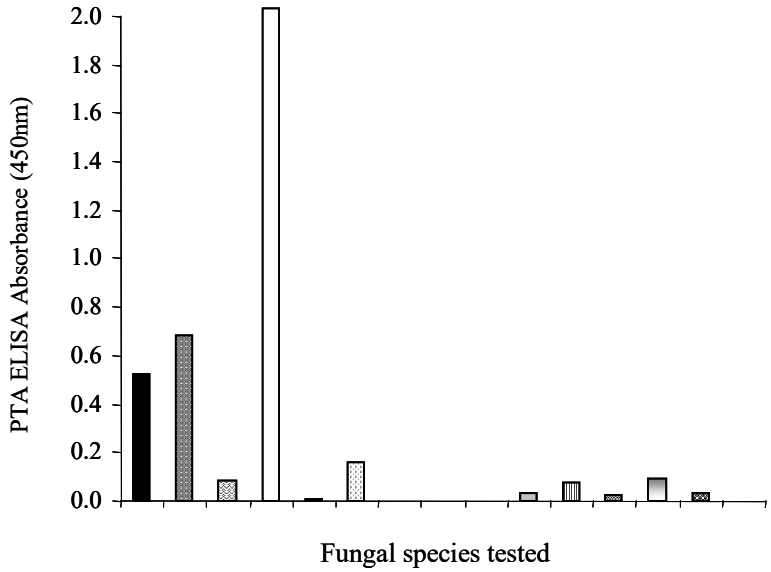


Figure 9c. Reactivity screening of HRI EMA 212 (positive control)

4.1.2.4 Conclusions

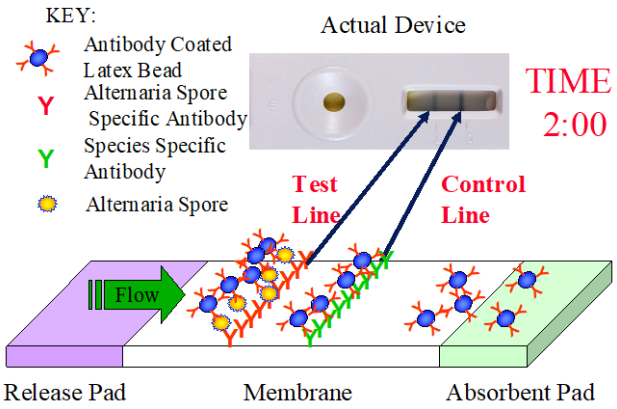
Previous work has shown that there was a close relationship between, the numbers of dark leaf spot lesions on plants exposed to dark leaf spot spores, and the ELISA values obtained when using antibodies Mab 220/1.D1.C2.E11 and Mab 220/2.G2.A7.D5. These results indicated that no further antibodies produced were selective enough to conidia of *A. brassicae*, and could not 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.

4.2 Development and optimisation of lateral flow for detection of *Alternaria brassicae* (dark leaf spot) and *Mycosphaerella brassicicola* (ringspot)

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

4.2.1.1 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 10). 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 10. Schematic drawing of a Double Antibody Sandwich (DAS) lateral flow device *(Positive result shown)*

4.2.1.2 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 10). 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.

4.2.1.3 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 6). 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 6. Antibody combinations used in the DAS lateral flow assessment

Latex conjugated antibody		Test line antibody
***Alternaria* polyclonal antiserum (PAb)** MAb 220/1.D1.C2.E11 MAb 220/2.G2.B7.D5 MAb 220/3.F10		MAb 191
Monoclonal Antiserum (MAb) MAb 220/1.D1.C2.E11 MAb 220/2.G2.B7.D5 MAb 220/3.F10	220/1.D1.C2.E11	MAb 191
Monoclonal Antiserum (MAb) MAb 220/1.D1.C2.E11 MAb 220/2.G2.B7.D5 MAb 220/3.F10	220/2.G2.B7.D5	MAb191
Monoclonal Antiserum (MAb) MAb 220/1.D1.C2.E11 MAb 220/2.G2.B7.D5 MAb 220/3.F10	220/3.F10	MAb 191
Monoclonal Antiserum (MAb) MAb 220/1.D1.C2.E11 MAb 220/2.G2.B7.D5 MAb 220/3.F10	191	MAb 191
Monoclonal Antiserum (MAb) MAb 220/1.D1.C2.E11 MAb 220/2.G2.B7.D5 MAb 220/3.F10	212	MAb191

4.2.1.4 Membranes and buffers used – CSL to complete

4.2.1.5 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

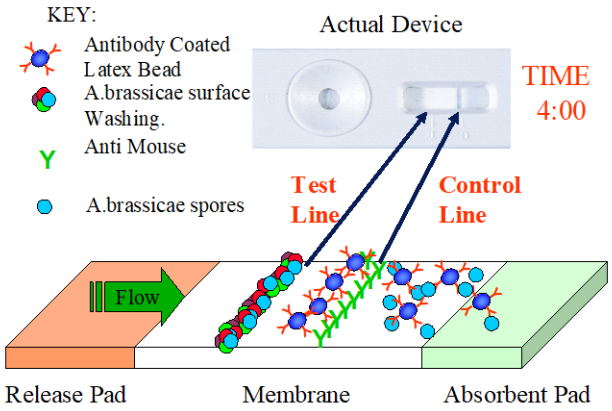
combination when mixed with an *A. brassicae* (dark leaf spot) positive sample no test line was observed.

4.2.1.6 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. The monoclonal antibodies tested in this format were of IgM class type. These antibodies are generally of low affinity requiring longer contact times with the target sample (dark leaf spot conidia) for successful binding to occur on 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 a 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 antibody species is rabbit.

4.2.1.7 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 11).** 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 11. 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 appliedto 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 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.

4.2.1.8 Membranes and buffers used – CSL to complete

4.2.1.9 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 7). *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 7. Assessment of antibodies for inclusion within a competitive lateral flow test format: test line development

Latex labelled	Membrane type					
detector antiserum	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

4.2.1.10 Results

Using 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 7). 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. Using test samples of conidial material of *Alternaria brassicae* a single control band on each device was readily observed, denoting dark leaf spot spore presence (positive test samples).

4.2.1.11 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.

4.2.2 Optimisation of the Competitive Lateral Flow Assay for *Alternaria brassicae*

4.2.2.1 Determination of the Competitive *Alternaria brassicae* Lateral Flow Assay detection threshold

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

4.2.2.2 Results

The results (Table 8) 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 4 - 6. No test lines are observed in plates 4 and 5 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 6). Weak test lines were observed in lateral flow devices tested with samples containing 47 dark leaf spot conidia (Plate 6). 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 12). Only those samples containing 23 - 0 dark leaf spot conidia per sample gave significant test line measurements on the reader device.

Table 8. Detection threshold for *Alternaria brassicae* using a competitive lateral flow assay

Total no. *A.brassicae* Spores applied to lateral flow device *(+ mycelial*	**Test line**	**Control line**	***A.brassicae* detected**

fragments)			
6000	-	+	++
3000	-	+	++
1500	-	+	++
750	-	+	++
375	-	+	++
187	-	+	++
94	-	+	++
47	+	+	+
23	+	+	-
12	+	+	-
0	+	+	-

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

Plate 4. Lateral flow tests for dark leaf spot conidia (6000 - 1500 conidia per sample)

Plate 5. **Lateral flow tests for dark leaf spot conidia (750 - 187 conidia per sample)**

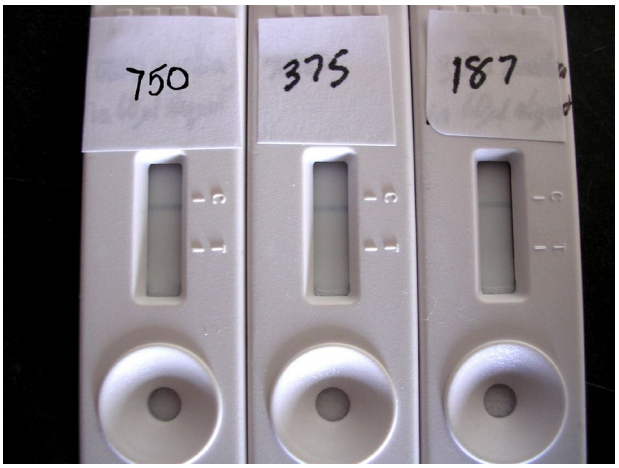
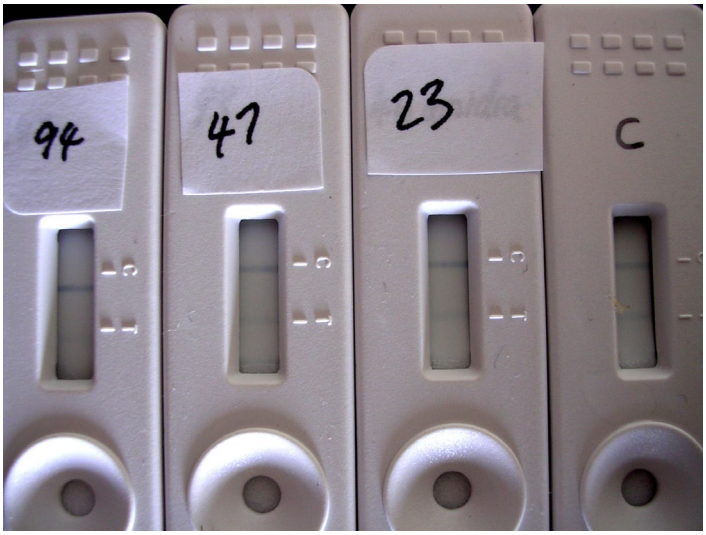


Plate 6. **Lateral flow tests for dark leaf spot conidia (94 - 23 conidia per sample + control)**



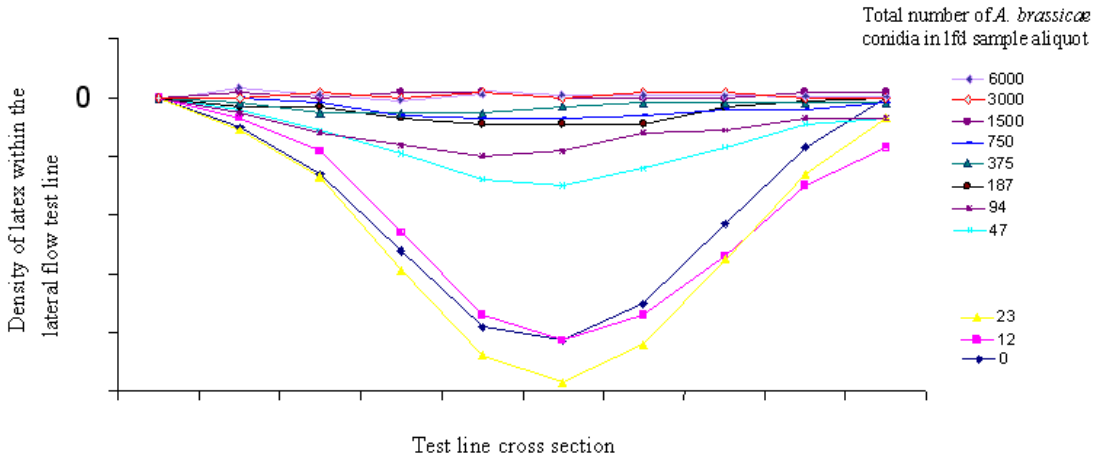


Figure 12. Detection threshold of *Alternaria brassicae* conidia employing lateral flow prototype

4.2.2.3 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.

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

4.2.3.1 Materials and Methods

A range of fungi, common in vegetable crops were grown on V8 media. Cultures were taken from the Warwick 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 9). 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 9. 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

4.2.3.2 Results

The competitive lateral flow format gave negative tests when samples containing different fungi (Table 9) were tested. Test lines were observed with all cultures tested (indicating a negative result). 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 13). Only those samples without dark leaf spot conidia gave significant test line measurements on the reader device (Figure 13).

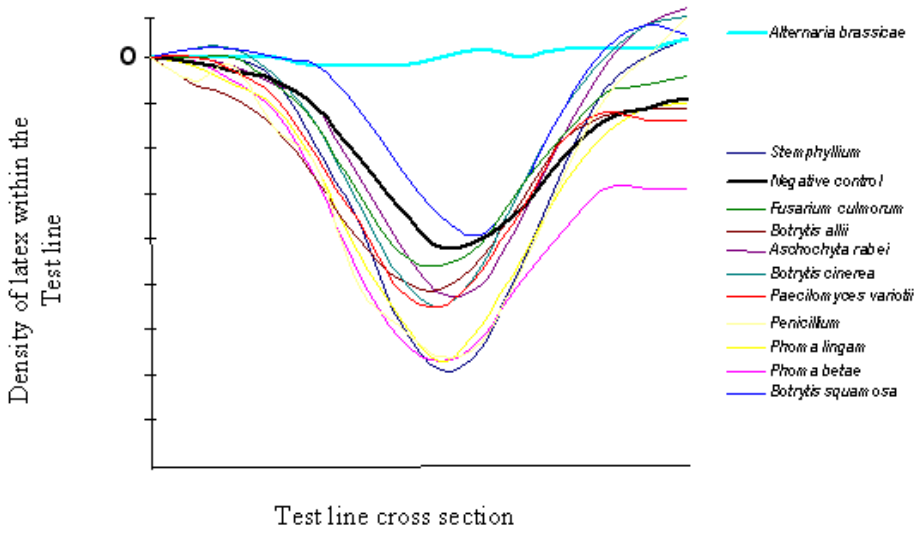


Figure 13. Reactivity of lateral flow prototype (BO2): Density of immobilised latex particles monitored on the test line of exposed lateral flow strips.

4.2.3.3 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. alternata* 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).

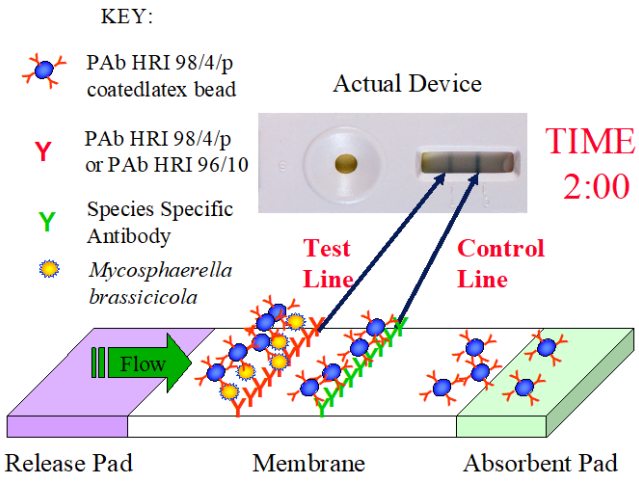
4.2.4 Development and optimisation of lateral flow device for detection of ascospores of ringspot

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.

4.2.4.1 Double Antibody Sandwich (DAS) test format

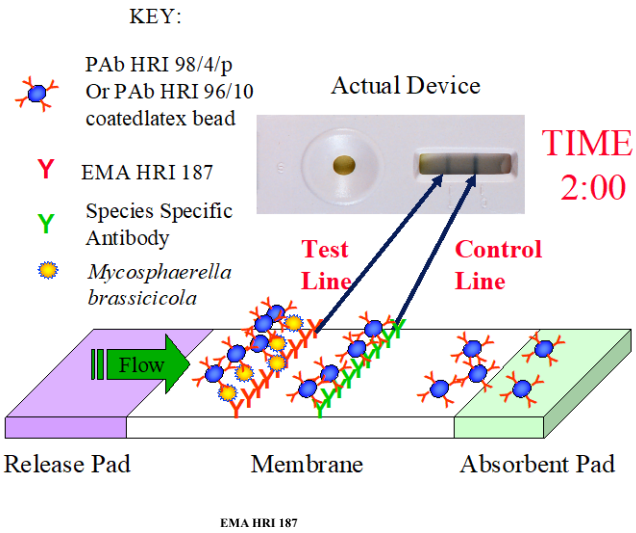
4.2.4.1.1 Materials and Methods

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) was examined (Figures 14a,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 14a). In the second format type B monoclonal antibodies (EMA HRI 187) were placed on the test line (Figure 14b). 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 14a. DAS lateral flow prototype A



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

Figure 14b. DAS lateral flow prototype B

4.2.4.1.2 Results and Conclusions

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. The double antibody sandwich lateral flow test (DAS lateral flow device) in the format used did not work using 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 using a range of membrane types and buffers may warrant further investigation. The DAS lateral flow device test proved 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.

4.2.4.2 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.

4.2.4.2.1 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 (Figure 15). 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.

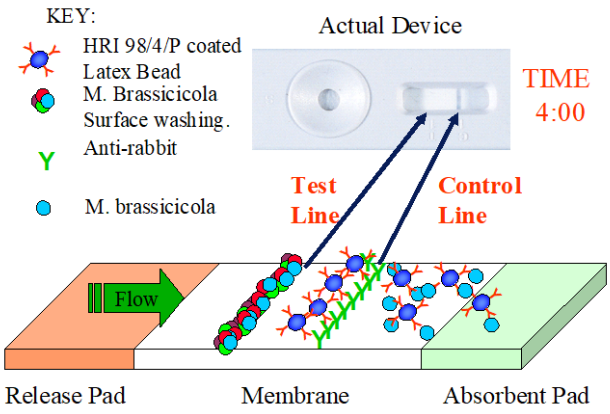


Figure 15. Competitive lateral flow format showing PAb HRI 98/4/p conjugated to latex beads and *M. brassicicola* mycelial washings sprayed at the test line.

4.2.4.2.2 Results and Conclusions

The competitive lateral flow test in the format used did not work using 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.

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.

4.3 Evaluation of lateral flow protoype (A01, B02) for monitoring airborne conidia of *Alternaria brassicae* within mixed air spora samples

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

4.3.1.1 Materials and Methods

4.3.1.1.1 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*), were 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 99% 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 sampling the eppindorf tube was removed, sealed and stored at -20ºC. This process was repeated for a further 10 sampling periods.

4.3.1.1.2 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 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.

4.3.1.2 Results

Significant quantities of dark leaf spot spores were collected within each sample tube as shown in Table 10. 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 10. Reaction of dark leaf spot lateral flow prototypes in a mixed spore sample

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	$6x10^3$	$8.4x10^3$	$1.9 x10^3$	✓

4.3.1.3 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. 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

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

4.3.2.1 Materials and Methods

4.3.2.1.1 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 sampler and microtitre 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 microtitre 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 at 16 C. 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).

4.3.2.1.2 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.

4.3.2.1.3 Immunoassay process

Enumeration of trapped *Alternaria* spp on the base of the microtitre wells was determined using an inverted binocular microscope (x 200) by bright field illumination. Exposed MTIST microtitre 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 microtitre 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. 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 had 100µl of 3,3', 5,5'- tetramethylbenzidene substrate (catalogue no. T-3405 and P-Sigma

4922 Sigma) added. 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).

4.3.2.2 Results

4.3.2.2.1 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 16 (Quadscan reading for each sampling period) and Table 11 (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 16). Low Quadscan values were obtained using lateral flow devices tested with material from vials containing particulates from air samples corresponding to these sampling period.

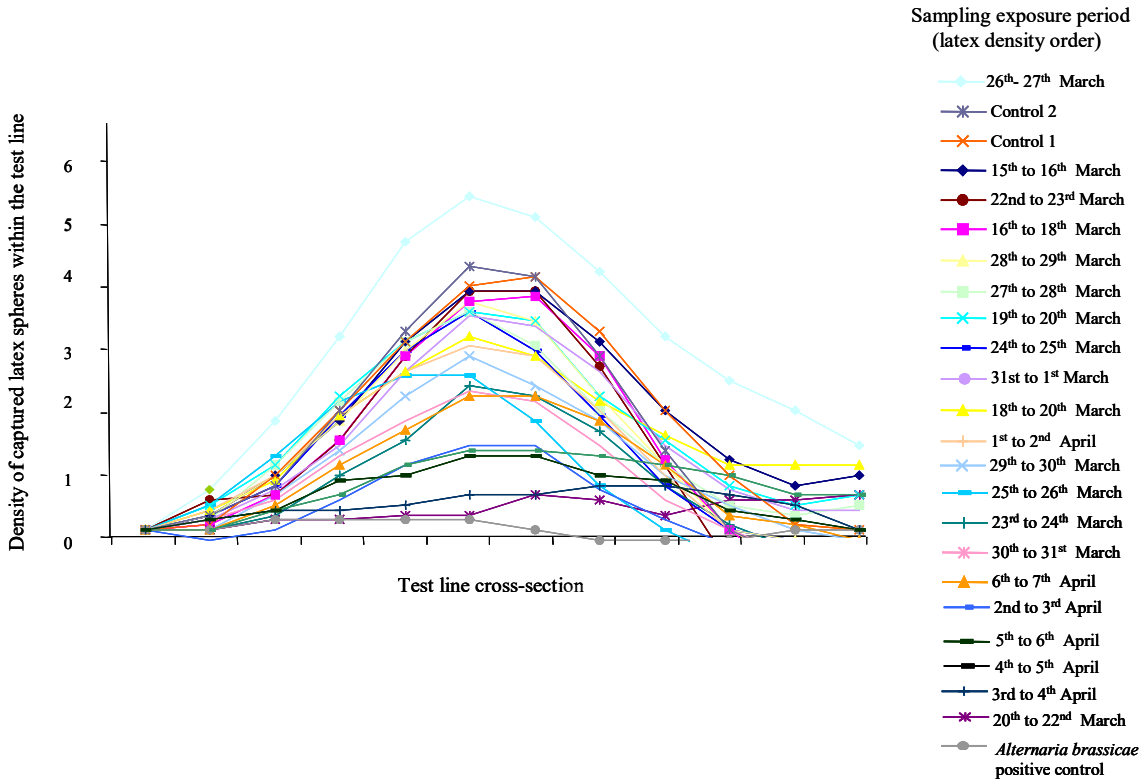


Figure 16. Quadscan readings of lateral flow test lines relative to control lines for dark leaf spot sampling periods

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 17). 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 11. 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

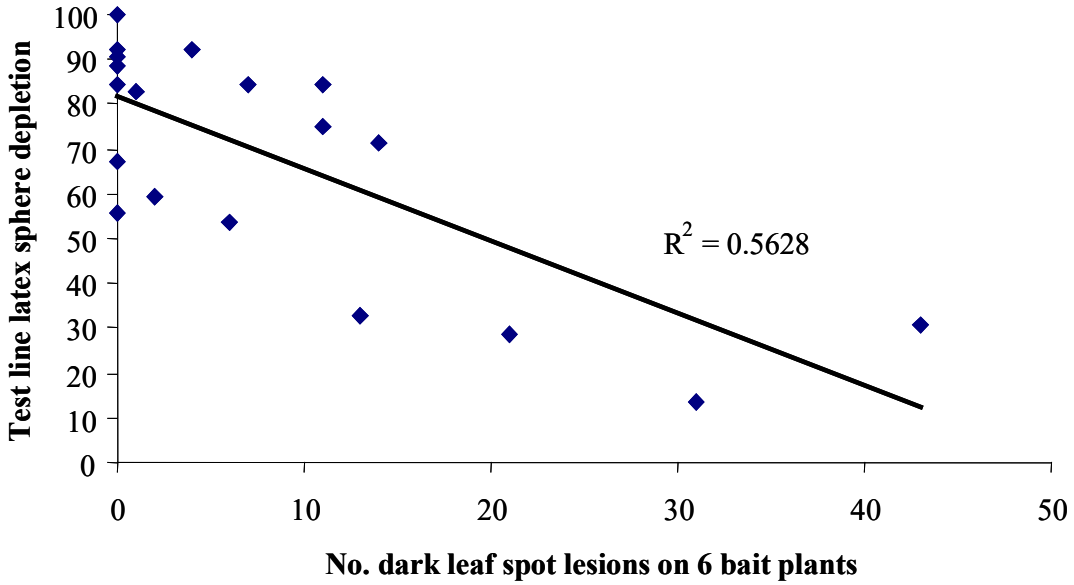


Figure 17. Maximum Quadscan readings for lateral flow test lines and corresponding number of dark leaf spot lesions on 6 trap plants.

4.3.2.2.2 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 18).

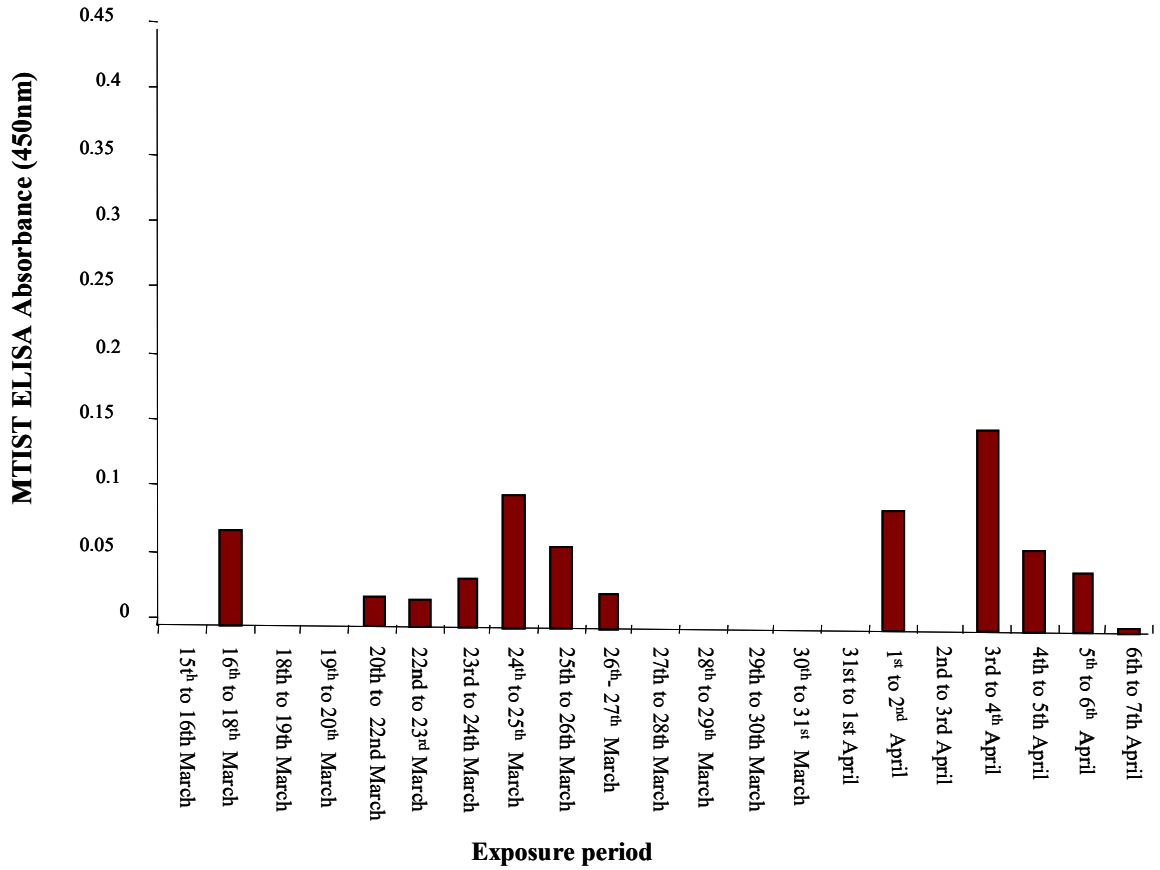


Figure 18. Detection of dark leaf spot conidia in air samples using ELISA

4.3.2.2.3 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 19. 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 19). 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.

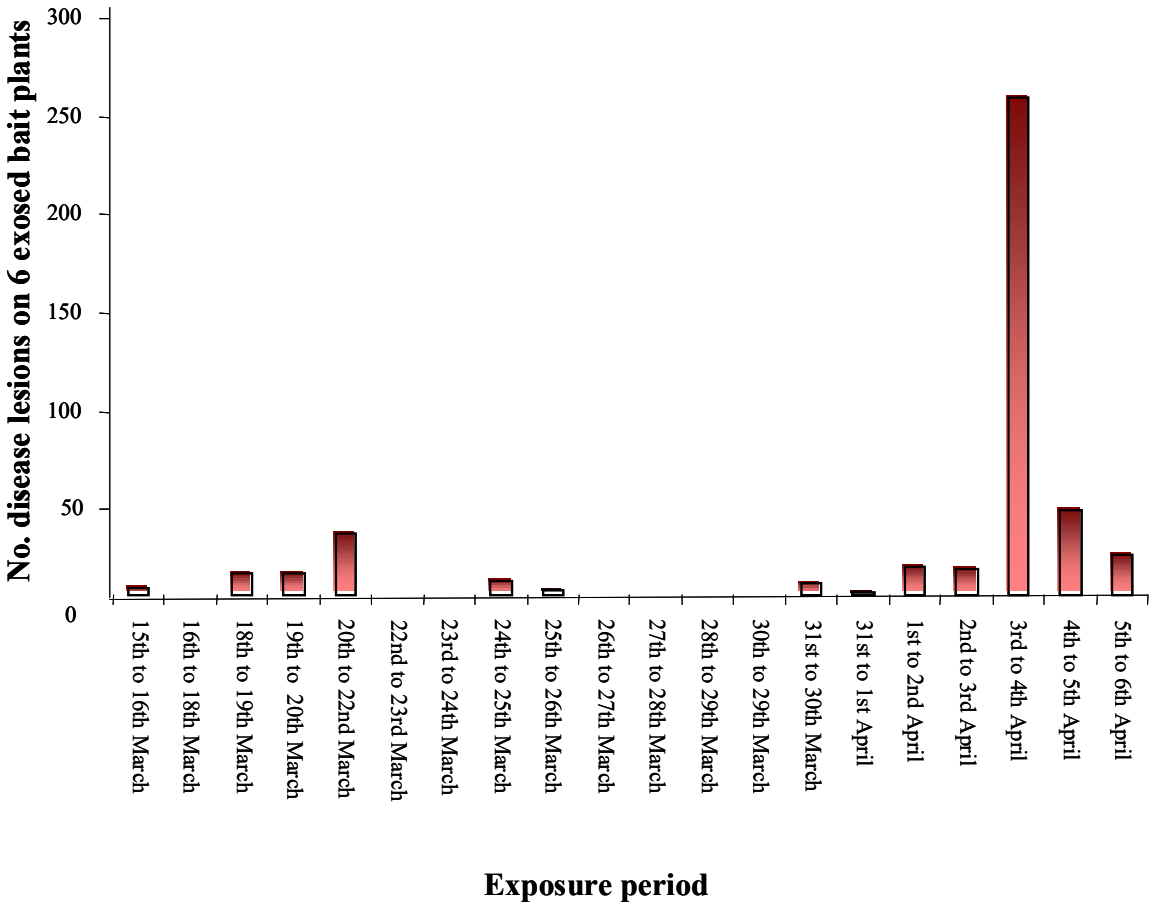


Figure 19. Number of dark leaf spot lesions on trap plants during air sampling periods (after 48 hrs of leaf wetness duration)

4.3.2.3 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.

5. YEAR 3 RESULTS - COMPARISON OF TRAPPING FORMATS AND WEATHER BASED DISEASE FORECASTS FOR DARK LEAF SPOT AND RINGSPOT IN AN OVER-WINTERED BRUSSELS SPROUT CROP

5.1 Monitoring dark leaf spot conidia and ringspot ascospores in air samples in a over-wintered crop of Brussels sprouts

The available trapping formats including the dark leaf spot lateral flow test were evaluated for their ability to detect dark leaf spot conidia in air samples in an over-wintered crop of Brussels sprouts at Warwick HRI, Wellesbourne. Three trapping formats were compared in this trial with disease development on trap plants exposed within the plot for 24 H periods .

5.1.1 Materials and Methods

A field experiment was conducted to compare different types of trapping systems for their accuracy in trapping dark leaf spot and ringspot spores.

5.1.1.1 Production of a ringspot and dark leaf spot seeding crop

An over-wintered, heavily infected (dark leaf spot, ringspot and white blister) field plot (20m x 10m) of Brussels sprouts (c.v. Golfer), was produced. The crop was planted during May 2004 (50 X 50 cm plant spacing) and established for 1 month prior to inoculation using infected ringspot and dark leaf spot dried leaf material removed from a heavily infected overwintered cauliflower crop in Lincolnshire. The crop became heavily infected with dark leaf spot and ringspot during 2004 and remained until spring 2005 when plants produced seeding heads which became infected with new lesions of dark leaf spot and ringspot.

5.1.1.2 Air samplers used in the trial

Three types of air sampler were used in a field trial to compare different trap types in their accuracy in trapping dark leaf spot and ringspot spores. An MTIST sampler and 7 day cyclone sampler were operated for 12 H periods form 06:00 H to 18:00 daily. These were compared to a Burkard 24 H volumetric trap which ran continuously over each 24 H period. The MIST trap containing microtiter strips (catologue no. 469957, Nunc Immunodiagnostics, Life Technologies Ltd, Paisley, Scotland) was changed daily after 18:00H. The 7 day cyclone sampler contained epindorf tubes for which a fresh tube was used automatically for each days sampling. Sample tube sets were changed at weekly intervals. The Burkard 24 H volumetric trap used a glass slide which had been coated with silicone (BDH Chemicals). In this trap air flow is directed on to the discreet areas of the slide which corresponded to different time intervals. Particulate matter from the airflow was directly impacted on to the glass slide. The glass slide was replaced daily after 18:00 H. The slide, epindorf tube and microtitre strips were stored at – 20 C after their removal from each air sampler.

MTIST sampler

A detailed description of the MTIST device can be found in Kennedy et al., (2000). In the outdoor version air is drawn through a manifold consisting of a plastic tube with a right angle bend placed over the sampler inlet. The manifold samples air through a 9cm diameter vertical circular inlet and directs it into the sampler body that is held horizontally. For field use the sampler (including manifold) is mounted on a wind vane so that the manifold inlet faces into the wind (Kennedy et al., 2000). Within the sampler the airflow is channelled through 32 trumpet-shaped nozzles each directed at the base of a microtiter well. The sampler contains four microtiter strips (catologue no. 469957, Nunc Immunodiagnostics, Life Technologies Ltd, Paisley, Scotland) each containing eight wells. Four types of well coating preparations were used (one on each strip): Poly-L-lysine (Sigma P-1524) diluted in distilled water to 0.1mg ml^{-1}, a 5:1 mixture of petroleum jelly (Vaseline) and paraffin wax which had been melted in a water bath and thoroughly mixed before being diluted with hexane (1:16), silicone which following melting was mixed with hexane as previously described and, a well coating of distilled water. One hundred µl of each single coating solution was applied to each well of 60 microtitre strips (catologue no. 469957, Nunc Immunodiagnostics, Life Technologies Ltd, Paisley, Scotland). This process was repeated for the remaining three coating preparations. After treatment the coated microtitre well strips were secured within ELISA multiframes (Catalogue No. 9503060, Life Technologies Ltd, Paisley, Scotland) and incubated at 20 ºC for 1 hour, after which any unbound material was removed by inverting the microtitre strips and tapping them down on to absorbent towelling. An inverted binocular microscope (Nikon model TMS) was used to check that the well coatings had been applied evenly. Prior to field exposure the microstrips were stored at 4ºC in a sealed container. 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 min-1. The MTIST sampler was operated daily for 12 H periods (06:00H – 18:00H) as previous studies had shown that conidia of dark leaf spot and ascospores of ringspot were present in air samples only during daylight hours.

Burkard 24 H volumetric traps

The Hirst-type trap Burkard 24 H volumetric trap (Burkard Manufacturing Co Ltd, Rickmansworth, Hertfordshire, England) is described elsewhere (British Aerobiology federation, 1995; Lacey & Venette, 1995). The samplers consisted of a metal body with a rectangular inlet slit (14 mm high and 2 mm wide) through which air was sampled at approximately 10 litre min-1 using a battery operated pump. The air-flow was controlled by critical orifices mounted just behind the traps, which were individually calibrated. Inside each sampler, spores were impacted on to a slide coated with silicone. The glass slide is attached to a 24 H clock which moves the slide corresponding to a 24 H period. The overall efficiency of the volumetric spore sampler is high (Stedman, 1978). After exposure, spore deposits on the slide at different points during the 24 H period were examined under a light microscope (x 400). Slides used in this trap were given a coating of silicone applied using a glass edge

coated with silicone drawn over the surface of the slide exposed to the air stream in the trap. This was used to enhance numbers of conidia of dark leaf spot trapped on the slide.

Burkard 7 Day cyclone sampler

The characteristics of the spore cyclone sampler, has been described by Ogawa & English (1995). Air is drawn through this sampler using a vacuum pump in the form of a cyclone. The characteristics of the trap namely the height of the cyclone, height of the air inlet, width of the air inlet, air exhaust diameter and the diameter of the cyclone with the length of the exhaust pipe influence the relative efficiency of the trap. These characteristics have been drawn together and standardised within the Burkard cyclone sampler. A separate sample container which is rotated automatically can be used for each sampling day. The trap can also be adjusted to sample for set durations each day. The quantitative efficiency of this type of trap is high as the sample volumes can be much higher than other types of trap. This type of trap is suitable for use with “in field” detection kits.

5.1.1.3 Enumeration of trapped spores in air samplers

Spore collecting tapes were removed from the Burkard 24 H volumetric trap and permanently mounted on glass microscope slides using "Mowoil" (supplied by Burkard Manufacturing Co., Rickmansworth, UK) and glass coverslips. The numbers of spores of each type impacted was determined by bright field microscopy using a Zeiss binocular microscope (x 400) and scanning an 8 mm^2 area of the tape by counting spores in transverse sections across the tape. Spore concentrations (spores per m^3 air sampled) were calculated from the spore counts using the trap flow rate, the time of exposure and, the fraction of the area counted for each tape (British Aerobiology Federation, 1995). The total number of individual spores of each spore type deposited on the base of each microtitre well was counted by using a Nikon model TMS inverted binocular microscope (x 200). Dark leaf spot conidia and ringspot ascospores in MTIST microtitre wells were also determined using ELISA (see section 6.1.1.3). 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.

5.1.1.4 Detection of ringspot and dark leaf spot in air samples using ELISA

Following bright field enumeration the strips were blocked with 200 µl of 1% Casein buffer (1% (w/v) casein PBS) and incubated at 37ºC for 45 min. Residual blocking buffer was removed and wells were washed four times for one min each with 200 µl PBS, 0.05 % Tween 20 and 0.1% Casein (PBSTw C). After which wells 1 and 3 of each strip each received 100 µl of monoclonal antibody EMA 187, wells 5 and 7 each received 100 µl of monoclonal antibody EMA 212 and the remaining wells of 2, 4, 6 and 8 each received 100 µl of PBS. 0.05% Tween 20 and 0.1% Casein. Following incubation in a Wellwarm shaker incubater (30°C) for a period of 45 mins as above, wells were washed three times for one min each with 200 µl PBSTincTw. After which a DAKO duet amplification system was used (DAKO Ltd, Angel Drive, Ely, Cambridge, UK; Cat no. K0492) to amplify the signal generated by bound tissue culture

supernatant antibodies. Wells were washed as described above and 100µl of 3,3', 5,5'-tetramethylbenzidene substrate (Sigma, Poole, Dorset UK; Cat. No. T-3405 and P-4922) was then added to each well. The reaction was stopped by adding 25µl of a 20% 1M H_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).

5.1.1.5 Monitoring dark leaf spot and ringspot spores in air samples in relation to plant infection

Plants were exposed in an over-wintered plot (20 x 20 m) of Brussels sprouts heavily infected with dark leaf spot and ringspot. For each of the sampling periods five pots of bait plants (Brussels sprouts c.v. Golfer, 5 true leaves), each containing one plants which had been grown in the absence of disease, were positioned adjacent to the spore traps. After each 24 H 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 and ringspot. 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 number of leaves infected and uninfected with dark leaf spot and ringspot counted.

5.1.1.6 Micro-climate measurements

Measurements of temperature, humidity, leaf surface wetness and rainfall were collected at 30 min intervals from when the logger was sited in the brassica crop using a SKYE Datahog II 7 channel logger. Measurements were collected by GSM portable phone Link (Skye Instruments Ltd, Llandrindod Wells, Powys). The logger was powered by a 12 V battery. Environmental data, was collected within MORPH and summarised within BRASSICA***spot***. Numbers of trapped conidia in the air could be directly compared with corresponding environmental conditions.

5.1.1.7 Prediction of dark leaf spot and ringspot infection in the field

Disease forecasting models (Brassica*spot*) were used to predict dark leaf spot and ringspot infection under prevailing environmental conditions. The development of the Brassica*spot* system has been described within HDC project FV53D. The Warwick HRI dark leaf spot and ringspot disease forecasting models were used to predict the occurrence of infection by both pathogens. The infection models use 30 minute environmental summaries of temperature, humidity, leaf wetness duration and rainfall to calculate the rate and hence the occurrence of infection. Environmental data was measured at 5 minute intervals using sensors positioned within the over-wintered Brussels sprout plot in the field.

5.1.2 Results

5.1.2.1 Disease observations from trap plant exposure within the over-wintered Brussels sprout crop

There were significantly higher numbers of ringspot lesions recorded daily on trap plants over the trial period (Figure 20). There were three main periods (17 – 23 May 2005, 14 – 18 June 2005 and 22- 30 June 2005) when ringspot was observed on trap plants. However low numbers of *Alternaria* lesions were recorded during the same periods however there were up to 10 times lower numbers of these lesions in comparison to ringspot. Due to the large numbers of lesions confirmation of dark leaf spot lesions could not be made using isolation of infected leaf material on agar. There were large numbers of powdery mildew lesions present and some white blister lesions on leaves (data not presented).

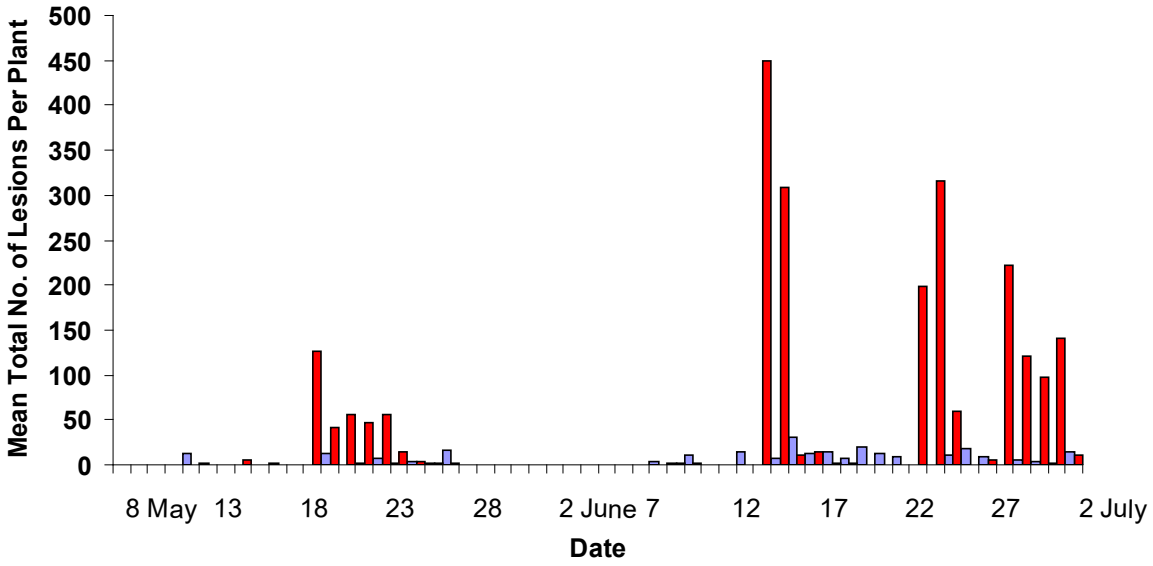
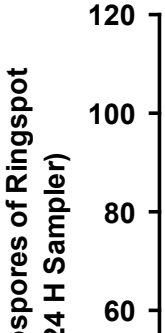


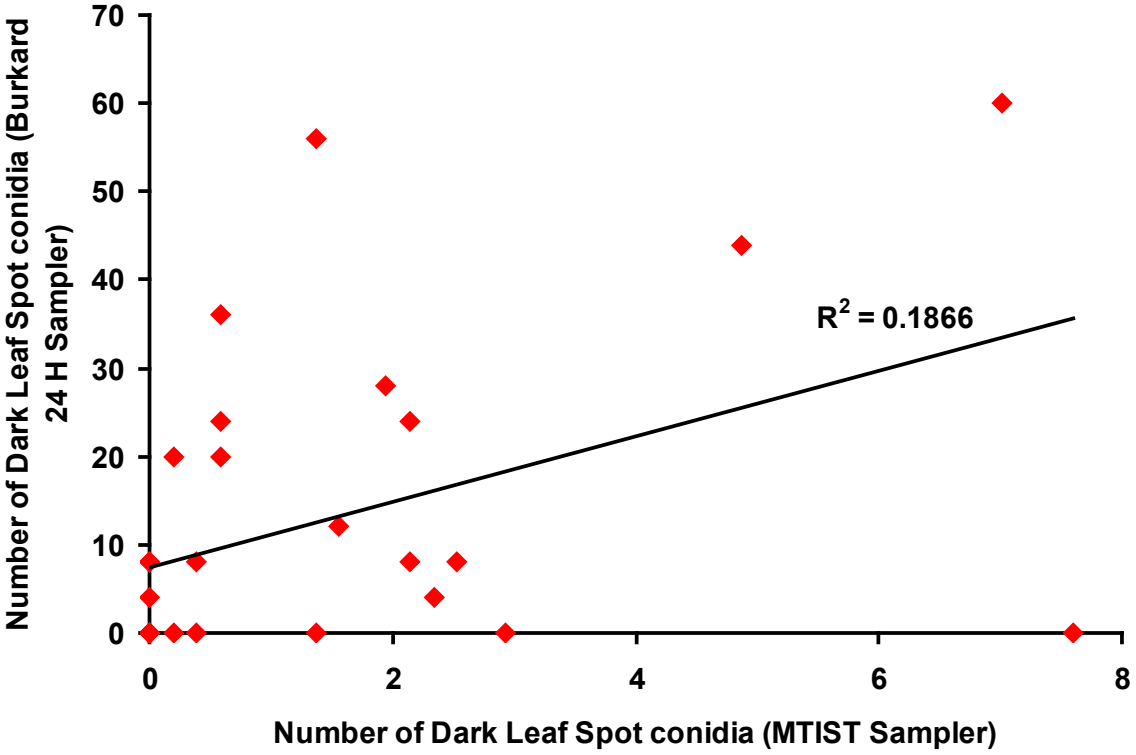
Figure 20. Mean total number of ringspot (■) and dark leaf spot (■) lesions per plant on trap plants exposed

5.1.2.2 Comparison of dark leaf spot and ringspot spore counts (m $^{-3}$) using 24 H volumetric and MTIST samplers

The number of ringspot ascospores trapped using the Burkard 24 H glass slide sampler was compared to ringspot ascospore counts taken within microtitre wells from the MTIST samplers using a microscope. The efficiency of each sampler in trapping ringspot ascospores and dark leaf spot conidia was compared per litre of air sampled Both air samplers were positioned in the crop at the same point and were air sampling over the same time periods. The number of dark leaf spot conidia were not counted in this trial because of the low numbers of lesions observed on trap plants exposed for 24 H periods in the crop (Figure 20). The results show (Figure 21) that there was a poor relationship between the two samplers in the number of ascospores of ringspot sampled per litre of air.



(a)



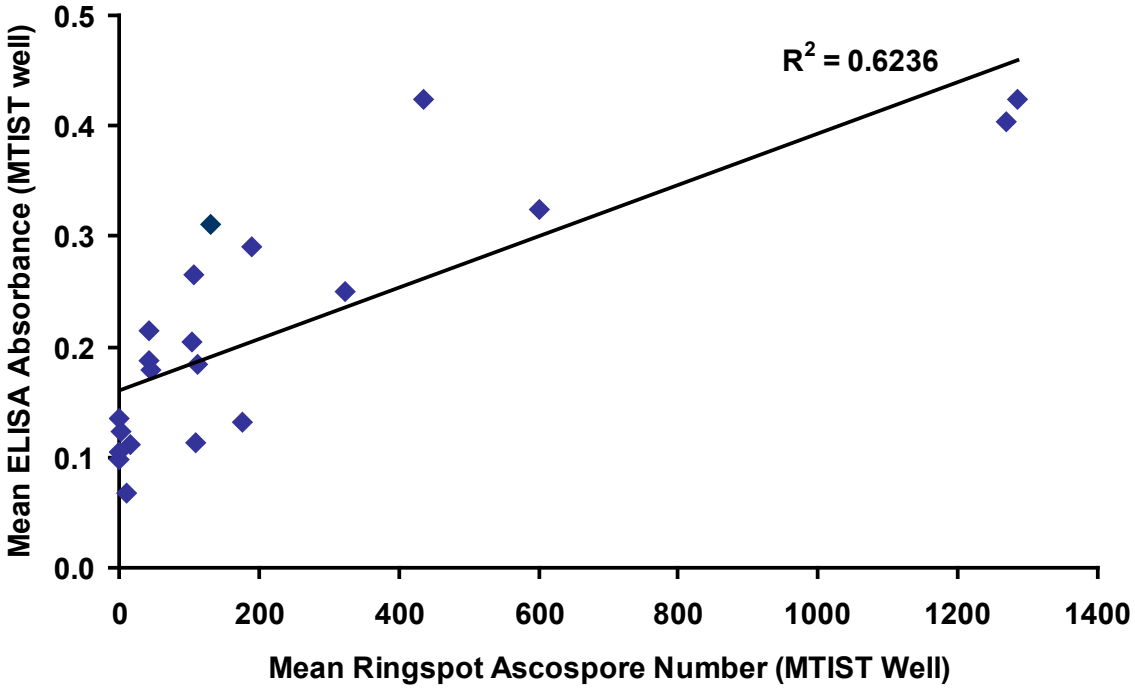
(b)

Figure 21. Comparison of the number of (a) ringspot ascospores m $^{-3}$ and (b) dark leaf spot conidia m $^{-3}$ in the field using a Burkard 24 H volumetric sampler and an MTIST sampler.

5.1.2.3 Comparison of daily ringspot ascospore and dark leaf spot conidial microscopic counts using an MTIST sampler and MTIST microtitre well PTA ELISA

Comparisons of the mean numbers of ringspot ascospores and dark leaf spot conidia from microscope counts against mean MTIST PTA ELISA of microtitre strips coated with silicone is shown in Figures 22a and 22b. There was a good linear relationship between numbers of ringspot ascospores in the MTIST wells (counted by microscopy) and the ELISA absorbance value (r^2 = 0.6236) shown in Figure 22. Untreated wells were counted for ascospore number and compared to the ELISA absorbance value of two assayed wells. There was a poor linear relationship between mean ELISA absorbance value and the mean number of dark leaf spot conidia counted per well using a microscope (Figure 22b). The maximum number of dark leaf spot conidia caught during the sampling period in one day was 178 (1 July 2005). However the maximum number of ringspot ascospores trapped in one day was 1270 (27 June 2005). The numbers of dark leaf spot conidia collected and retained in wells given a silicone coating was low. On most days an average of only 10 dark leaf spot conidia were trapped and retained in the MTIST wells. Dark leaf spot conidial numbers were too unreliable for further comparisons of trapping systems to be made with the exception of trap vessel coatings.

(a)



(b)

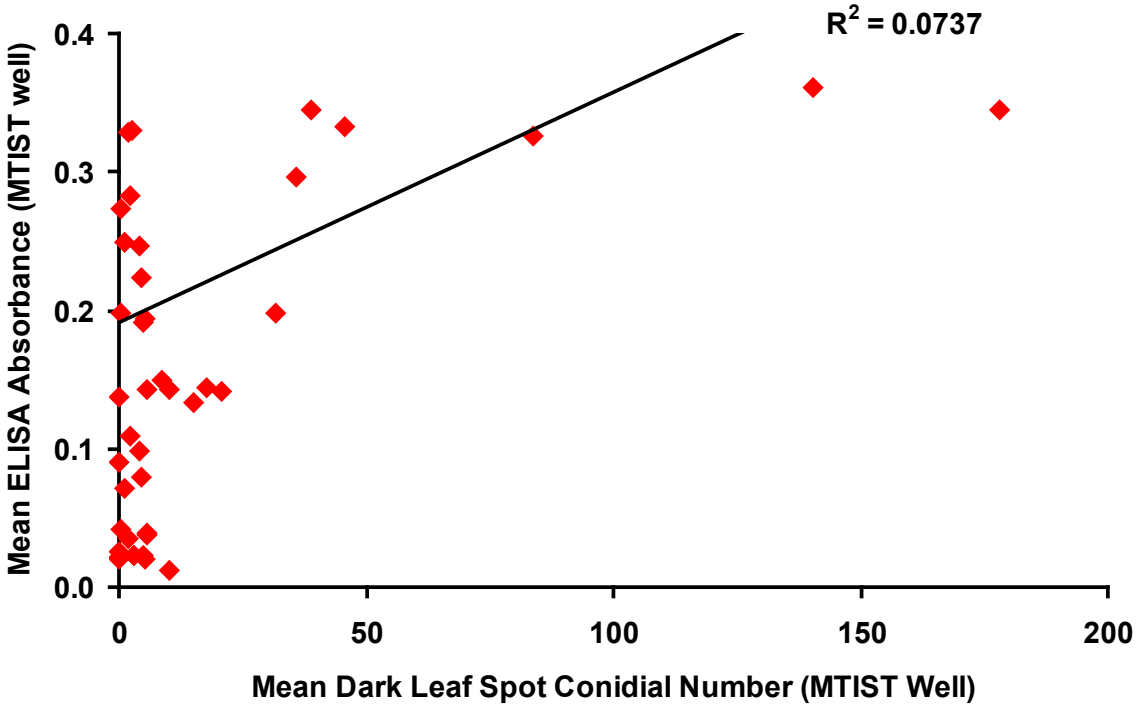
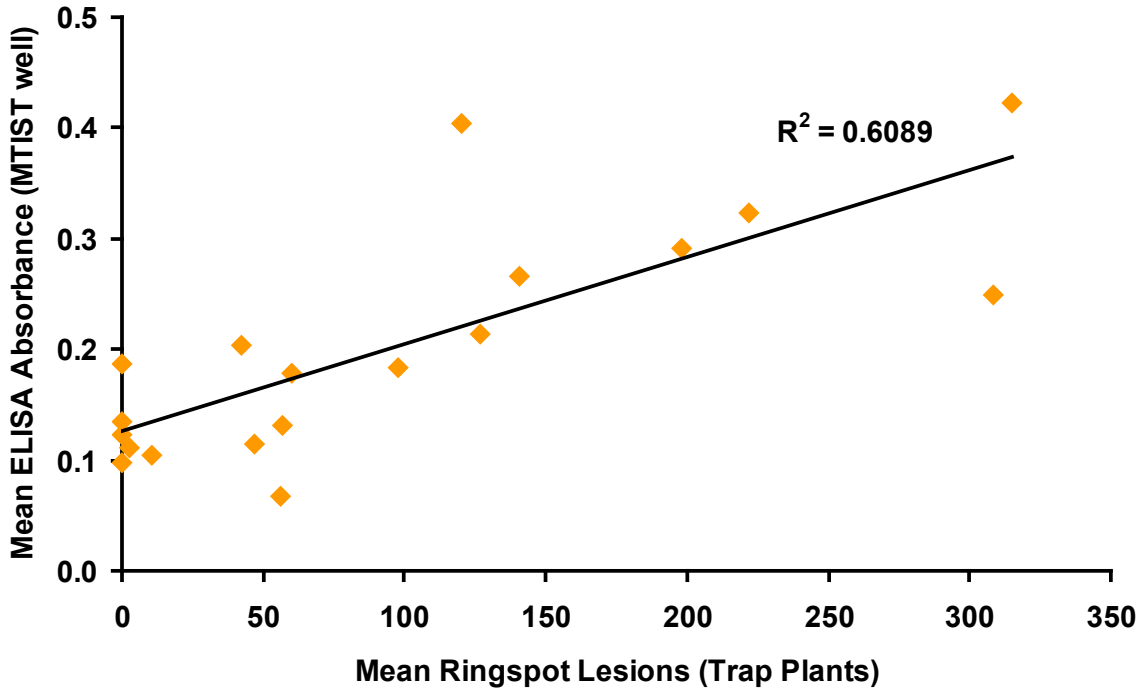


Figure 22. Relationship between PTA ELISA and number of air-borne spores of (a) ringspot (b) dark leaf spot

5.1.2.4 Comparison of ringspot lesion number on exposed trap plants and (a) mean MTIST ascospore number (microscopic count) (b) MTIST microtitre well PTA ELISA

The numbers of ringspot lesions on exposed trap plants, ascospore number and corresponding PTA ELISA values were compared (Figure 23). The relationship between PTA ELISA and mean ringspot lesion number per plant for 24 H exposure periods is shown in Figure 23a. The relationship between mean ringspot ascospore number per MTIST well and mean ringspot lesion number per plant is shown in Figure 23b. There appeared to be a closer linear relationship between ELISA absorbance and mean lesion number per plant in comparison to microscope counts from MTIST wells.

(a)



(b)

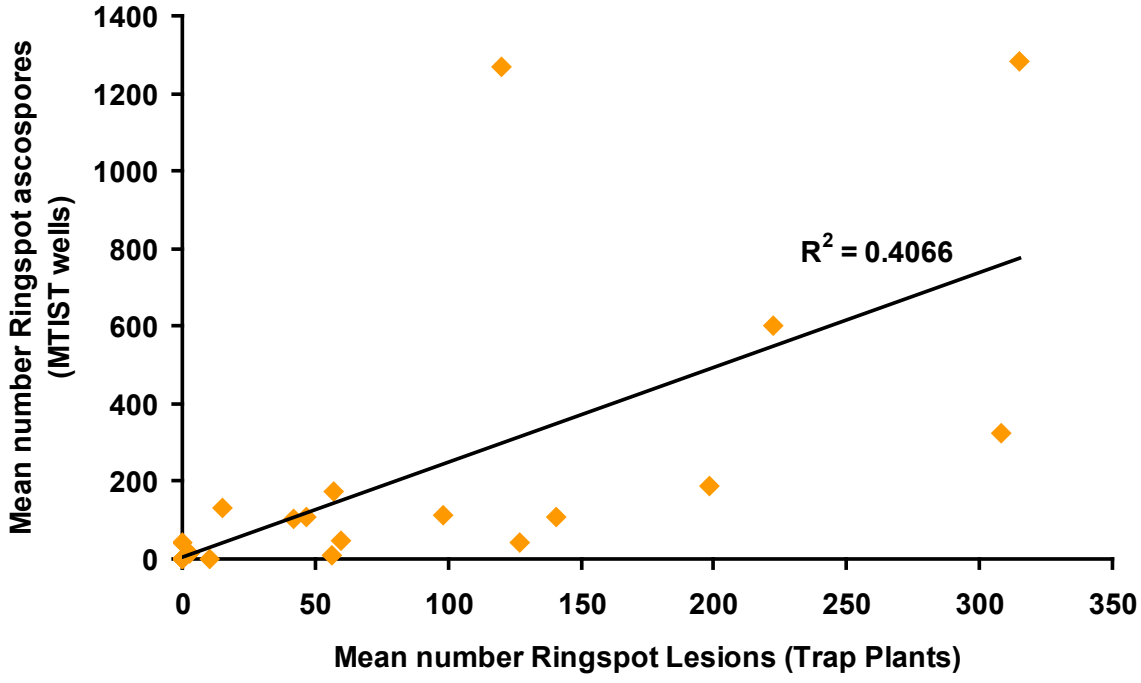


Figure 23. Relationship between trap plant lesion number (a) mean absorbance per MTIST (PTA ELISA) and (b) mean ringspot ascospore counts per MTIST well (microscopy).

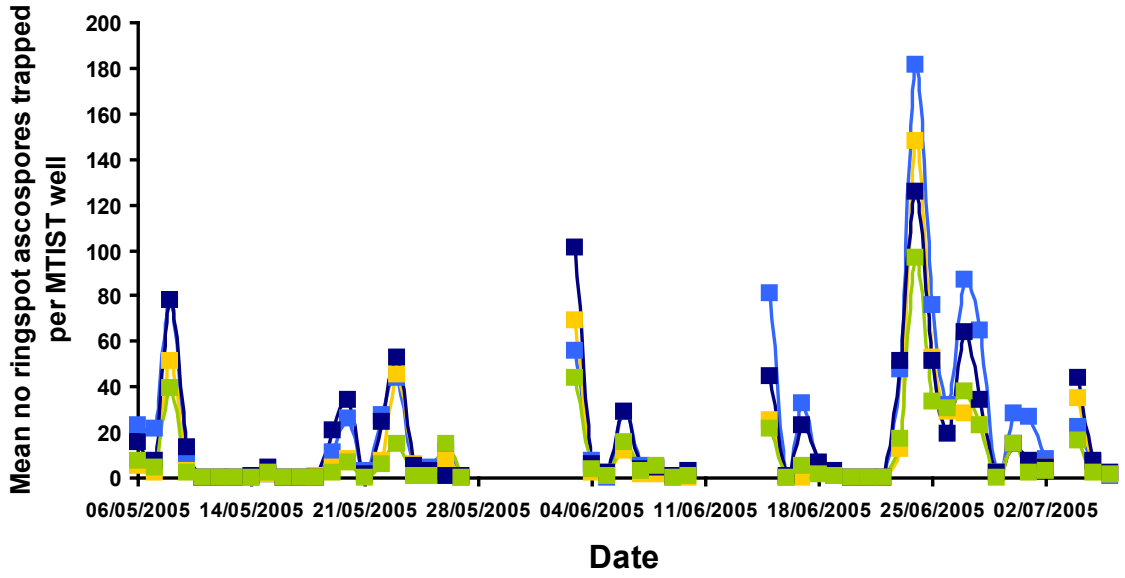
The maximum number of ringspot lesions per plant recorded on any day during the trial was approximately 300 – 350. Plants were given a 48 H wetting period after exposure to ensure

maximal ringspot ascospore infection. There were two days during the trial period when ascospore numbers in excess of 1200 were trapped within MTIST wells however only one of these periods resulted in high numbers of ringspot lesions on trap plants (Figure 23b).

5.1.2.5 Comparison of MTIST well coating material on the collection and retention of ringspot ascospores and dark leaf spot conidia

The collection and retention efficiency of several well coatings were compared to investigate if the number of dark leaf spot conidia and ringspot ascospores could be increased during sampling periods. This was carried out using an MTIST trap where each of four microtitre wells in the trap was coated with a different well coating prior to the start of sampling periods. The four well coatings consisted of an untreated (control), poly-l-lysine, paraffin wax and silicone. Tests were conducted simultaneously over the same trapping periods enabling direct comparisons to be made between well coatings on the number of dark leaf spot conidia and ringspot ascospores retained in each treatment. The mean numbers of ringspot ascospores and dark leaf spot conidia trapped in wells with different coatings are shown in Figure 24 a and b.

(a)



(b)

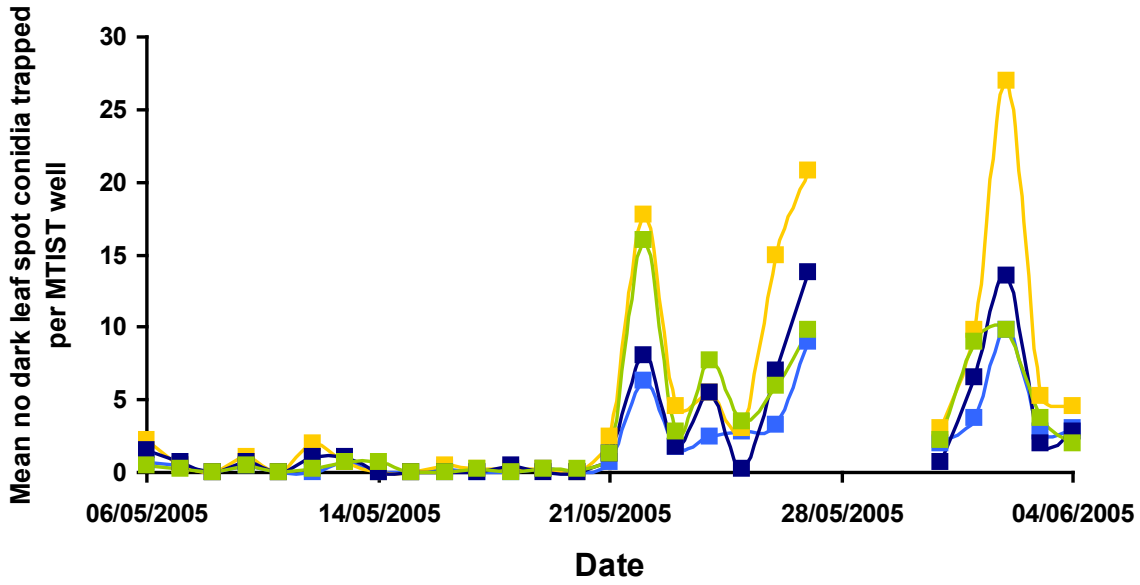


Figure 24. Comparison of MTIST well coatings comprising of untreated (■), silicone (■), Paraffin (■) and Poly-l-lysine (■) material on the collection and retention of (a) ringspot ascospores (b) dark leaf spot conidia.

The results show that coating microtitre wells treated with silicone gave consistently higher collection and retention of dark leaf spot conidia (Figure 24b). On many days during test

periods higher numbers of dark leaf spot conidia were observed in microtitre wells coated with silicone in comparison to all other well coatings. In contrast there was no consistent effect of well coating on collection and retention of ringspot ascospores in microtitre wells. On many days during the trial period high numbers were observed in untreated wells in comparison to other well coatings employed (Figure 24a). There was no detrimental effect of well coating on ELISA absorbance values in this trial (data not presented).

5.1.2.6 Prediction of dark leaf spot and ringspot infection conditions in the field

The results of using the Brassica$_{spot}$ disease forecasting system using collected environmental data is shown in Figure 25. There were few periods where dark leaf spot and ringspot infection were predicted in the field on trap plants. Infection conditions for both pathogens were fulfilled when an infection score of greater than 150 was recorded as designated with a red coloured bar (high risk) on the day where infection was predicted as having occurred. Days represented by a yellow bar (moderate infection risk – day with an infection score of 100-150) also occurred. A major high risk period occurred from the 22 - 24 June 2005. Other moderate risk periods were recorded on the 4 and 25 June 2005 and the 5, 6, and 7 July 2005.

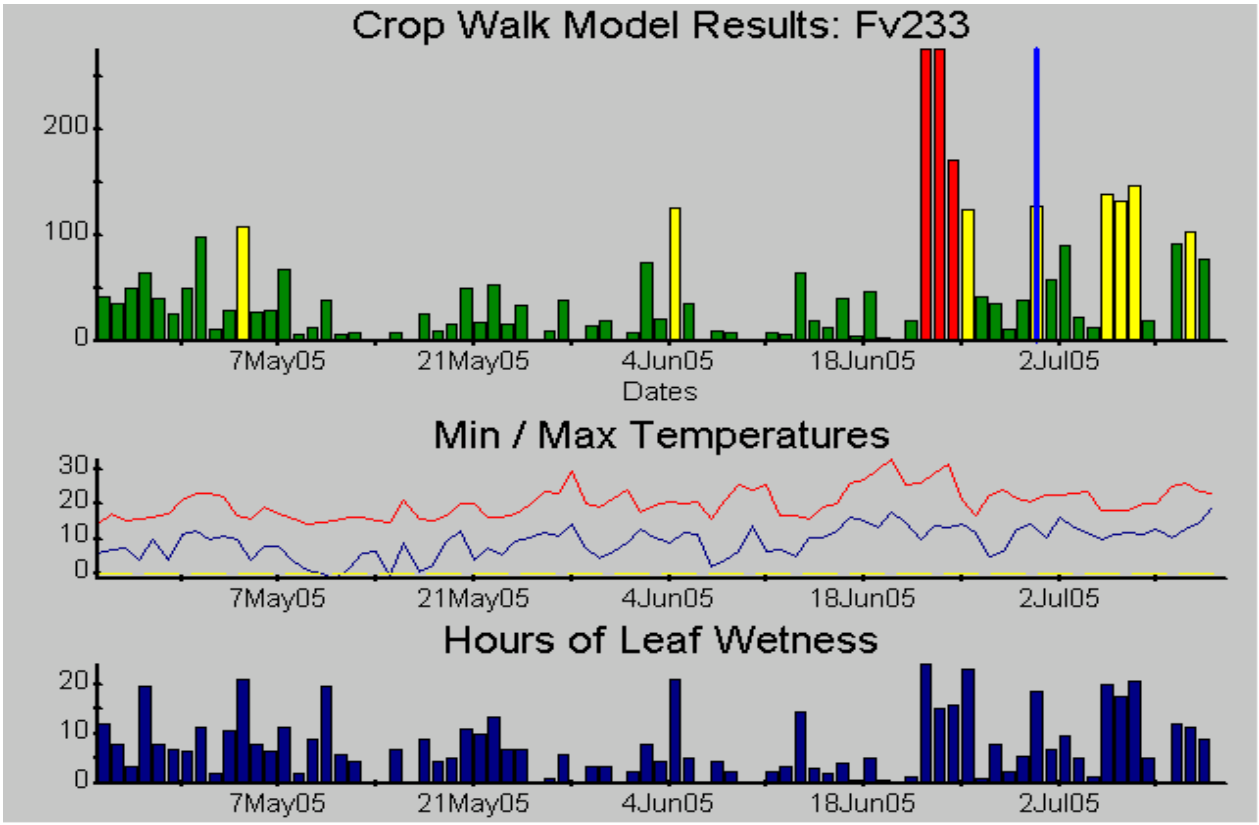


Figure 25. Prediction of dark leaf spot and ringspot infection in the field **.**

5.1.3 Conclusion

Development of accurate and rapid “in field” tests depends on the component parts of the system used. The lateral flow test is one important part of the system representing the end point in the detection of different target inoculum. However another important component in the development of a test is the sampling system used for the detection of inoculum. It is important that this aspect is properly optimised if the accuracy of the system is to be maintained. A number of trapping formats exist which were compared in this investigation. The MTIST trap which was used for testing the reaction of antibodies used in the development of lateral flow tests was compared to other formats (Burkard 24 H volumetric sampler). The results of this study suggest that the MTIST trap is more efficient at sampling smaller spore sizes in comparison the Burkard 24 H sampler. This resulted in the MTIST sampler proving more efficient at sampling ringspot ascospores however the Burkard sampler was more efficient at sampling dark leaf spot spores. The results are comparable to previous studies using a range of organisms with varying spore sizes (Wakeham et al., 2004). Some of these differences can be attributed to the presence or absence of well coatings in the trapping vessel. While ringspot ascospores did not require a well coating conidia of dark leaf spot were trapped more efficiently by the presence of a well coating in the trapping vessel (Figure 24b). Generally the numbers of dark leaf spot conidia present was low during the trial and only small numbers of lesions were observed on exposed trap plants over the duration of the trial (Figure 20). There was a good linear relationship between the ELISA value from MTIST wells and the numbers of ringspot ascospores. A poor linear relationship was observed between dark leaf spot conidial number and ELISA absorbance however there were low numbers of conidia observed and this may have affected these results (Figure 22a and b). It is possible that there is a non linear relationship between ELISA absorbance and dark leaf spot conidial number due to the size of dark leaf spot conidia. The ELISA absorbance mirrored closely the number of lesions present on exposed trap plants (Figure 23a). These tests indicated that ELISA and (lateral flow tests) would be suitable for predicting both dark leaf spot and ringspot inoculum in the field and that this could be equated to a disease risk. No well coating within trapping vessels will be necessary for ringspot detection (Figure 24a). However dark leaf spot detection sampling could be optimised further by employing a silicone coating on the trapping vessel (Figure 24b). The comparisons using numbers of ringspot lesions will to some extent be affected by the presence of infection risks on some days during the trial (Figure 24). This resulted in some plants receiving different wetting durations during exposure in the field which may explain some of the results relating to dark leaf spot conidial detection where long periods of wetness are required.

6. ASSESSMENT OF THE COMPETITIVE LATERAL FLOW SYSTEM FOR THE RAPID DETECTION OF FIELD TRAPPED INOCULUM OF *ALTERNARIA BRASSICAE*

6.1 Introduction

Eight hundred *Alternaria* competitive lfd prototypes were supplied by CSL for commercial field trial usage. Evaluation of a range of these lfd prototypes was carried out to determine that the detection threshold was the same as that observed with earlier prototypes supplied by CSL (Year 2 Annual report 4.2.2)

6.2 Evaluation of CSL competitive lfd prototypes for field commercial trials using an *Alternaria brassicae* calibration series

6.2.1 Materials and Methods

Isolates of *A. brassicae* (Table 1), taken from the Warwick 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 (including mycelial fragments) of *A. brassicae* were harvested, collected and mixed in a lateral flow Type C extraction buffer. A serial doubling dilution series was made to provide 60µl aliquots of conidial/mycelial material of *A. brassicae* ranging from 9.6 x 10^4 to 24 dark leaf spot conidia present. Each 60µl conidial suspension was applied to a separate lateral flow device (prototype BO2, A03 (produced and tested in Year 2), DO2, D08, D10 and D13 (new lfds produced for Year 3 commercial trials) and, following the 4 minute assay processing time the development of Test and Control lines were recorded. A negative control of extraction buffer alone was included for each lfd prototype.

6.2.2 Results

The results (Table 12) show that the developed competitive lateral flow device had the potential to detect in an *Alternaria* mycelial soluble extract 47 conidia per sample. This was observed however only when employing prototype B02. In an *Alternaria* soluble mycelial extract containing 24 spores per sample full test line development was observed. Test line development denotes limit of detection ie *Alternaria* not detected. Employing prototype A03 a weak test line was barely visible when a mycelial soluble extract of 94 *Alternaria* conidia was applied to the device and, with full test line development observed at a soluble mycelial extract of 47 *A. brassicae* conidia applied. Using the new batch of *Alternaria* lfds (prototypes DO2, DO8, D10 and D13) test detection sensitivity was markedly reduced with weak test line development observed when a mycelial extract containing 1500 *A. brassicae* conidia was applied to lfd prototypes D10 and D13. For prototypes DO2 and D08 weak test line development was at a soluble mycelial extract of 750 *A. brassicae* conidia. A clear control line was observed in all tests showing that each device had operated successfully.

Table 12. Detection threshold for *Alternaria brassicae* using a competitive lateral flow assay

Alternaria test sample appled to each lfd		Competitive lfd protype					
Alternaria soluble mycelial extract doubling dilution series	**No Alternaria conidia present in lfd test sample**	**A03**	**B02**	**D2**	**D8**	**D10**	**D13**
Neat	**9.6 x 10^4**	**C**	**C**	**C**	**C**	**C**	**C**
1 in 2	**4.8 x 10^4**	**C**	**C**	**C**	**C**	**C**	**C**
1 in 4	**2.4 x 10^4**	**C**	**C**	**C**	**C**	**C**	**C**
1 in 8	**1.2 x 10^4**	**C**	**C**	**C**	**C**	**C**	**C**
1 in 16	**6x10^4**	**C**	**C**	**C**	**C**	**C**	**C**
1 in 32	**3x10^4**	**C**	**C**	**C**	**C**	**C**	**C**
1 in 64	**1500**	**C**	**C**	**C**	**C**	**C t**	**C t**
1 in 128	**750**	**C**	**C**	**Ct**	**Ct**	**C T**	**C T**
1 in 256	**375**	**C**	**C**	**C T**	**C T**	**C T**	**C T**
1 in 512	**188**	**C**	**C**	**C T**	**C T**	**C T**	**C T**
1 in 1024	**94**	**C t**	**C**	**C T**	**C T**	**C T**	**C T**
1 in 2048	**47**	**C T**	**C t**	**C T**	**C T**	**C T**	**C T**
1 in 4096	**24**	**C T**	**C T**	**C T**	**C T**	**C T**	**C T**
CSL buffer alone	**0**	**C T**	**C T**	**C T**	**C T**	**C T**	**C T**

C	Control line observed
T	Test line observed (*Alternaria* not detected)
t	Weak test line development (Low level *Alternaria* detected)

6.2.3 Conclusion

Lateral flow test prototypes were supplied by CSL. The reaction of some of these prototypes is outlined in earlier in this report (see section 4.3). Dark leaf spot lateral flow prototypes supplied by CSL on subsequent occasions gave variability in the concentration of dark leaf spot conidia detected (lateral flow positive tests). It was important that the sensitivity of the test was maintained when new batches of tests were supplied as the required threshold of dark leaf spot spores would not be detected as a positive result if less sensitive tests were used. The problem in sensitivity was ascribed primarily to the antibody conjugate used in the test. Prototypes supplied by CSL used a latex/antibody conjugate however it was decided that the required sensitivity of the lateral flow test could be improved by using a immunogold/antibody conjugate within the lateral flow test.

6.3 Development of lateral flow device for detection of field inoculum of dark leaf spot (*Alternaria brassicae*)

6.3.1 Materials and Methods

Competitive lateral flow components

Preliminary tests were carried out using lateral flows comprised of a Millipore 135 HiFlow™ cellulose ester membrane direct cast on to 2ml Mylar backing (Cat No. SHF2400225, Millipore Corp, USA.), an absorbent pad (Cat No. GBOO4, Schleicer and Schuell, Germany) and a sample pad (Cat No. T5NM, Millipore Corp., USA). Following lateral flow construction (Figure 26) control lines of an anti-mouse serum were sprayed directly on to the membrane surface using a flat bed air jet dispenser (Biodot Ltd, The Kingley Centre, West Sussex, UK). A collected *Alternaria brassicae* soluble mycelial suspension, prepared (see section 4.3) was adjusted to a protein concentration of 500µg ml^{-1} in PBS and applied as a test line to the membrane using a flat bed air jet dispenser (Biodot). Membranes were air dried at 35ºC for a period of 4 hours. The test and control line labelled lateral flows were cut in to 4 mm strips and each strip housed within a plastic case (Schleicer and Schuell, Germany).

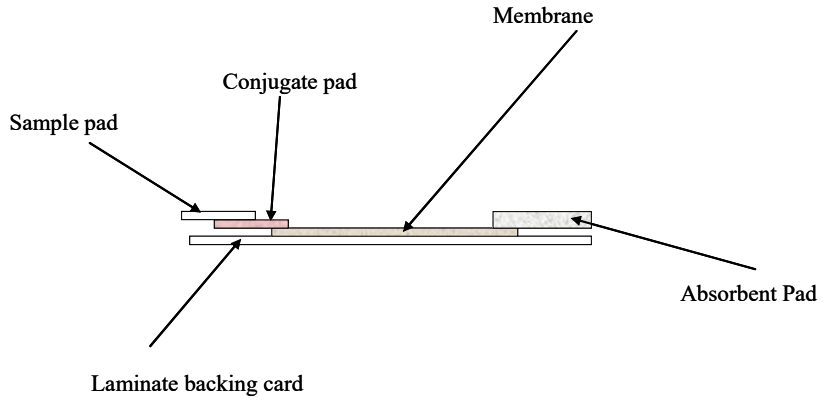


Figure 26. Schematic overview of a lateral flow device.

Antibody conjugation

A volume of 500 µl purified IgM monoclonal antibody, produced at Warwick HRI Wellesbourne to *Alternaria brassicae* (coded HRI EMA 212), 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 2 ml in Phosphate buffered saline buffer (PBS) and incubated on a roller incubator for 3 hours. After which the antibody bound gold beads were collected by centrifugation (4000 xg) and resuspended to a final volume of 1.625ml in Warwick HRI application buffer (20mM Sodium phosphate buffer, 100Mm Sodium Chloride, 0.25%

Trehalose, 0.1% Sucrose, pH 7.2). Each sample pad of each lateral flow device had 60µl of the antibody gold conjugate solution added before air drying at 27ºC. Following air-drying the lateral flows devices were mounted within a plastic housing device (Schleicer and Schuell).

Detection of Alternaria by competitive lateral flow technology

Alternaria brassicae conidial plate washings (including soluble mycelial fragments) were prepared as described above but this time in Warwick HRI extraction buffer (0.05M Tris HCL, 0.15M NaCl, 0.4% Triton X100, 0.2% Tween 20, 0.2% BSA, 0.12% Geropan). A dilution series was made (1:800 to 1:2000) to provide 60µl aliquots of conidial / mycelial material of *A. brassicae* ranging from 120 to 50 conidia. Following a 6 minute assay processing time the development of test and control lines were recorded. A negative control of extraction buffer alone was included.

6.3.2 Results

The results (Table 13, Plate 7) show that the developed Warwick HRI competitive lateral flow device has a comparable level of sensitivity to the original CSL prototypes supplied (CSL A and B prototypes, Table 1). A clear control line was observed in all tests showing that each device had operated successfully.

Table 13. Detection threshold for dark leaf spot conidia using the biodiagnostics competitive lateral flow prototype

Alternaria **soluble mycelial extract doubling dilution series**	**No. *Alternaria* conidia present applied in lfd test sample**	**HRI prototype competitive lfd**
1 in 800	120	C
1 in 1000	100	C
1 in 1400	80	C t
1 in 1600	70	C t
1 in 2000	50	C T
HRI buffer alone		C T

C	Control line observed
T	Test line observed (*Alternaria* not detected)
t	Weak test line development (Low level *Alternaria* detected)

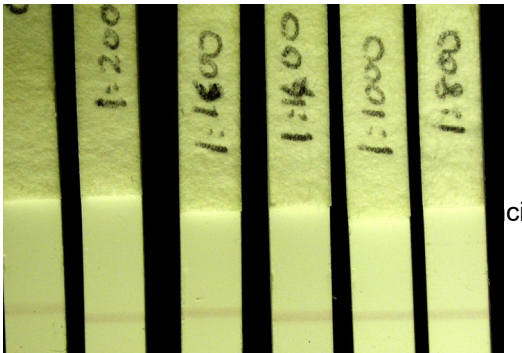


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Plate 7. ***Alternaria*** **competitive lateral flow device using an immunogold carrier**

6.3.3 Conclusions

The results show that using the immunogold antibody conjugate a competitive lateral flow device could be constructed which had a visual sensitivity at a dilution of 1:1000 dark leaf spot. This was the required sensitivity for dark leaf spot conidial detection in the field and equated to 23 – 94 conidia of dark leaf spot (see section 4). This approximates the level of dark leaf spot required in the field for disease development on vegetable brassica crops. However using a lateral flow reader device where the amount of gold conjugate on the test line could be quantified the sensitivity of the device could be taken down to a dilution of 1:2000 dark leaf spot which is much lower than the level of dark leaf spot inoculum required for dark leaf spot development in vegetable brassica crops.

6.4 Optimisation of dark leaf spot competitive lateral flow format (*clfd*) for commercial usage

6.4.1 Direct conjugation of EMA 212 to gold spheres

Three ml of EMA 212 was supplied at 0.5mg ml^{-1} to British Biocell International (BBI, Golden Gate, Ty Glas Avenue, Cardiff, UK) and conjugated directly to 40nm gold in a 2mM borax buffer, Ph 8.2 containing 0.095% azide. Of the gold conjugate processes tested only one conjugate proved optimal for conjugation of EMA 212. This conjugate was supplied to Warwick HRI at an OD 10.1.Tests carried out at BBI determined that the conjugate bound to 5ng ul^{-1} Goat anti-mouse IgM (BBI stocks). BBI have retained a protocol for future conjugation of EMA 212.

6.4.2 Optimisation of lateral flow phase

Bio-Diagnostics (www.bio-diagnostics.co.uk) a UK's leading specialist in the field of autoimmune diagnosis, was supplied with the Warwick HRI developed *Alternaria* competitive lateral flow for optimisation in a dry assay format for use in commercial studies. The EMA212 40nm gold conjugation (OD 10.1) was supplied for use within the dry assay format.

Bio-Diagnostics replicated the Warwick HRI assay format but reported poor background and control line development which was too slow. Seven extraction buffers (Bio-diagnostics) were tested however no improvement was observed in comparison to the developed Warwick HRI buffer system. Bio-Diagnostics investigated a range of solid phase material

and were able to produce a clean streak free membrane with positive results visible within 5 minutes. Further improvement of the test and control line is recommended to fully optimise line definition. Bio Diagnostics supplied Warwick HRI with 120 dark leaf spot lateral flow devices for laboratory and field studies which are reported in section 7 of this report .

7. TESTS WITH LATERAL FLOWS FOR DARK LEAF SPOT DETECTION IN COMMERCIAL TRIALS

7.1 Monitoring airborne inoculum of the dark leaf spot pathogen (*A. brassicae*) in commercial vegetable brassica crops

7.1.1 Introduction

Tests were conducted using the dark leaf spot lateral flow device to monitor the number of dark leaf spot conidia in samples and compare this with dark leaf spot disease development in commercial crops of Brussels sprouts and cabbage. Trials were conducted during 2005 at two vegetable brassica production sites in different areas of the UK.

7.1.2 Materials and Methods

7.1.2.1 Crop experimental design and crop disease observations

Trials were conducted in commercial crops at Skegness (T. A. Smith & Co, The Elms, Croft, Skegness, Lincs.), Alphagrow (Hesketh Bank, Preston, Lancashire) and run in conjunction with grower/consultants. At Skegness a Brussels sprout crop was monitored and assessed for disease development. At Hesketh bank the trial was located in an over-wintered cabbage crop. The trial design consisted of single plots (15 x 15 m) from which data on environmental conditions were taken using an Aardware data logger (Aardware Design, Walton on Thames). Trials were located in an unsprayed part of the crop. Observations were taken on disease levels on 5 – 10 marked plants which had their leaves tagged and numbered. Disease observations on tagged leaves were taken weekly. The Brassica$_{spot}$ system was used to determine disease (infection) risk on each day during the trial. The Aardware data logger was positioned adjacent to the trial site and provided information on temperature wetness duration, humidity and rainfall at 30 min intervals (with a 5 minute log interval).

7.1.2.2 Air sampling at each trial site

Air samples were taken continuously over a period of 6 weeks using a Burkard 7 day cyclone sampler at both trial sites. This sampler automatically changes the trapping vessel each day at a preset time period. The seven tubes within the sampler (one for each day) can be changed weekly by using fresh tubes. The sampler was operated for 12 H per day between 05:00 H and 17:00 H at a sampling volume of 16.5 litre of air min ^{2}. Addtionally a weekly cyclone sample was collected (at the Hesketh Bank site only) using another Burkard cyclone sampler where the trap vessel was replaced on a weekly basis by hand. The weekly cyclone sampler was operated for 12 H per day between 05:00 H and 17:00 H at a sampling volume of 10 litre of air min ^{2}. The eppindorf sample collection vessels (from the cyclone samplers) were stored prior to assay development at -20ºC. Sample vessels from all traps were tested in the laboratory using the prototype dark leaf spot lateral flow device and the responses recorded.

7.1.2.3 Detection and quantification of dark leaf spot using lateral flow devices

Approximately 110µl 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 100µl aliquot of each spore suspension was then applied to a sample pad of an individual competitive lateral flow device (Biodiagnostics prototype). Determination of test line development was made by visual assessment and, using an EVL one step Reader (EVL P.O. Box 198, 3440 AD Woerden, The Netherlands). Each device was scanned on two occasions using the EVL one step Reader. Using a dark leaf spot conidial and mycelial dilution series it was approximated that a reader value on the test line of below 0.8 indicated a positive result for the required amount of dark leaf spot inoculum necessary for dark leaf spot disease development. Due to the limited number of prototypes available it was not possible to carry out lateral flow tests on all sampling periods. However by utilising disease forecasts at the site a number of risk days could be compared with non risk days for the presence or absence of dark leaf spot inoculum at the required concentration for disease development to occur.

7.1.2.4 Visual microscopic counts of dark leaf spot from air samples

Samples used in tests with lateral flows were checked visually for the presence or absence of dark leaf spot conidia. Approximately 10µl of extraction buffer was removed from each sample vessel prior to testing with the lateral flow device and placed on a microscope slide. Estimates of the numbers of dark leaf spot conidia were taken by counting the number of dark leaf spot conidia in each 10µl sample before multiplying by 10. The total number of dark leaf spot conidia per sample was express per m^3 of air sampled.

7.1.2.5 Method of prediction of dark leaf spot infection in the field

(see section 5.1.1.8)

7.1.3 Results

7.1.3.1 Detection of dark leaf spot conidia in air samples using lateral flow devices and disease development at Skegness 2005

The results of using dark leaf spot lateral flow device on air samples collected at Skegness (Biodiagnostics prototype) are shown in Table 14. The EVL one step reader device gives an optical reading of the amount of captured immunogold on the test and control line. When more immunogold is captured on either line the visualisation of the line is more discrete. However use of the reader enables the device to used semi-quantitatively to determine the amount of dark leaf spot present in the sample. It was not possible to test all days where air samples were taken due to the restricted numbers of lateral flow devices available.

Table 14. Visual daily assessment of test line for sampling periods at Skegness in 2005

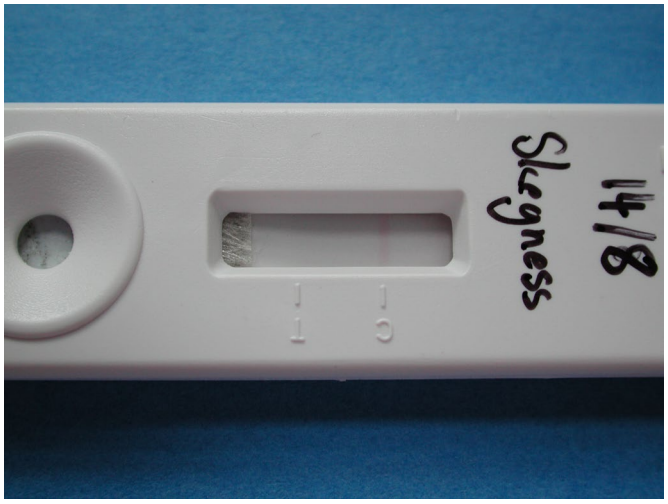
Field exposure period	Test line observation (Positive =No)
13th August	No
14th August	No
20th August	Yes
23th August	No
25th August	Yes
26th August	Yes
27th August	Yes
31st August	Yes
2nd September	Yes
6th September	Yes
7th September	Yes
10th September	Yes

Table 15. Dark leaf spot conidial concentration per m^3 at Skegness in 2005

Field exposure period	Dark leaf spot conidia m^3	Immunogold Line Reading Test	Control
13th August	31.9	0	0.7
14th August	37.0	0	1.0
20th August	18..5	1.1	1.8
23th August	32..8	0.6	1.3
25th August	6. 7	1.7	2.4
26th August	26.9	1.6	1.7
27th August	13.4	0.8	1.1
31st August	2.5	1.5	1.7
2nd September	2.5	1.1	1.2
6th September	NA	1.1	1.2
7th September	2.5	1.3	2.8
10th September	NA	1.1	2.2

Using the dark leaf spot lateral flow prototype gave positive results using samples collected on the 13, 14, and 23 August 2005 (the absence of the test line). There was a close relationship between the amount of dark leaf spot conidia in each sample and the degree of accumulation of gold on the test line on each lateral flow test. The results for the 14 August 2005 are also shown in Plate 8a were there was no visualisation of the test line on the lateral flow device. Although there was no visualisation of the test line on the 23 August 2005 a small amount of immunogold was accumulated on the test line as measured by the EVL one step reader device.

(a)



(b)

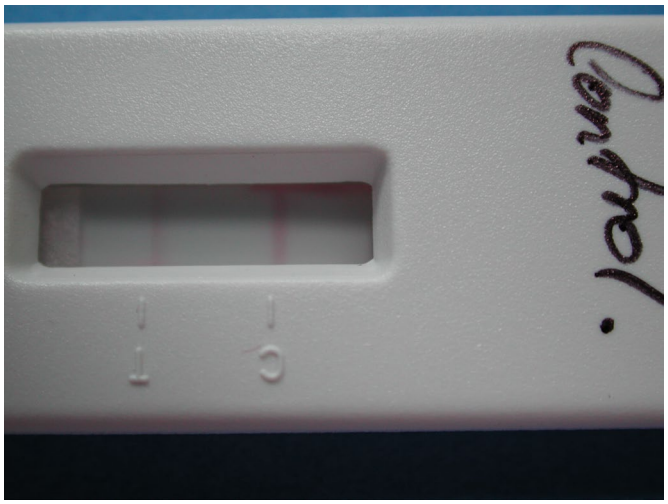


Plate 8 Dark leaf spot lateral flow test Skegness 2005 (a) 14 August 2005 - positive (b) control test – negative

7.1.3.2 Development of dark leaf spot during the trial period at Skegness 2005

The development of disease at the Skegness site is shown in Figure 27. Dark leaf spot lesions were already present in the Brussels sprouts crop at the beginning of the trial. There were two periods of disease development by dark leaf spot in the crop. These occurred after the 11 August 2005 and again after the 31 August 2005. There was one period of ringspot development in the crop which occurred after the 17 August 2005 (Figure 27). Both ringspot and dark leaf spot development on unsprayed plants at the Skegness site was high as approximately 90 - 100 lesions were recorded per plant by the beginning of September 2005.

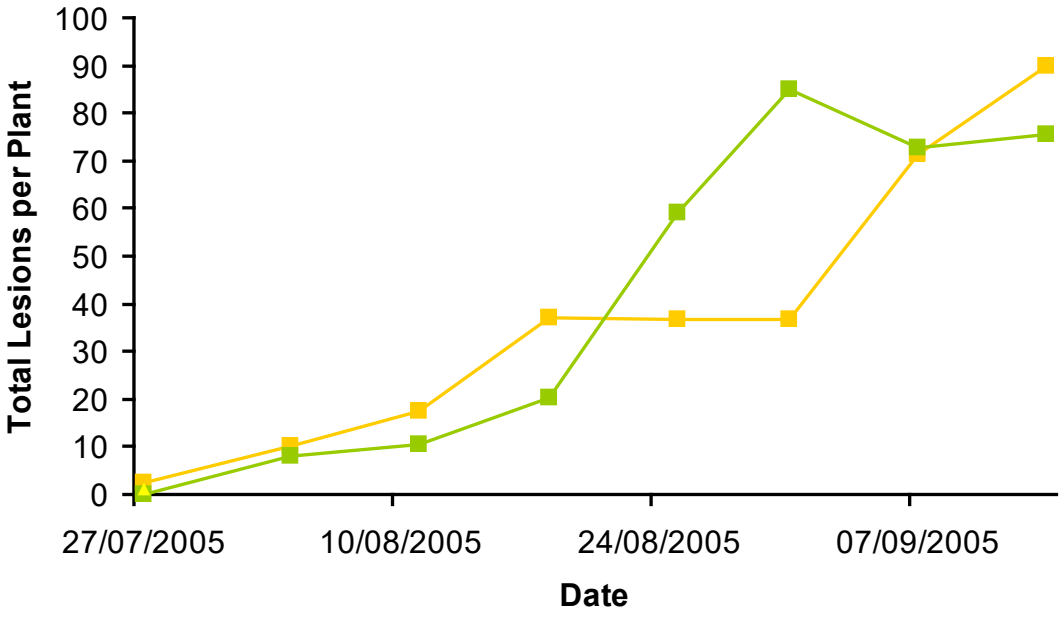


Figure 27. Ringspot (■) and dark leaf spot (■) disease development at Skegness in 2005

7.1.3.3 Prediction of dark leaf spot and ringspot infection conditions at Skegness in 2005

The results of using the Brassica$_{spot}$ disease forecasting system at the Skegness site in 2005 are shown in Figure 28. There were many periods when dark leaf spot and ringspot infection was predicted in the field. Infection conditions for both pathogens were fulfilled when an infection score of greater than 100 was recorded as designated with a red coloured bar (high risk) on the day where infection was predicted as having occurred. High risk periods occurred on the 14, 20, 23, 25, 26, 27 31 August 2005 and the 2, 6, 7 10, 11, 12 14, and 16 September 2005. Moderate infection risks (yellow bars) were recorded on the 12, 13 17, 22 August 2005, 1 3, 4, 5, 9, 13 September 2005 for both pathogens (Figure 28). Other risk period recorded (Figure 28) were outside the period when the trial was operating.

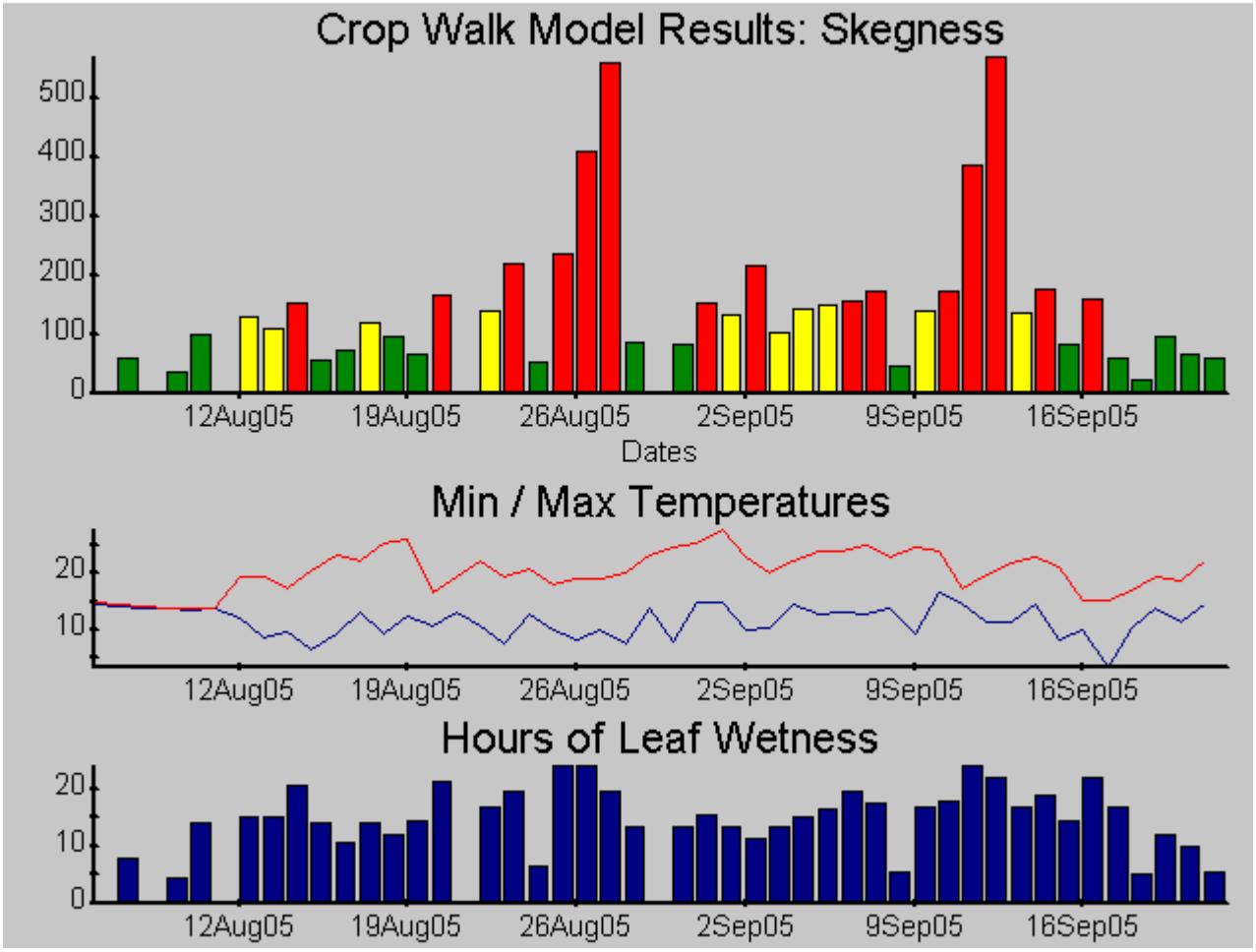


Figure 28. Prediction of dark leaf spot and ringspot infection at Skegness 2005

7.1.3.4 Detection of dark leaf spot conidia in air samples using lateral flow devices and disease development at Hesketh Bank 2005

Results of testing air samples collected at Hesketh Bank (Biodiagnostics prototype) are shown in Table 16. The EVL one step reader device was used to give an optical reading of the amount of captured immunogold on the test and control line on the lateral flow device. When more immunogold is captured on either line the visualisation of the line is easier however low levels of reaction cannot be visualised. Use of the reader enables the device to used semi-quantitatively to determine the amount of dark leaf spot present in samples at Hesketh Bank. Additionally weekly air samples were taken using a, 7 day cyclone sampler were tested for the presence of dark leaf spot conidia and test line visualisation on the lateral flow device. It was not possible to test all days where air samples were taken due to the restricted numbers of lateral flow devices available.

Table 16. Visual assessment of test line for sampling periods at Hesketh Bank in

2005 (a) daily samples (b) weekly samples

(a) Daily sampling periods

Field exposure period	Test line observation (Positive =No)
14th August	Yes
16th August	Yes
22td August	Yes
24th August	Yes
25th August	Yes
31st August	Yes
2nd September	Yes
9th September	Yes
10th September	Yes

a) Weekly sampling periods

Field exposure period	Test line observation (Positive =No)
10 - 17th August	Yes
17 - 24th August	Yes
24td August – 1 st September	Yes
1 st - 8 th September	Yes
8 th - 15th September	Yes

Table 17. Dark leaf spot conidial concentration per m^3 at Hesketh Bank in 2005

(a) Daily sampling periods

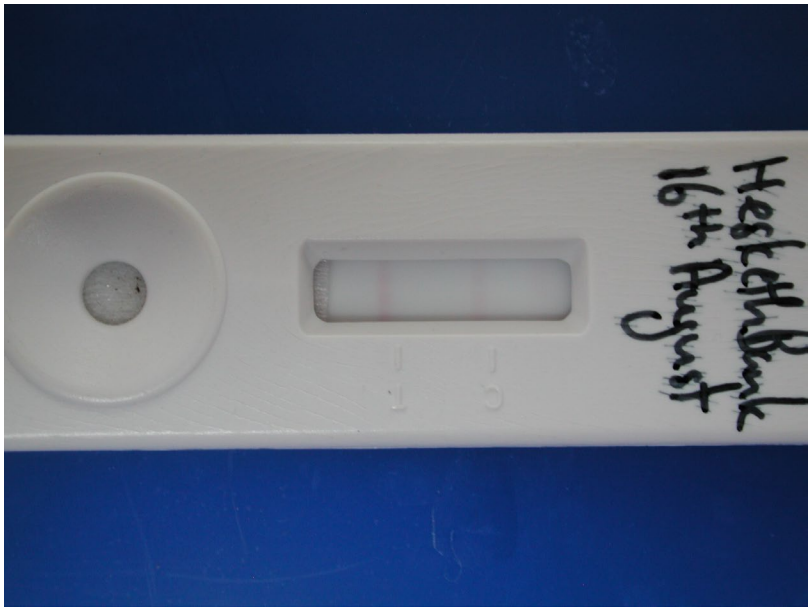
Field exposure period	Dark leaf spot conidia m^3	Immunogold Line Reading	
		Test	Control
14th August	1.0	2.3	2.2
16th August	0	2.1	2.8
22td August	3	2.1	2.8
24th August	2	1.2	2.2
25th August	3	1.0	2.0
31st August	0	2.0	1.5
2nd September	5	2.4	2.7
9th September	0	0.6	1.7
10th September	0	2.5	3.7

(b) Weekly sampling periods

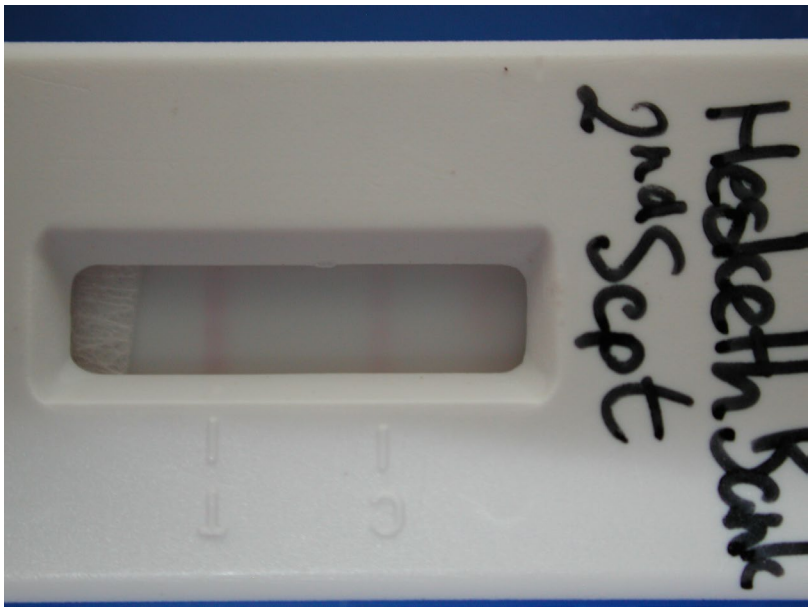
Field exposure period	Dark leaf spot conidia m^3	Immunogold Line Reading	
		Test	Control
10 - 17th August	7	0.4	1.0
17 - 24th August	3	0.0	1.1
24td August – 1 st September	0	1.3	1.8
1 st - 8 th September	27	0.4	1.2
8 th - 15th September	12	0.9	1.6

Using the dark leaf spot lateral flow prototype gave negative results using samples collected on all days tested (the presence of the test line, Table 16). There was a close relationship between the amount of dark leaf spot conidia in each sample and the degree of accumulation of gold on the test line on each lateral flow test. The results for the 16 August 2005 and the 2 September 2005 are also shown in Plate 9a and 9b. There was visualisation of the test line on the lateral flow device on both days indicating a negative result. A low reading on the test line as measured by the EVL one step reader device was recorded for the 9 September 2005 however no dark leaf spot conidia were recorded in the sample tube for that period (Table 17). Weekly samples had higher levels of dark leaf spot conidia within the samples however these did not produce a positive result using the dark leaf spot lateral flow device due to blockage of the sample pad (Plate 9c).

(a)



(b)



(c)

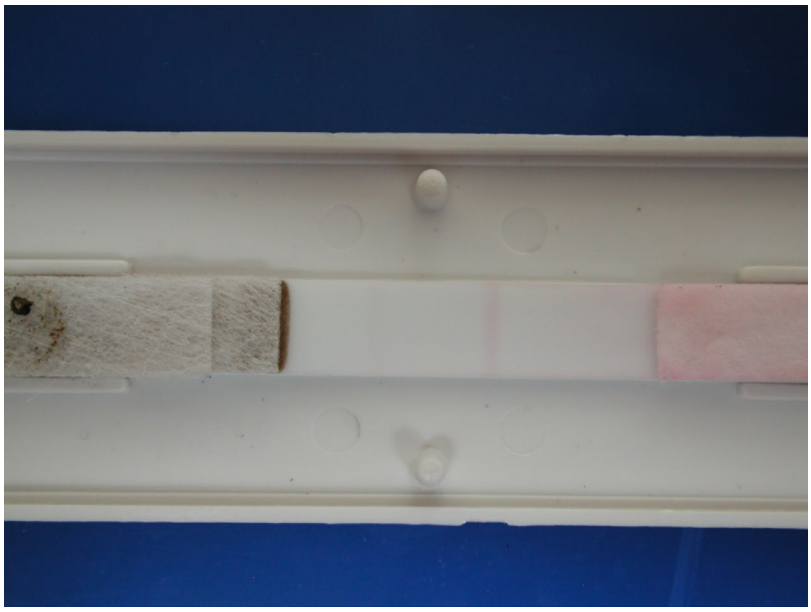


Plate 9 **Dark leaf spot lateral flow tests Hesketh Bank 2005 (a) 16 August 2005 – negative (b) 2 September 2005 – negative (c) Membrane blocking on the weekly test**

7.1.3.5 Development of dark leaf spot during the trial period at Hesketh Bank 2005

The development of disease at the Hesketh Bank site is shown in Figure 29. Dark leaf spot lesions were already present in the cabbage crop at the beginning of the trial. There were low numbers of dark leaf spot lesions present in the trial site over the duration of the trial (approximately 10 lesions per plant). Dark leaf spot and ringspot development occurred at a low level in the crop after the 8 September 2005. However, only 15 lesions per plant of both dark leaf spot and ringspot were recorded by the middle of September 2005. It could not be ascertained if all these spots resulted from the dark leaf spot pathogen (due to time and resource restrictions).

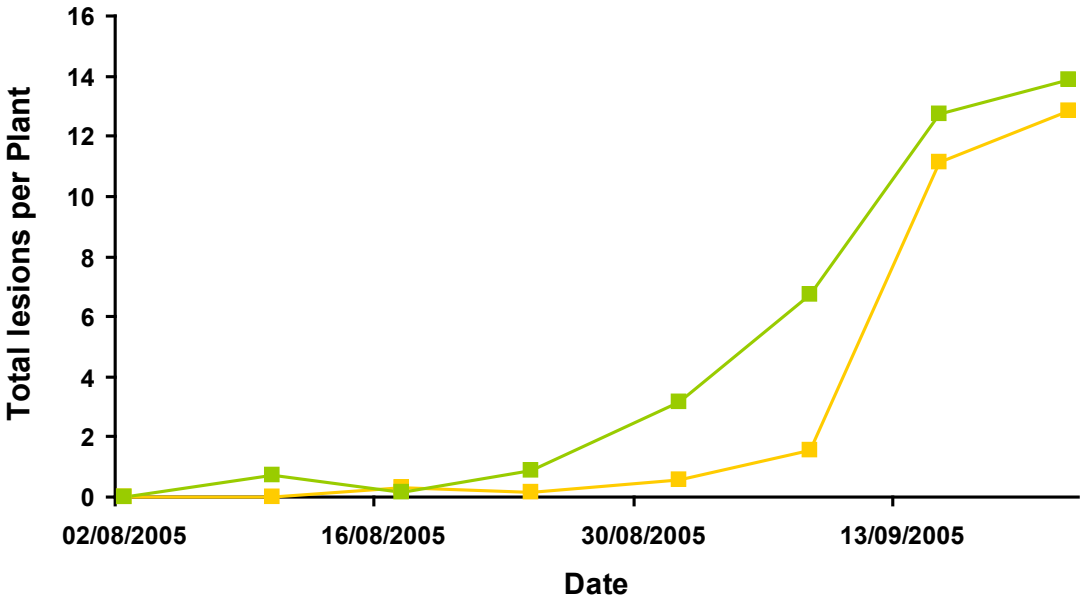


Figure 29. Ringspot (■) and dark leaf spot (■) disease development at Hesketh Bank in 2005

7.1.3.6 Prediction of dark leaf spot and ringspot infection conditions at Hesketh Bank in 2005

The results of using the Brassica$_{spot}$ disease forecasting system at the Hesketh Bank site in 2005 are shown in Figure 30. This predicted few periods when conditions were suitable for dark leaf spot and ringspot infection during the trial period. Over the trial period only the 24 – 31 August 2005 gave significantly high risk of dark leaf spot and ringspot indection. Some moderate risk periods were also recorded on the 22 August and the 6, 9, 10, 13 and 16 September 2005.

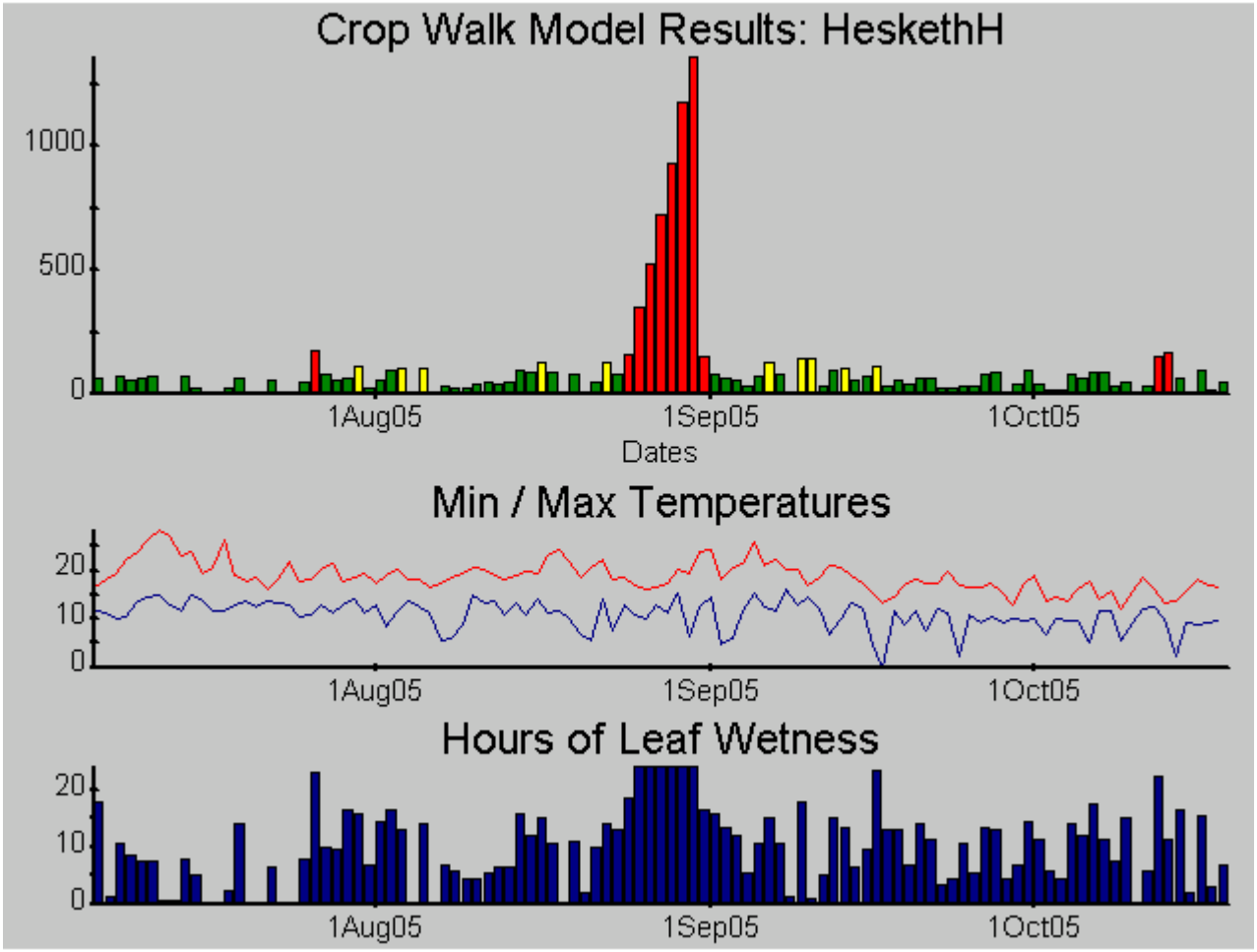


Figure 30. Prediction of dark leaf spot and ringspot infection at Hesketh Bank in 2005

7.1.4 Conclusions

Using the Biodiagnostics dark leaf spot lateral flow device gave positive results for dark leaf spot development at the Skegness trial site in 2005. These could have been obtained before disease development was observed on the crop. The lateral flow device used at Hesketh Bank gave a negative result when tested on air samples collected from the crop. No disease development was recorded on the cabbage crop during the trial. Usage of the dark leaf spot lateral flow device gave accurate predictions of development of dark leaf spot at each site. However weekly samples gave blocking of this prototype and changes to the prototype would be required if weekly samples were to be processed in the future.

8. DEVELOPMENT OF A COMPETITIVE LATERAL FLOW SYSTEM FOR THE RAPID DETECTION OF FIELD TRAPPED INOCULUM OF RINGSPOT

8.1 Introduction

Development of a lateral flow device for ringspot ascospores posed different problems to that encountered with dark leaf spot. A major problem was caused by ringspot ascospore production in culture. This process can take up to 6 weeks. Ascospores were necessary for the production of antibodies since these are the main targets for detection. During year one and two of the project CSL were unable to produce antibodies to ascospores of the ringspot pathogen supplied by Warwick HRI. However other antibodies specific enough to the ringspot pathogen were available from previous MAFF projects. These were not ideal for lateral flow production but nevertheless were used in the development of a ringspot lateral flow device.

8.2 Development of a ringspot competitive lateral flow format (*clfd*) for detection of *M. brassicicola*

8.2.1 Materials and Methods

8.2.1.1 Production of test line antigen

A UK field isolate of *M. brassicicola* (ringspot) (CH195001), was taken from the Warwick HRI culture collection and grown on Sprout decoction agar (SPD). Following a two week growth period a 5cm cube of actively growing mycelium was excised and inoculated on to a SPD agar medium, which had been pre-covered with a PN6026 Supor 450 90mm diameter membrane disc (Gellman Sciences, Cat No. 60206). The fungal isolate was incubated at 18° C and 40mm of the mycelial radial growth on the membrane was removed and 3ml of PBS was added. By using sweeping motions across the surface of the membrane with a sterile glass rod the filamentous structure of the ringspot fungus was disrupted. The liquid phase was collected and 500µl aliquots mixed with 0.5g Ballotoni glass beads in a 1.5ml eppindorf tube. Using a Fast Prep machine (FP 120, Thermosavent, Holbrook, NY, USA) the collected fungal material was processed. After which the aqueous phase was removed and, the protein concentration of the collected sample determined using a Bio-Rad protein assay (Bio-Rad, Cat. No. 500-0006), as specified by the manufacturer. The ringspot collected material was adjusted to protein concentrations of 1,0.8 and 0.4mg ml^{-1} and, stored at -20°C.

8.2.1.2 Competitive lateral flow construction

Tests were carried out using lateral flows constructed from a Millipore 240 HiFlow™ cellulose ester membrane direct cast on to 2ml Mylar backing (Millipore Corp, USA.), an

absorbent pad (Cat No. GBOO4, Schleicer and Schuell, Germany) and a sample pad (Cat No. T5NM, Millipore Corp., USA). Following lateral flow construction (Figure 31) a control line of an anti-mouse serum was sprayed directly on to the membrane surface using a flat bed air jet dispenser (Biodot Ltd, The Kingley Centre, West Sussex, UK). A 0.4mg ml-1 test line of the prepared ringspot antigen, (as described above) was applied to the membrane 5mm below the control line using a flat bed air jet dispenser. This process was repeated on a new competitive lateral flow membrane but this time the test line applied was at a protein concentration of 0.8mg ml^{-1}. This process was again repeated and a test line concentration of 1mg ml^{-1} *M. brassicicola* soluble antigen applied. The membranes were air dried at 18°C for a period of 24 hours. After which the test and control line labelled lateral flows were cut in to 4 mm strips, labelled and each strip housed within a plastic case (Schleicer and Schuell, Germany).

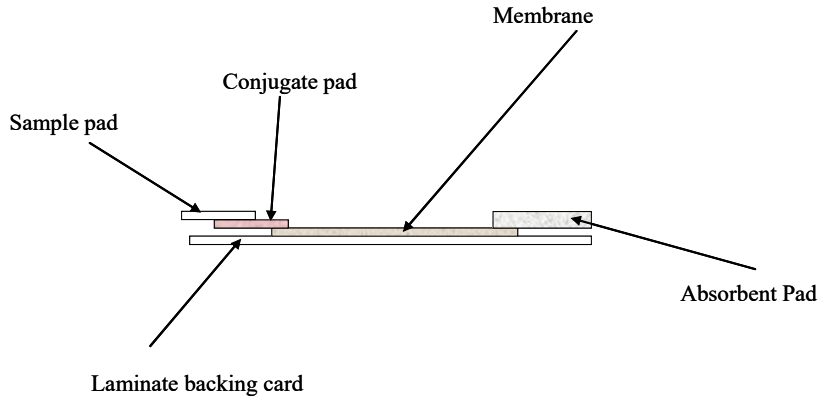


Figure 31 Schematic overview of a lateral flow device

8.2.1.3 Antibody conjugation

An antibody dilution of IgM monoclonal antibody, produced at Warwick HRI, Wellesbourne, to ascosporic inoculum of *Mycosphaerella brassicicola* (see section 3.1) coded HRI EMA 187), was prepared in Warwick HRI application buffer mixed (0.05M Tris HCL, 0.15M NaCl, 0.4% Triton X100, 0.2% Tween 20, 0.2% BSA, 0.12% Geropan) at a ratio of 1 part IgM EMA 187 to 80 parts Warwick HRI application buffer. Further dilutions of 1:320, 1:640 and 1:800 were prepared as described above. A 60µl volume of each antibody (Ab) dilution was mixed with 12µl of a goat anti-mouse IgM 40nm gold conjugate (Code BA GAMM 40, British Biocell International, Cardiff,UK) and incubated with agitation for 1 hour.

8.2.1.4 Competitive Lateral Flow assay

To determine the ability of the antibody EMA 187 to bind to the test line antigen the mixed EMA 187 gold conjugates at concentrations of 1:80. 1:320, 1:640, 1:800 were aliquoted on

to individual sample pads of the prepared competitive lateral flow devices (Table 18). After a run-time of 10 minutes, the lateral flow devices were recorded for the presence of test and control line using an EVL lateral flow reader.

Table 18 Effect of concentration of EMA187 /gold conjugate on test line visualisation.

	Millipore 240 HiFlow™ lateral flow device		
EMA 187 gold conjugate (72µl aliquot applied)	Test line at 1 mg ml^{-1}	Test line at 0.8 mg ml^{-1}	Test line at 0.4 mg ml^{-1}
1:80	✓	×	×
1:320	✓	✓	✓
1:640	✓	✓	✓
1:800	✓	✓	✓
1:1000	✓	✓	✓

✓ EMA 187 gold conjugate applied to sample pad of Millipor 240 HiFlow lateral flow device

8.2.2 Results

Lateral flow devices which had received a test line application concentration of 1 mg ml^{-1} *M. brassicicola* (ringspot) antigen gave test line formation for each of the EMA 187 antibody dilution gold conjugates applied. However test line intensity was poor when EMA 187 was at its most concentrated activity (1:80) and at its weakest at the lowest dilution (1:1000).

Lateral flow devices which had received a test line application of 0.8 mg ml^{-1}, gave test line formation at all dilutions with the exception of the weakest dilution (1:1000). However good line formation was observed only when EMA 187/conjugate was applied at dilutions of 1:320 and 1: 640 (Table 18).

At test line application of 0.4 mg ml^{-1}, clear test line development was observed only when an EMA 187 /conjugate application dilution of 1:320 was applied. For all tests a control line was observed to indicate that the test had run successfully.

8.2.3 Conclusions

The combination of a Millipore 240 HiFlow™ lateral flow device, striped with a test antigen of mycelial components of the ringspot antigen demonstrates a potential for use in rapid detection systems for ringspot ascospores when using the test components as described

above. However the results indicate that for optimal usage the test line antigen should be applied at or above 0.8 mg/ml^{-1} in PBS and, for clear test line development an antibody dilution of 1:320 to 1:640 should be used. Increasing antibody activity (EMA 187, 1:80) proved inhibitory to test line sensitivity and this is likely to result from stearic hindrance during antibody / test line antigen binding.

8.3 Evaluation of ringspot competitive lateral flow format (*clfd*) for detection of *M. brassicicola*

8.3.1 Materials and Methods

Lateral flow devices were prepared as described above using test line applications of *M. brassicicola* (ringspot) at 1 mg ml^{-1} and 0.8 mg ml^{-1}. The ringspot monoclonal antibody (EMA 187) was used at an activity of 1:320, 1: 640 and 1:800 and, was conjugated to the BBI gold spheres as described previously. An antibody/conjugate sample (10ug of *M. brassicicola* mycelial antigen, prepared as described above) was applied in 10µl Warwick HRI application buffer. As a control 10µl of Warwick HRI application buffer alone was applied to a duplicate set of antibody conjugate samples. All antibody sample conjugates were then aliquoted to individual sample pads of the prepared competitive lateral flow devices. A run-time of 10 minutes was used for the lateral flow devices. Test and control line formation was recorded using an EVL one-step reader.

8.3.2 Results

For clear control and test line development the application of *M. brassicicola* antigen at a test line protein concentratin of 1 mg ml^{-1} and, an increased antibody activity of EMA 187 proved optimal (Table 19). Test and control line signal depletion was noted for all lateral flows tested when the sample contained *M. brassicicola* antigen (ringspot). Visually the clearest differentiation of test line depletion, when a spiked *M. brassicicola* sample was used, was at an antibody dilution of 1:640. Nevertheless little difference was noted between digital readings when analysed by the EVL reader at antibody dilutions of 1:320 and 1:640 (Table 19). Poor test and control line development was again noted when a dilution of 1:800 EMA 187 gold conjugate was used. This was also reflected in the digital values observed by using the EVL reader.

Table 19 Test and control line development of competitive lateral flow devices in the development of a rapid test for detection of ringspot (*M. brassicicola*)

EMA 187	Test line at 1 mg ml-1	Test line at 1mg ml-1	Test line at 0.8 mg ml-1	Test line at 0.8mg ml-1

gold conjugate			Sample spiked with 10µg M. brassicicola mycelial antigen				Sample spiked with 10µg M. brassicicola mycelial antigen	
	Test	Control	Test	Control	Test	Control	Test	Control
1 : 320	4.8	10.2	3.0	5.4	2.5	7.7	1.3	3.2
1 : 640	4.1	3.2	2.5	1.3	2.1	2.4	1.0	1.1
1 : 800	1.3	1.3	0.9	1.1	1.2	1.0	0.5	1.4

8.3.3 Conclusions

Visually an antibody dilution of 1:640 gave greatest clarity between test line formation and depletion and, in a commercial test may prove best format. Using a lateral flow device sprayed with a test line at 1 mg ml $^{-1}$ gave clear test and control lines when sample application did not contain *M. brassicicola* antigen (ringspot). However near test line depletion was observed using a spiked sample of *M. brassicicola* when the membrane test line comprised of 0.8 mg ml^{-1} ringspot antigen. With further optimisation between antibody activity (EMA 187) test line protein concentration and buffer type the development of a suitable lateral flow device for field detection of *M. brassicicola* was investigated.

8.4 Evaluation of the developed *M. brassicicola* competitive lateral flow prototype for the rapid detection of ringspot field inoculum

8.4.1 Materials and Methods

Samples used in these tests were derived from a 7 day cyclone spore trap which had operated in a ringspot infected overwintered Brussels sprouts crop during the summer of 2005 (see section 5). Sampling dates where no disease, low, medium and high disease levels were recorded on Brussels sprout trap plants (Chapter 5.1.2.1) were identified and the relevant sampling reservoirs (eppindorf tubes) were selected. To each of these tubes EMA 187 gold antibody conjugate (60µ of 1:60 EMA 187 conjugated to 12µ BBI anti-mouse IgM gold spheres), was dispensed and incubated for 30min at room temperature. After which the aqeous phase of each of the eppindorf tubes was removed and aliquoted to separate sample pads of prepared competitive lateral flow devices. The lateral flow devices had been prepared as described above employing a test line application of either 1 mg ml^{-1} or 0.8 mg ml^{-1} *M. brassiciciola* antigen. Following a 10 min. run time the lateral flows were recorded using an EVL one-step-reader.

8.4.2 Results

A trend in test and control line depletion was observed as disease risk increased both visually (Plate 10) and, using the digital values generated by the EVL one-step reader (Table 20a,b). For both competitive lateral flow types (test lines at 1 mg ml^{-1}, 0.8 mg ml^{-1} *M. brassicicola*) complete depletion of the test line was observed when air samples, collected in the field at the same time as exposed trap plants gave high disease index ratings (Section 5, Figure 20), were assessed by visual observation. The depletion of the test line for these two periods (24/06/2005 and 15/06/2005) would predict high disease risk of ringspot. Sensitivity of the test was improved by using a test line application of 0.8 mg ml^{-1} *M. brassicicola* (Table 20a) with test line depletion observed (Table 20b) when a moderate ringspot lesion rating was recorded (Section 5, Figure 20). However overall line strength was low for both the control and the test line and, in the absence of a digital reader would prove problematical in reliable detection.

Plate 10 Ringspot lateral flow prototype tests using samples of known ringspot ascospore numbers collected from the field

Table 20a Field assessment of test and control line formation of competitive lateral flows (Clfd) using a test line application of 1mg ml^{-1} *M. brassicicola*

Field 24hr exposure period	Ring spot lesion rating on exposed trap plants	Clfd EVL Line Reading Test	Control
31/05/2005	0	1.9	1.9

23/05/2005	Low	2.2	1.9
19/05/2005	Moderate	1.3	0.5
24/06/2005	High	0.4	0.1

Table 20b Field assessment of test and control line formation of competitive lateral flows (Clfd) using a test line application of 0.8 mg ml^{-1} *M. brassicicola*

Field 24hr exposure period	**Ring spot lesion rating on exposed trap plants**	**Clfd EVL Line Reading**	
		Test	**Control**
3/06/2005	0	0.4	0.8
25/06/2005	Low	0.7	1.6
29/06/2005	Moderate	0.0	0.2
15/06/2005	High	0.0	0.1

8.4.3 Conclusions

The development of a ringspot lateral flow device presented many problems. The ringspot ascospore can adhere to test vessels therefore optimisation of the sampling regime proved the most difficult task in the development of a ringspot lateral flow assay. The modified assay worked best when it was recognised that part of the reaction should be carried out within the trapping vessel. However vessel coatings can now be employed which may reduce this source of error. With the developed protocol it will be easier to optimise the lateral flow device to give positive reactions at the required sensitivity. The lateral flow reader device can also be used to provide quantification of ascospore numbers over a wider concentration range than by using visual estimates alone.

9. DISCUSSION

Production of “in field” tests for pathogenic inoculum requires the development of tests which can be visualised by end users and which are reliable in detecting epidemiological important levels of inoculum. The format which seemed most appropriate in this respect was the lateral flow device. This report details the development of lateral flow devices for ringspot ascospores and dark leaf spot conidia. In these tests the visualisation of test lines indicated positive results or negative results and equated to an epidemiological significant level of inoculum. The use of lateral flow reader technology enables the device to be used more accurately and can extend its usage to cover reactions which are difficult to visualise. This effectively makes the lateral flow tests more reliable since they do not depend on visualisation of the reaction on a test line. It also offers the potential to build into the test information on other components in the plant pathogen interactions such as cultivar resistance. Additionally the number and variability of pathogenic lesions can be predicted which makes the test useful in providing estimates of disease over localities.

9.1 Optimisation of lateral flow device for dark leaf spot conidia

In tests 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. Further development of the test in year three saw a switch to using immunogold as the carrier system rather than latex primarily for reasons associated with the mass production of tests by a new manufacturer other than CSL. The test remained at the original sensitivity which in year two and year three tests has enabled the lateral flow device to be used predictively in the field producing an excellent level of accuracy.

9.2 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. Results using the EMA 212 antibody (which was used in the final lateral flow test format) has shown a low cross reactivity 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. The information would be important in determining potential errors which might occur under field conditions. The test has been shown to give accurate results under field conditions and it is unclear what circumstances would give rise to false positive or negative result. 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 using lateral flow reader technology (no visual reactions) which means its usage can be extended to most situations where information on absolute amounts of spores is required. The tests will also be used in conjunction with disease forecasts which will provide the background risk at each location.

9.3 Lateral flow test formats for detecting ascospores of ringspot

In year three of the project a working prototype lateral flow device for the ringspot pathogen has been developed using a competitive lateral flow format with 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. However these were never considered ideal for lateral flow development since they reacted to wall components of the ringspot ascospore. To use these antibodies entailed changes to the procedure of the test so that this type of reaction could be accommodated within the ringspot inoculum test. Additionally the ringspot ascospore sticks to the wells of collection vessels when sampled and they are difficult to remove without the usage of a suitable well coating. For these reasons the working ringspot lateral flow prototype carried out some reactions in the sample tube. While not ideal these changes overcame some of the problems associated with the development of the test. A working prototype which gave a visualisation in ascospore number at levels of between 50 and 100 was produced. With some further work this could be improved on enabling the detection of ringspot ascospores in air samples at low levels especially if lateral flow reader technology was employed.

9.4 Practical usage of the dark leaf spot lateral flow test under field conditions in commercial crops.

Usage of the tests developed within this project 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). 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 or could be assessed using a lateral flow reader device. This would indicate the presence of dark leaf spot or ringspot in the sample. In commercial trials using the dark leaf spot lateral flow device with a EVL lateral flow reader predictions could be made about the likelihood of disease development by dark leaf spot at each site. These proved extremely accurate in predicting periods when dark leaf spot would develop in crops. 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.

9.5 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. This is particularly important with regard to dark leaf spot where inoculum from oilseed rape is blown during very narrow windows of disease transmission into vegetable brassica crops. By utilising the dark leaf spot test these periods can be identified and the disease can be eradicted early in its development on the crop or stopped completely from appearing in the crop at all. 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 also be useful early in the season as a method of preventing disease transfer between over wintered crops and freshly transplanted crops.

The approach can be easily extended to include all diseases affecting vegetable brassica crops. For example 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.

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