# ANNUAL REPORT

To:
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**BRASSICAS: FURTHER DEVELOPMENT AND VALIDATION OF DETECTION TESTS FOR DARK LEAF SPOT AND RINGSPOT**

**HDC PROJECT FV 233a**

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June 2007

Commercial - In Confidence

# Grower Summary

**FV 233a**

**BRASSICAS: FURTHER DEVELOPMENT AND VALIDATION OF DETECTION TESTS FOR DARK LEAF SPOT AND RINGSPOT**

**Annual report 2007**

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| **Project title:** | **Brassicas: Further development and validation of detection tests for dark leaf spot and ringspot** |
| **Project number:** | FV 233a |
| **Project leader:** | Dr Roy Kennedy |
| **Year of project:** | Annual |
| **Date of report:** | |
| **Locations:** | Warwick HRI, University of Warwick, Wellesbourne<br>Growers' holdings in Lincolnshire and Lancashire |
| **Coordinator:** | Andy Richardson |
| **Project commenced:** | 01 June 2006 |
| **Completion:** | 01 June 2008 |
| **Key words:** | Air-borne spores, dark leaf spot, ringspot, detection, disease control, fungicides, disease, Alternaria brassicae, Mycosphaerella brassicicola, Brassica, Brussels sprouts, cauliflower, fungicides. |

**Signed on behalf of:** **Warwick HRI**

***Signature***:……Simon Bright…… Date: …2 October 2007…

***Name:*** Professor Simon Bright
Director and Head of Department

The results and conclusions in this report are based on an investigation conducted over one year. The conditions under which the experiment was carried out and the results obtained have been reported with detail and accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results especially if they are used as the basis for commercial product recommendations.

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# BRASSICAS: FURTHER DEVELOPMENT AND VALIDATION OF DETECTION TESTS FOR DARK LEAF SPOT AND RINGSPOT

## GROWER SUMMARY

### Headline

Detection tests for conidia of dark leaf spot of vegetable brassicas have been used successfully in commercial crops by consultants in 2006. Detection tests for ringspot ascospores have been successfully validated at Warwick HRI.

### Background and expected deliverables

Detection tests for dark leaf spot conidia have been produced. The competitive lateral flow device was described in HDC report FV 233 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. The dark leaf spot lateral flow device was produced at the appropriate sensitivity and used successfully to predict disease development in a series of trials conducted in commercial brassica crops 2005. A working lateral flow prototype for ringspot ascospore detection was also successfully produced within HDC project FV 233 but was not tested in the field. The current project (FV233a) will further validate the use lateral flow tests developed within HDC project FV 233 under field conditions in commercially grown crops of vegetable brassicas. This will involve mass production of both dark leaf spot and ringspot LFD tests and their use in conjunction with 8 day cyclone air samplers in the field. Additionally the use of the tests in comparison with the output from the Brassica$_{spot}$ disease forecasting system for dark leaf spot and ringspot will also be investigated. Year one of the work has concentrated on the release and usage of the dark leaf spot lateral flow test. However during year two both tests will be used under field conditions in commercial crops. The effectiveness of the information in conjunction with fungicidal control regimes for dark leaf spot and ringspot will also be evaluated

The expected deliverables from this project are:

- Monoclonal antibodies which recognise conidia of dark leaf spot conidia and ringspot ascospores.
- Detection and sampling systems for dark leaf spot and ringspot inoculum which will provide information on disease problems in crops before they are visible.
- Assessment of 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.

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

 

**2006**

Use of the Warwick HRI dark leaf spot lateral flow device gave mostly negative results for dark leaf spot conidia in air samples at Hesketh Bank ((Lancashire) and Bicker (Lincolnshire) during 2006. These tests were conducted by end users (crop consultants). There were not many positive results observed for dark leaf spot inoculum because during 2006 environmental conditions were unfavourable for dark leaf spot and ringspot development during June, July and August 2006. Usage of the dark leaf spot lateral flow device gave accurate predictions of dark leaf spot conidia at Hesketh Bank. At Hesketh Bank the levels of dark leaf spot were too low in air samples for disease development on crops especially in spring and autumn harvested cauliflowers. However crops such as Brussels sprouts which have more favourable crop microclimates may be more susceptible to dark leaf spot development at Hesketh Bank.

**Validation of ringspot lateral flow tests for detecting ascospores of ringspot at Warwick HRI in 2006**

Use of the MTIST trap at both Bicker and Hesketh Bank in 2006 indicated the prevalence of high levels of ringspot ascospores in air samples at these sites at the end of the 2006 growing season (November 2006 – February 2007). The results show that the ringspot lateral flow tests corresponded closely to the ringspot ELISA test at Warwick HRI. There was also a good relationship between the results of the lateral flow tests and the numbers of ringspot lesions on trap plants exposed in the field adjacent to a field plot heavily infected with ringspot. The testing of the ringspot lateral flow device was hampered by the unfavourable environmental conditions for ringspot disease development which occurred during June, July and August 2006 at Warwick HRI. This was shown in the number of risk periods for infection by dark leaf spot and ringspot during October and November 2006. Epidemic development was only observed during October 2006. However lower risk periods did occur during mid October 2006. This indicates that presence of inoculum appears to be a very accurate indicator for ringspot and dark leaf spot epidemic development.

**Action points for growers**

- Growers can use the in field inoculum tests to determine when disease development will occur in their crop.
- The system will be useable within localities to determine which areas are more disease prone at the start of the growing season.

**Anticipated practical and financial benefit**

The successful use of the dark leaf spot test kit will help improve the uptake of this technology. It will now be possible for the grower to obtain data on the disease risk to crops due to pathogenic inoculum in a rapid and inexpensive way. By using spore traps in conjunction with forecasts the grower or consultant will be able to assess the risks precisely from dark leaf spot and ringspot to their crops. This offers the possibility of guaranteed disease free crops using minimum crop protection inputs. By using this approach the grower will be able to cope with reduced numbers of actives available in brassica production and any subsequent decline.

 

- 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 fungal pathogens in vegetable brassica crops.
- There will be a lower requirement for and reliance on expensive eradicants.
- Disease control in vegetable brassica crops using this technology (once confidence has been established) may help reduce fungicide residues in crops at harvest.

Growers can access these tests via Roy Kennedy at Warwick HRI. Contact Roy via roy.kennedy@warwick.ac.uk or 024 7657 5024.

 

## SCIENCE SECTION

## INTRODUCTION

### Dark leaf spot and ringspot air borne disease problems affecting vegetable brassica crops

Ringspot caused by *Mycosphaerella brassicicola* and dark leaf spot (*Alternaria brassicae*) are two of the most important vegetable brassica diseases in the UK. 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. Fungal disease can be particularly difficult to control in Brissels sprout crops. 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. These diseases are less important on cauliflower and broccoli crops. Both ringspot and dark leaf spot are also present on oilseed rape crops. Disease transmission from oilseed rape during harvest and from unsprayed cauliflower and broccoli crops on to long season crops such as Brussels sprouts is frequent. 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. 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.

### Forecasting diseases in vegetable brassica crops

 

Monitoring environmental conditions necessary for infection can be used to determine risk of infection by dark leaf spot and ringspot. 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 infection may also be associated with windspeed which could be another useful criteria used to determine infection risk. Ringspot also 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 such as Brassica $_{spot}$. 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 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. In order to avoid these problems new and rapid methods of detecting and quantifying pathogenic inoculum are required. The development of rapid tests for dark leaf spot and ringspot inoculum has been described in HDC project FV 233 which was successfully completed in October 2005.

**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.

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 1). The fluid sample is added to the well, releasing the specific antibody bound coloured particles, which then begin to flow across the membrane. If the target antigens (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). This would result in one visible line on the device indicating a positive result. Two lines of equal colour intensity indicate a negative result.

 

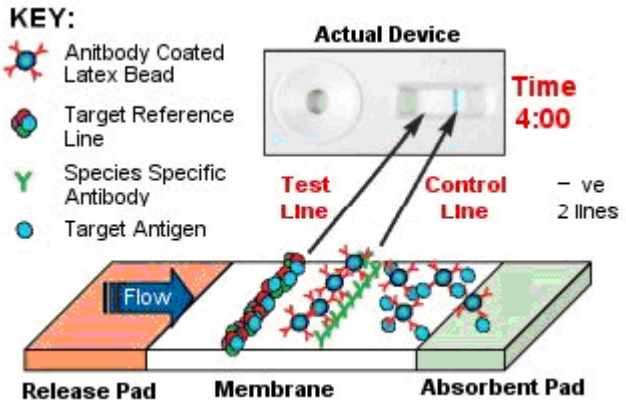


**Figure 1:** The Competitive lateral flow assay format

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

**Epidemiological advantages of testing for detecting inoculum**

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

Given these constraints there are major periods when inoculum at high levels is present within crops but those crops are largely disease free. The grower will have no information other than weather on which to base his decisions because his crop is largely disease free and he has no information on the risk posed to his crop by surrounding control practices. This often accounts for disease levels in crops moving from a very low level to a very high level in a short period of time. At certain times of year (e.g. harvest and during harvesting of oilseed rape crops and autumn cauliflower crops) long season vegetable brassica crops are very susceptible to increased disease risks and the grower is vulnerable to outside pressures on his crop which he has no way of measuring consistently. By using “in field” inoculum tests developed within this project the grower can measure these risks in his locality both at the spatial and temporal scale. This will enable disease risk to be correctly measured and dealt with in many instances using protectant fungicides.

 

**Objectives of the current study**

The work within this project will validate the use of lateral flow tests developed within HDC project FV 233 under field conditions in commercial grown crops of vegetable brassicas. This will involve mass production of LFD tests and their use in conjunction with 8 day cyclone air samplers in the field. Additionally the use of the tests in comparison with the output from the Brassica$_{spot}$ disease forecasting system for dark leaf spot and ringspot will also be investigated. Year one of the work will concentrate on the release and usage of the dark leaf spot lateral flow test. However during year two both tests will be used under field conditions in commercial crops. The effectiveness of the information in conjunction with fungicidal control regimes for dark leaf spot and ringspot will also be evaluated

**Evaluation of Dark Leaf Spot and Ringspot lateral flow devices for monitoring airborne conidia of *Alternaria brassicae* and *Mycosphaerella brassicicola* in field trials at Warwick HRI**

**Monitoring airborne inoculum of the dark leaf spot pathogen** (*A. brassicae*) **and ringspot (*M. brassicicola*) in inoculated overwintered Brassica crops**

**Materials and Methods**

**Monitoring dark leaf spot and ringspot 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 month for the presence of dark leaf spot and ringspot spores in air samples. Air samples were taken using two Burkard 8 day cyclone samplers and a microtitre immuno-spore trap (MTIST). A daily sample of micro-organisms in the air was collected daily in each eppindorf sample tube within each 8 day cyclone spore trap. The tubes were removed from the traps weekly. The eppindorf tubes of both spore traps were stored prior to assay development at -20ºC. Prior to field exposure the microstrips for the MTIST trap were stored at 4ºC in a sealed container. Air flow through the MTIST sampler was estimated in still air by measuring the air speed at different points across the inlet manifold using a hot film anemometer (air velocity transducer model number 8460, TSI Incorporated, St Paul, MN, USA) and integrating over the area of the inlet. In the tests reported here, the volume flow rate through the device was measured at 57-litre min$^{-1}$. The MTIST sampler and the 7 day cyclone 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. Light is a requirement for ascospore release (Kennedy, 1999).

For each of the sampling periods twelve *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 six plants (chosen at random) were placed in an environment of 100% humidity for 48 hrs at 16 C. 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. The remaining six plants were left unmisted under the same conditions. 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).

**Competitive lateral flow device for dark leaf spot conidia**

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

Approximately 200µl of extraction buffer was added to each of the collected eppindorf vessels (cyclone spore sampler) 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. 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.

**Detection of ringspot in air samples using ELISA**

Field exposed microtitre strips 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, 0.05 % Tween 20 and 0.1% Casein (PBSTw C). After which wells 1 - 4 of each strip received 100 µl of monoclonal Ab EMA 187 (raised at Warwick HRI to *M. brassicicola*), with the remaining wells of 5 - 8 each receiving 100µl of PBS. 0.05 % Tween 20 and 0.1% Casein. Following incubation in a Wellwarm shaker incubater (30° C) for a period of 45 mins as above, wells were washed three times for one min each with 200 µl PBSTincTw. A DAKO duet amplification system was used

(DAKO Ltd, Angel Drive, Ely, Cambridge,UK; Cat no. K0492) to amplify the signal generated by bound tissue culture supernatant antibodies. Wells were washed as described above and 100µl of 3,3',5,5'- tetramethylbenzidene substrate (Sigma, Poole, Dorset UK; Cat. No. T-3405 and P-4922) was then added to each well. The reaction was stopped by adding 25µl of a 20% 1M $H_2S0_4$ solution to each well. Absorbance at 450nm was determined with a Biohit BP800 ELISA plate reader (Alpha Laboratories, 40 Parham Drive, Eastleigh, Hampshire, UK).

**Optimisation and testing the ringspot lateral flow devices using daily air samples collected using an eight day cyclone sampler at Warwick HRI.**

*Ringspot lateral flow device Assembly*

A Millipore High Flow 180 membrane was sprayed with a soluble ringspot extract at a protein concentration of 1mg /ml and at a delivery rate of 25 m/s. The sprayed membranes were then air dry overnight at room temperature. A control line of anti-mouse FITC (60µl: 600µl 0.5M PBS) was applied at rate of 50 m/s to the same membrane. Antibody EMA 187 semi-purified at 7 ng/ml was diluted in conjugate buffer (2% sucrose, 2% trehalose, 2% BSA in 0.2M PBS) at 1 in 150 and 1 in 200. Volumes of 25 µl were mixed with 5µl BBI gold anti-mouse aliquots for 45 minutes in a Wellwarm incubator shaker (30°C). Conjugate pads (5 x 10 mm) were dipped in to each aliquot mixture and the conjugate allowed to travel across the whole of the pad. The pads were then removed and air-dried at 37°C for no more than 45 mins. Treated pads were stored in a McCartney bottle in darkness at room temperature prior to use in the ringspot lateral flow device.

Two types of assay (direct and indirect) were preformed on duplicate tubes from two eight day cyclone samplers on days when ringspot lesions were recorded on trap plants misted after field exposure (see section 3.1.1.1) to optimise the lateral flow device**.** The cyclone samplers were operated from 06:00 H to 18:00H on the tests days chosen. A coating of 0.05% Sodium Azide was used in each test tube. Due to the limited availability of samples where ringspot was known to be present within the tube only limited numbers of tests could be carried out to optimise the ringspot lateral flow device.

*Direct field assay*

One hundred and fifty µl aliquots of running buffer (0.25% PVP, 0.4% Casein PBS 0.05% sodium azide (PVPC)) were transferred to field exposed eppindorf tubes and vortexed for 1 minute at high speed. A 2µl aliquot was pipetted in to a glass multiwell slide and assessed for *M. brassicicola* (ringspot) presence both by light and immunofluoresence microscopy. The remaining volume was distributed between two competitive ringspot lateral flow prototypes. One prototype had a conjugate pad at 1 in 150 whilst the other was at 1 in 200 antibody conjugate. Time taken to run completion was 20 minutes and a final 20µl flow of running buffer was aliquoted to each strip to complete membrane clearing. A non-field exposed eppindorf was used as a control

*In Direct field assay*

An 80µl aliquot of running buffer (PVPC) and a pad containing 1: 200 antibody conjugate was applied to each field exposed eppindorf (same field exposed eppindorfs as previously

used and the procedure carried out as in the direct lateral flow assay assay). Using a Gilson pipetter the liquid was gently moved around the eppindorf to release the gold conjugate in to solution. Each eppindorf was incubated at room temperature for aprox 3 mins. prior to transferral of the liquid phase to a lateral flow device. A non-field exposed eppindorf was used as a control.

*Combined Ringspot and dark leaf spot field Lateral flow protocol*
Approximately one hundred and ten µl *Alternaria* buffer added, mixed and removed (to simulate the sample for the *Alternaria* lateral flow test). One hundred and twenty µl HRI PVPC buffer was added to field exposed eppindorf tubes along with ringspot conjugate pad (1:200 antibody conjugate dilution). The conjugate pad was covered with liquid which was gently moved up and down to solubilise gold conjugated antibody in to solution. Movement of liquid around sides of eppindorf allowed gold conjugated antibody to come in to contact with the deposited eppindorf bound spores. The conjugate pad was incubated for a minimum of 5 minutes. The sample was then dropped on to (ensuring that all liquid was removed from conjugate pad and the eppindorf) the ringspot lateral flow device. The device was read after 25 minutes. The presence of a red dot denoted that spore levels are below those required to initiate ringspot disease. Absence of red dot indicates that spore levels are above threshold required for ringspot disease.

**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 vegetable 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.

**Prediction of dark leaf spot and ringspot infection in the field**
Disease forecasting models (Brassica$_{spot}$) were used to predict when 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 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.

**Visual microscopic counts of dark leaf spot from air samples**
Air samples collected using the MTIST air sampler were checked visually for the presence or absence of dark leaf spot conidia. Microtitre well stips for each sample day were examined visually by using a microscope. The microtitre strip was inverted on the microscope stage and

counted directly on the base of each well. Estimates of the numbers of dark leaf spot conidia were taken by counting the number of dark leaf spot conidia in each well for each day. The total number of dark leaf spot conidia per sample date was express as the volume of air sampled on each day was constant.

## Results

### Disease observations from trap plant exposure within the over-wintered Brussels sprout crop at Warwick HRI in 2006

There were low numbers of dark leaf spot lesions present on trap plants exposed within an infected plot during September and October 2006 and given a 48 H period at 100% relative humidity post exposure (Figure 2). Approximately 25 and 37 dark leaf spot lesions were observed on trap plant on the 9 and 12 September 2006 respectively. Approximately 9 dark leaf spot lesions were observed on trap plants on the 7 September 2006 however on all other days where dark leaf spot lesions were present fewer than five lesions per day were observed on trap plants. Numbers of ringspot lesions observed on trap plants were much higher on plants given a 48 H high humidity period after exposure. The 13 September 2006 had the highest total number of ringspot lesions of any exposure period with 129 lesions recorded. Approximately 117 ringspot lesions were observed on trap plants exposed on the 19 October 2006. The 1, 5, 6, 9, 11 and 20 of October 2006 had a total of over 40 ringspot lesions on exposed trap plants.

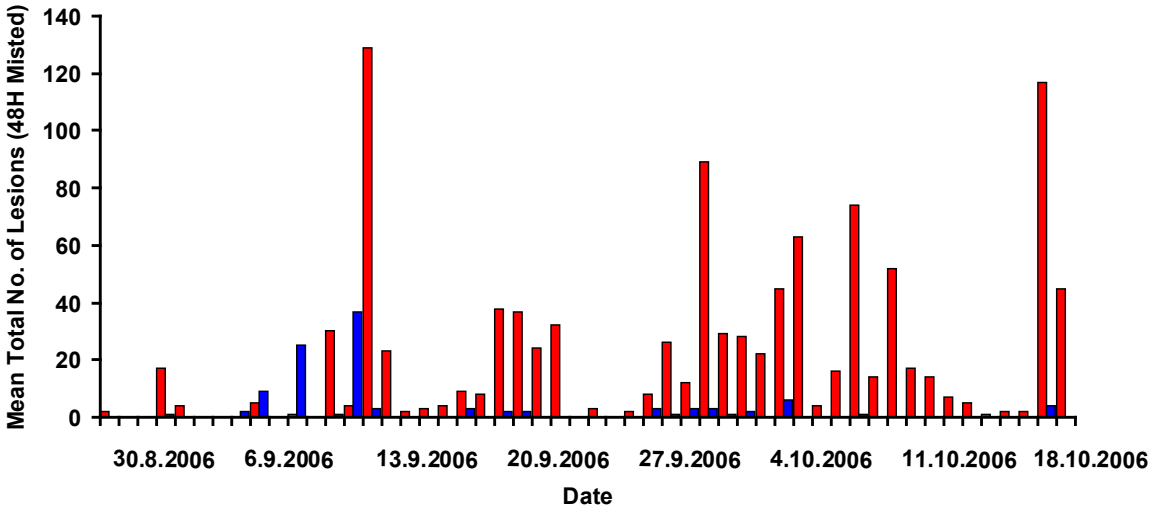


**Figure 2:** Mean total number of ringspot (■ ) and dark leaf spot (■ ) lesions per plant on trap plants exposed in the field and given a 48 H misting period post exposure

Plants which were unmisted after field exposure had lower number of ringspot lesions and extremely low numbers of dark leaf spot lesions. The 12 September 2006 had a total of two dark leaf spot lesions on the exposed trap plants (Figure 3). However, the total daily number of ringspot lesions observed on field trap plants on that date, without additional exposure to high humidity, was 21. There were three days over the experimental period when there was

 

over 50 ringspot lesions on trap plants not given additional wetting. These occurred on the 4, 6 and 11 October 2006 (Figure 3). The 11 October 2007 had the highest number of ringspot lesions (156) on exposed plants given no additional wetting period after exposure.

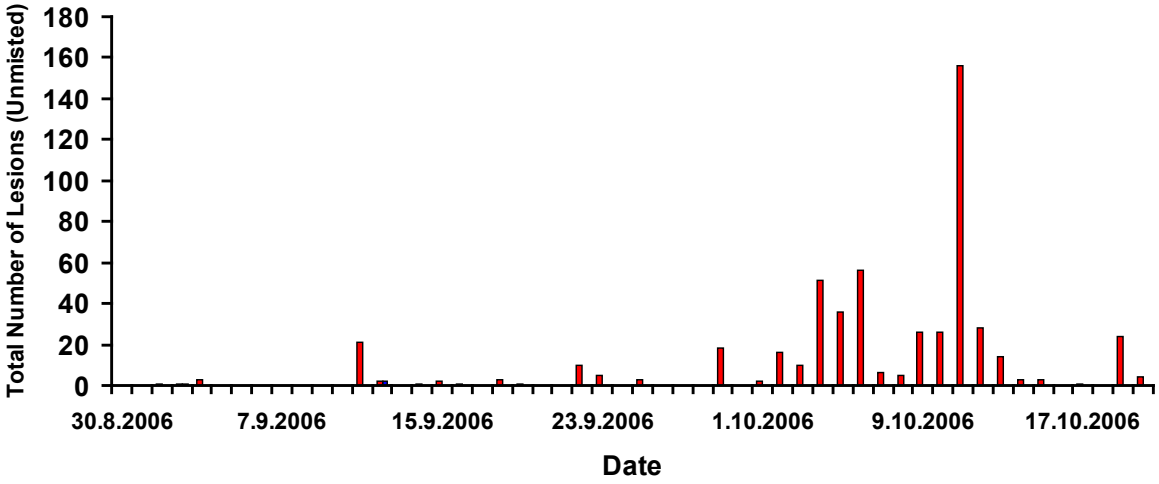


**Figure 3:** Mean total number of ringspot (■ ) and dark leaf spot (■ ) lesions per plant on trap plants exposed in the field and given a 48 H misting period post exposure

**Detection of dark leaf spot conidia in air samples using lateral flow devices in disease plots at Warwick HRI**

The results of using the dark leaf spot lateral flow device are shown in Table 1. Not all days were tested using the dark leaf spot lateral flow device due to the low levels of dark leaf spot found in the field (see section 3.2.2.1). A number of days were tested based on the results of the dark leaf spot infection model (see section 3.2.2.6). Due to the number of high risk infection days and the requirements for supplying commercial trial users with dark leaf spot tests the results of infection levels on trap plants (see section 3.2.2.1) were also used to determine which days were tested. Positive tests for dark leaf spot were recorded on the 12 September 2006 only. However the 21 and 28 September 2006 were also test periods which gave semi positive results. The use of a scanning densitometer gave readings on these trial days which were lower that those of an unexposed control sample. This indicated a weaker test line and a semi positive result.

**Table 1. Visual assessment of test line for sampling periods using the dark leaf spot lateral flow device in field trials at Warwick HRI**

| Field exposure Period | Model Risk Observation | Test line | No. of conidia | Immunogold Line Reading |
|---|---|---|---|---|

| | | | | |
|---|---|---|---|---|
| **8 September** | **Green** | **Yes** | **0** | **7.6** |
| **9 September** | **Green** | **Yes** | **0** | **7.9** |
| **15 September** | **Green** | **Yes** | **0** | **6.8** |
| **19 September** | **Green** | **Yes** | **0** | **6.3** |
| **03 October** | **Green** | **Yes** | **0** | **6.6** |
| **04 October** | **Green** | **Yes** | **0** | **NA** |
| **08 October** | **Green** | **Yes** | **0** | **6.4** |
| | | | | |
| **3 September** | **Red** | **Yes** | **0** | **NA** |
| **12 September** | **Red** | **No** | **40** | **1.6** |
| **14 September** | **Red** | **Yes** | **0** | **6.8** |
| **21 September** | **Yellow** | **Yes *** | **38** | **4.0** |
| **22 September** | **Yellow** | **Yes** | **10** | **5.8** |
| **28 September** | **Red** | **Yes *** | **31** | **4.0** |
| **12 October** | **Yellow** | **Yes** | **9** | **3.4** |

(***** - Faint line present)

**Detection of ringspot ascospores in air samples using ELISA**

Results of detecting of ringspot ascospores in air samples using ELISA is shown on Figure 4. The ELISA test indicated many days when ringspot ascospores were present in air samples. However the majority of ringspot lesions on trap plants given no misting period after field exposure occurred on days when an ELISA absorbance value of approximately 0.1 was observed. Values of approximately 0.1 absorbance were recorded on the 12, 23, 30 September, 1, 6, 9, 12, 13, 16, 22 and 27 October 2006. An additional day with an ELISA absorbance value of 0.1 and above occurred on the 6 November 2007 (Figure 4). The highest absorbance values were recorded 12 September (0.163), 27 October (0.203) and the 6 November 2006 (0.164). A high environmental risk was recorded on the 12 September 2006 and trap plant infection was observed. On the 27 October 2007 and 6 November 2006 there was a zero risk of ringspot infection based on model predictions. No trap plants were exposed on the 27 October 2006 and no trap plant infection was observed on trap plants exposed on the 6 November 2006.

 

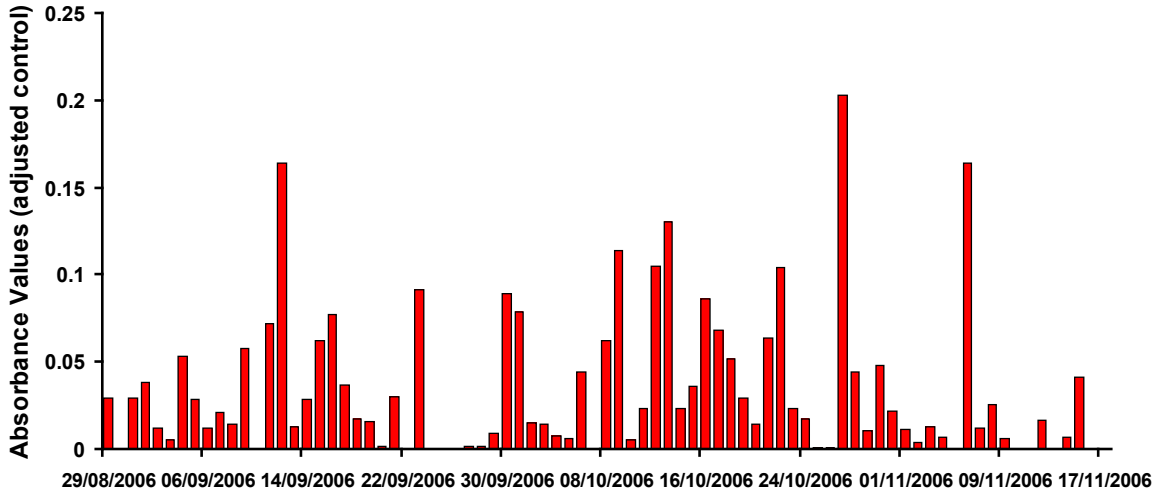


**Figure 4:** Detection of ringspot ascospores in air samples using ELISA

### Detection of *Alternaria brassicae* conidia in air samples using microscopic counts

The microscopic counts of *A. brassicae* conidia in air samples is shown on Figure 5. The number of dark leaf spot conidia recorded throughout the sampling period was low. The highest number of dark leaf spot conidia in air samples was recorded during September 2006. There were over 15 conidia in air samples collected from 06:00H until 18:00H on the 5, 9 and 12 September 2006. This corresponded closely to trap plant infection by *A. brassicae*. There were approximately 10 conidia observed in air samples collected on the 23 September 2006. Lower numbers of *A. brassicae* conidia were recorded on most other days during September 2006. Approximately 5 conidia of dark leaf spot were observed in daily air samples collected between the 8 and the 16 October 2006.

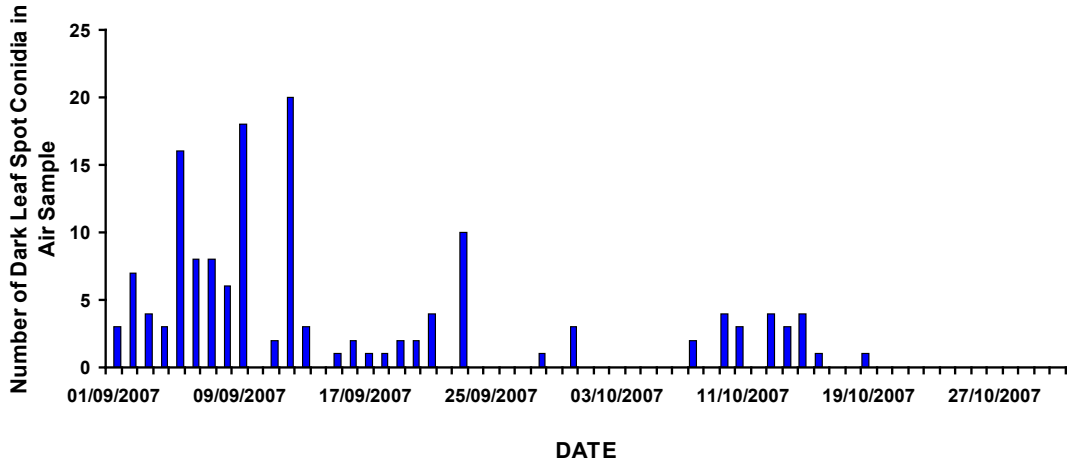


**Figure 5:** Detection of conidia of dark leaf spot in air samples using microscopy

 

**Detection of ringspot ascospores in air samples using the ringspot lateral flow prototype in samples from the field at Warwick HRI**

Both the direct and indirect assay format gave similar results when tested on a limited number of cyclone tubes from an air sampler in a ringspot infected field plot at Warwick HRI. The eppindorf tubes collected on the 6 and 9 October 2006 from the field contained significant numbers of ringspot ascospores. However only tests conducted on tubes collected on the 9 October gave total line depletion (Table 2). Using a scanning densitometer on lateral flow tests carried out on tubes exposed on the 6 and 9 October 2006 gave similar results. Tests using the a format which allowed testing of the same tube for ringspot and dark leaf spot gave positive results on the 20 September and the 19 October 2006 (Table 2).

**Table 2. Visual assessment of test line for sampling periods using two ringspot lateral flow device prototypes in field trials at Warwick HRI**

| Field exposure Period | Test line | No. of lesions (Misted Trap Plants) | Immunogold Line Reading |
|---|---|---|---|
| ***Direct Field Assay*** **(200 Conjugate pad)** | | | |
| **26 September** | **Yes*** | **0** | **0.8** |
| **06 October** | **Yes*** | **63** | **0.5** |
| **09 October** | **No** | **74** | **0** |
| **Control** | **Yes** | **0** | **4.6** |
| ***In Direct Field Assay*** **(200 Conjugate pad)** | | | |
| **26 September** | **Yes*** | **0** | **2.9** |
| **06 October** | **Yes*** | **63** | **0.9** |
| **09 October** | **No** | **74** | **0.7** |
| **Control** | **Yes** | **0** | **3.9** |
| ***Combined Assay (for ringspot and dark leaf spot from the same sample tube)*** | | | |
| **20 September** | **No** | **38** | **NA** |
| **19 October** | **No** | **63** | **NA** |
| **05 November** | **Yes** | **0** | **NA** |

(* - Faint line present : NA – Not available)

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

The results of using the Brassica$_{spot}$ disease forecasting system are shown in Figure 6. There were many periods when dark leaf spot and ringspot infection was predicted in the field over the trap plant exposure period. 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. Days represented by a yellow bar (moderate infection risk) also occurred. A major high risk period occurred from the 17 - 21 August 2006 and on the 23 and 24 August 2006. Other high risk periods occurred on the 3, 12, 14, 23 and 28 September 2006 and the 23 November 2006. Other moderate risk periods were recorded on the 3, 13, 14, 26, 27 and 28 August, and the 5, 6, 7, 13, 21, 22, 25,

26, 27 and 29 September 2006. The 1, 11, 12, 13, 14, 15, 17, 18, 19 and 23 October 2006 were also moderate risk periods for ringspot and dark leaf spot infection.

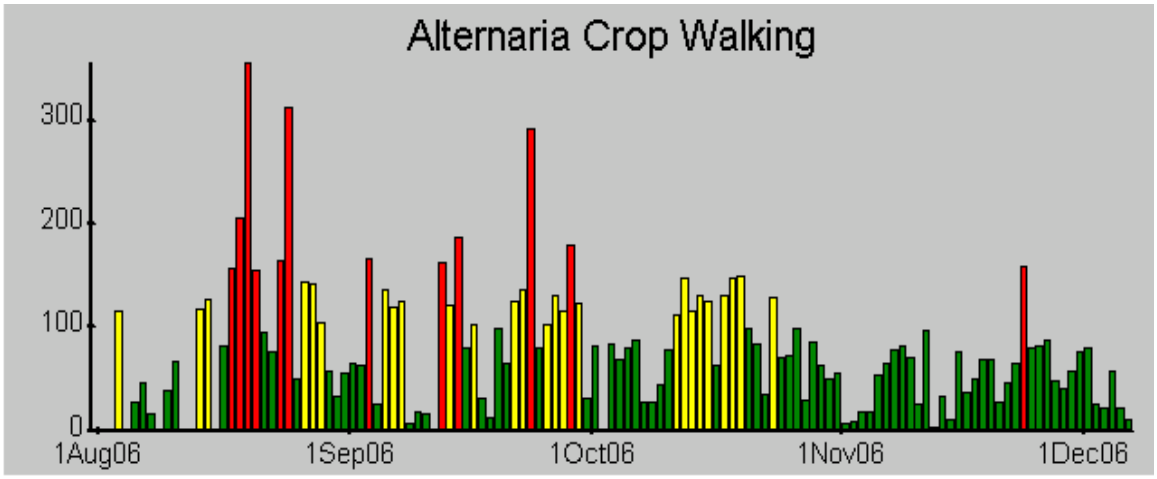


**Figure 6:** Prediction of dark leaf spot and ringspot infection in the field at Warwick HRI 2006

**Conclusion**

The results show that the dark leaf spot and ringspot lateral flow tests corresponded closely to dark leaf spot counts from tubes and the ringspot ELISA test. There was also a good relationship between the results of the lateral flow tests and the numbers of dark leaf spot and ringspot lesions on trap plants exposed in the field adjacent to a field plot heavily infected with ringspot and dark leaf spot. Other diseases present in the plot did not interfere with the results of the lateral flow tests. There was a substantial amount of both powdery mildew (*Erysiphe cruciferarum*) and *Botrytis cinerea* present in the plot. There were more false positive results observed using the ELISA test format for estimation of ringspot infection than using the ringspot lateral flow device. This resulted from the absorbance value (amount of inoculum) used to predict infection. The results indicate a potential interaction with environment in the determination of the ELISA value used to signify infection. It is therefore more valid to compare the ELISA results with trap plants given a 48 H misting period after field exposure. It should be noted that there were few high risk periods for infection by dark leaf spot and ringspot during October and November 2006. This was due to climatic conditions observed in the field during 2006. High temperatures reduced the development of dark leaf spot and ringspot epidemics. Epidemic development was only observed during October 2006. However lower risk periods did occur during mid October 2006. This indicates that presence of inoculum appears to be more accurate as a risk indicator for ringspot and dark leaf spot epidemic development.

## TESTS WITH LATERAL FLOWS FOR DARK LEAF SPOT DETECTION IN FIELD TRIALS AT COMMERCIAL SITES

**Monitoring airborne inoculum of the dark leaf spot pathogen (*A. brassicae*) and ringspot in sprayed vegetable brassica crops during 2006**

**Introduction**

Field tests were conducted using the dark leaf spot lateral flow device within commercial crops of cauliflower and Brussels sprouts. The number of dark leaf spot conidia within air samples was measured during each sampling period. Trials were conducted during 2006 at two vegetable brassica production sites in different areas of the UK. Ringspot inoculum was also monitored at each site at the end of the growing season (2006 – 2007) using an MTIST trap in laboratory ELISA tests. This test was compared where possible with the ringspot lateral flow test.

**Materials and Methods**

**Crop experimental design and environmental monitoring at each trial site**

Trials were conducted in commercial crops at Bicker (Allium and Brassica Centre, Wash Rd, Kirton), Alphagrow (Hesketh Bank, Preston, Lancashire) and run in conjunction with grower/consultants. At Bicker a Brussels sprout crop was monitored and assessed for disease development. At Hesketh bank the trial was located in an over-wintered cabbage crop initially and then a summer cauliflower crop. The ringspot tests were carried out during December 2006 – February 2007 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. Trials were located in a sprayed parts of the crop. 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).

**Air sampling at each trial site**

Air samples were taken continuously over a period of 30 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) were 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 $^{2}$. These samples were used for tests with the dark leaf spot lateral flow device. Tests using the ringspot lateral flow device were carried out in the laboratory on air samples from the Hesketh Bank test site (January 2007 – February 2007). At both the Hesketh Bank and Bicker test site an MTIST air sampler was used for comparisons with the ringspot lateral flow device using an ELISA test (see section 3.1.1.3).

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

The dark leaf spot lateral flow device used is described in section 3.1.1.2. Sample days for tests were chosen using the output from the Brassica$_{spot}$ dark leaf spot and ringspot models. Sampling days were selected in three categories (no risk, moderate risk and high risk days). 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 (as described in FV 233 Final Report). 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) where necessary.

**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 estimated.

**Prediction of dark leaf spot infection in the field**

(see section 6.1.1.8)

**Results**

**Detection of dark leaf spot conidia in air samples using lateral flow devices at Hesketh Bank 2006**

The results of using dark leaf spot lateral flow device on air samples collected at Hesketh Bank are shown in Table 2. The EVL one step reader device gives an optical reading of the amount of captured immunogold on the test and control line. Use of the reader enables the device to be used semi-quantitatively to determine the amount of dark leaf spot present in the sample. The Brassica$_{spot}$ dark leaf spot was used to determine those days when lateral flow tests could be used for detection of dark leaf spot.

**Table 3. Visual assessment of test line for sampling periods using the dark leaf spot lateral flow device in field trials at Hesketh Bank**

| Field exposure Period | Model Risk Observation | Test line | No. of conidia | Immunogold Line Reading |
|---|---|---|---|---|
| 1 September | Green | No | 36 | 0.2 |

| | | | | |
|---|---|---|---|---|
| **2 September** | **Green** | **Yes** | **0** | **6.5** |
| **8 September** | **Green** | **Yes** | **1** | **6.8** |
| **9 September** | **Green** | **Yes** | **0** | **6.3** |
| **15 September** | **Green** | **Yes** | **2** | **6.5** |
| **19 September** | **Green** | **Yes** | **0** | **5.5** |
| **03 October** | **Green** | **Yes** | **0** | **6.5** |
| **04 October** | **Green** | **Yes** | **0** | **5.8** |
| **07 October** | **Green** | **Yes** | **0** | **6.5** |
| **08 October** | **Green** | **Yes** | **4** | **5.9** |
| **3 September** | **Red** | **Yes** | **0** | **6.5** |
| **12 September** | **Red** | **Yes** | **0** | **6.5** |
| **14 September** | **Red** | **Yes** | **0** | **6.0** |
| **21 September** | **Yellow** | **Yes** | **3** | **6.3** |
| **22 September** | **Yellow** | **Yes** | **0** | **6.5** |
| **23 September** | **Red** | **Yes** | **0** | **6.1** |
| **25 September** | **Yellow** | **Yes** | **1** | **6.5** |
| **26 September** | **Yellow** | **Yes** | **0** | **6.5** |
| **28 September** | **Red** | **Yes** | **0** | **6.5** |
| **12 October** | **Yellow** | **Yes** | **0** | **6.5** |
| **13 October** | **Yellow** | **Yes** | **0** | **6.7** |

A selected number of tests were carried out on days when there was high, moderate and zero environmental risk of dark leaf spot infection at Hesketh Bank. In 2006 only tests conducted on samples taken on the 1 September 2006 gave positive results (Table 3). All other days tested gave negative results for the presence of dark leaf spot conidia (Table 3). The scanning densitometer reading on the test line confirmed the positive result observed on the 1 September 2006. A value of 0.1 was obtained on this date however the scanning densitometer value obtained for readings with negative lateral flow test results was over approximately 6. Microscopic observation of sub samples from the 1 September 2006 confirmed the presence of dark leaf spot conidia in samples collected on the 1 September 2006. The environmental risk was zero for this time period (Figure 8).

**Detection of ringspot ascospores in air samples using ELISA and the ringspot lateral flow device at Hesketh Bank 2006**

 

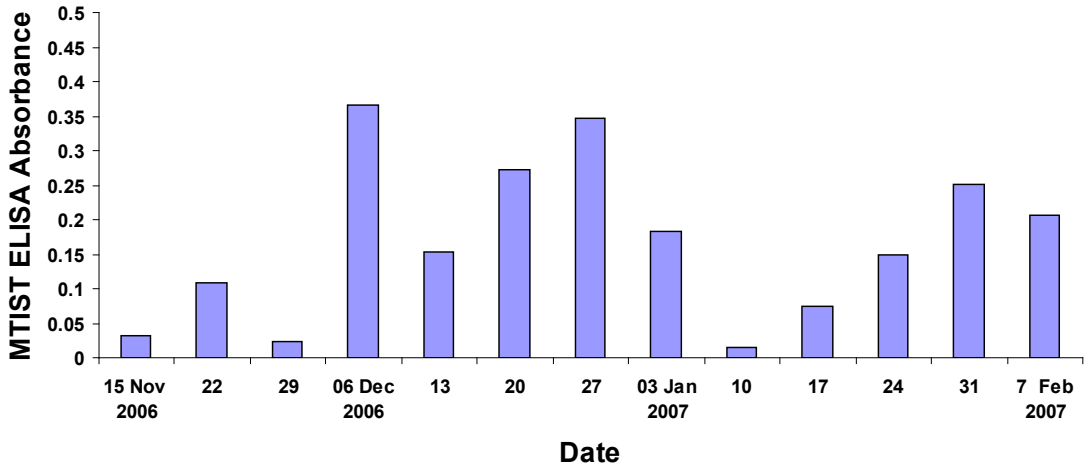


**Figure 7:** Absorbance values for ELISA tests taken on MTIST wells sampled weekly at Hesketh Bank from November 2006 to February 2007

Ringspot inoculum was monitored using an MTIST trap at Hesketh Bank from the 16 November 2006 to the 14 February 2007. The results of the weekly sampling (daily sampling 06:00 H – 18:00 H for 7 days) are shown in Figure 7. High ELISA absorbance values were recorded during the weeks 6 December 2006 to the 10 January 2007. The highest absorbance values (0.4) were recorded on the 6 – 13 December 2006 and the 27 December 2006 – 3 January 2007 indicating high levels of airborne ringspot ascospores (Figure 7). High absorbance values were also observed in the weeks from the 24 January 2007 – 14 February 2007. After this time the crop was within its harvest interval and monitoring of ringspot ascospores in the air ceased.

**Prediction of dark leaf spot and ringspot infection conditions at Hesketh Bank in 2006**

The results from Brassica$_{spot}$ disease prediction system at the Hesketh Bank site in 2006 are shown in Figure 8. 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 by the model. However high risk period were designated as having occurred if scores of over 150 had occurred. This was designated with a red coloured bar (high risk) on the day where these scores occurred. Days with scores of 100 – 150 were designated as having a low to moderate risk of infection. Moderate risk days occurred on the 9, and 13 July 5, 14, 18, 26 August 6, 9, 18, 23, 26, 30, and 31 October 23, 25, 26 November 5, 16, 20 and 21 December 2006 and the 1, 2, 6 and 7 January 2007. High risk periods were recorded on the 16, 17, 19, 20, 21, August and the 1, and 30 September 1, 28, and 29 October 6, 7, 8, 9 and 13 November 2006. There were many risk periods recorded during December 2006. These occurred on the 6, 7, 8, 9, 10, 11, 17, 18, 23, 24, 25, 26, 27, 28, and 29 December 2006. Additional high risk periods occurred on the 9 and 10 January 2007.

 

**Figure 8:** Prediction of dark leaf spot and ringspot infection at Hesketh Bank 2006

**Detection of dark leaf spot conidia in air samples using lateral flow devices at Bicker 2006**

Limited numbers of vials were available for testing during 2006 because tests were carried out directly by members of the Allium and Brassica Centre. Only four weeks (34 days) were available for testing at the beginning of July 2006 (14 July – 17 August 2006). Tests on this limited number of test tubes proved negative. No further test results were available from the Bicker site during 2006. Environmental infection risk during this period was relatively low (Figure 10).

**Detection of ringspot ascospores in air samples using ELISA and the ringspot lateral flow device at Bicker 2006**

Lateral flow tests for detection of ringspot ascospores could not be used at Bicker in 2006. No vials were available for testing with the ringspot lateral flow prototype as the crop was harvested before the prototypes became available during November/December 2006. However an MTIST trap was used at the Bicker site during November 2006. Results from the MTIST trap (Figure 9) indicated the presence of ringspot ascospores in air samples at high levels from the 3 – 9 November 2006. Estimated numbers of ringspot ascospores in air samples dropped to approximately 0.1 during the 9 – 20 November 2006. Absorbance values of 0.1 and below indicated a low probability of ringspot lesion occurance on crop plants. Absorbance values of approximately 0.01 were recorded during the 20 November to the 1 December 2006 (Figure 9).

 

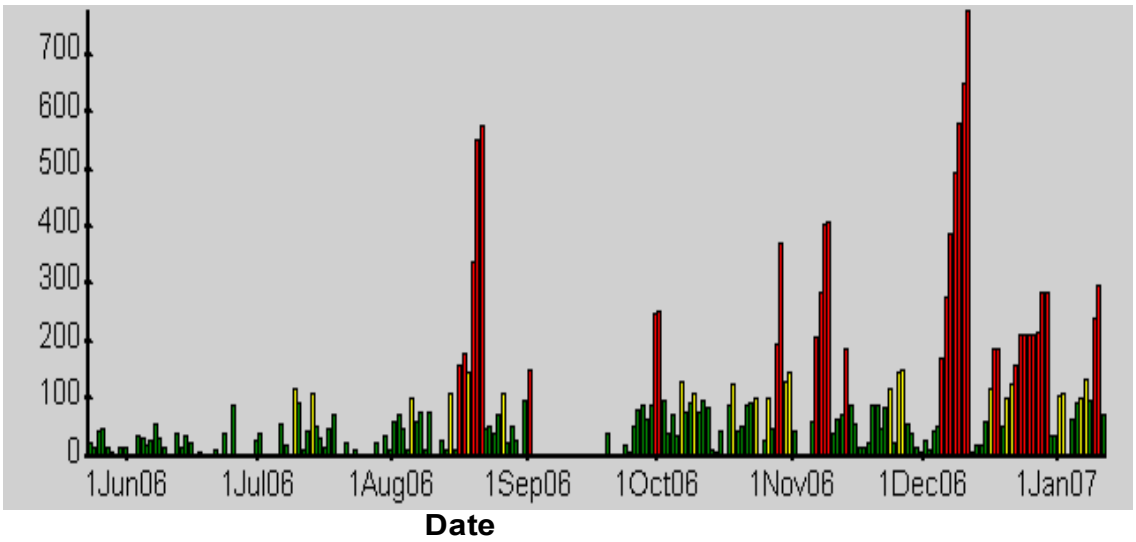


**Figure 9:** Absorbance values for ELISA tests taken on air samples collected in MTIST wells sampled at Bicker during November 2006

**Prediction of dark leaf spot and ringspot infection conditions at Bicker in 2006**

The results of using the Brassica$_{spot}$ disease forecasting system at the Bicker site in 2006 are shown in Figure 10. This predicted relatively few periods when conditions were suitable for dark leaf spot and ringspot infection during the trial period. Over the trial period high risk infection periods occurred on the 15 and 16 June and the 23 July 2006. High risk periods were also recorded on the 3, 13, 18 and 19 August 2006. Some moderate infection risk periods were also recorded on the 26 June 11, 20, 21, 27 July and the 1, 7, 12, 22, 24, 26 and 27 August 2006. No information from the Brassica$_{spot}$ disease forecasting system at the Bicker was available during September, October and November 2006.

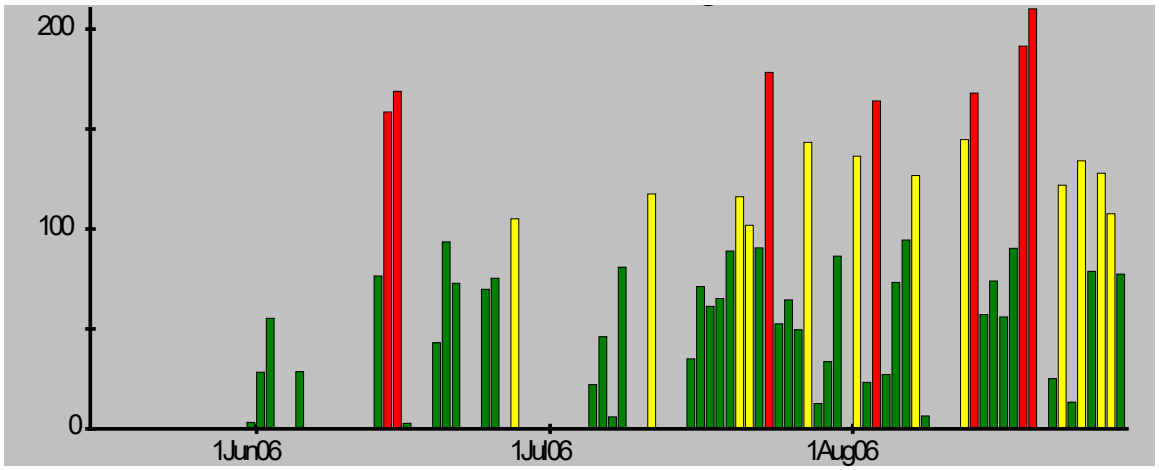


**Figure 10:** Prediction of dark leaf spot and ringspot infection at Bicker in 2006

**Conclusions**

Using the Warwick HRI dark leaf spot lateral flow device gave positive results for dark leaf spot conidia in air samples at Hesketh Bank and Bicker during 2006. However during 2006 environmental conditions were unfavourable for dark leaf spot and ringspot development during June, July and August 2006. Usage of the dark leaf spot lateral flow device gave accurate predictions of dark spot conidia at Hesketh Bank. The results indicate a clear link between dark leaf spot and oilseed rape harvesting. However at Hesketh Bank the levels of dark leaf spot are too low in air samples for disease development on crops especially spring and autumn harvested cauliflowers. This was similar to the Bicker site in Lincolnshire although not enough data could be obtained at Bicker in 2006 to determine a pattern of dark leaf spot development in vegetable brassica crops. Low levels of dark leaf spot in air samples at Hesketh Bank which maybe spatially discrete indicate that conditions are unfavourable for dark leaf spot development on cauliflower crops in this area. However crops such as Brussels sprouts which have more favourable crop microclimates may be more susceptible to dark leaf spot development at Hesketh Bank. Use of the MTIST trap at both Bicker and Hesketh Bank in 2006 indicated the prevalence of high levels of ringspot ascospores in air samples at these sites at the end of the 2006 growing season.

## DISCUSSION

Detecting pathogenic spores before they can infect crops is a useful approach in controlling air borne diseases of vegetable or arable brassicas (Kennedy, *et.al*., 2006). If the technique is to be of value practically then methods of detecting spores in the field are a necessary prerequisite for uptake. The system also needs to be economic and in relation to the value of the crop and the cost of fungicide applications. Producing field devices which can detect conidia of dark leaf spot and ascospores of ringspot was successfully investigated within HDC contract FV233. These devices require sensitivity testing in relation to the appearance of ringspot symptoms and validation under field conditions in conjunction with air sampling devices. Air samplers are available which can trap airborne particulates within vessels. These vessels can be sampled for dark leaf spot and ringspot spores using the ‘in field’ spore detection devices.

### Field tests with the dark leaf spot lateral flow device in commercial crops

Field tests with the dark leaf spot lateral flow device in Lancashire and Lincolnshire were largely negative. In the dark leaf spot lateral flow competitive format the presence of a test line indicates the absence of dark leaf spot spores in a sample. However the climatic conditions occurring during 2006 were not favourable for dark leaf spot development. Only one positive test (12 September 2006) was observed for dark leaf spot using the device at Hesketh Bank in Lancashire. Environmental conditions were not favourable for infection on that date. Given the number of conidia was relatively high and was not replicated it is likely that the conidia of in the sample came from outside the crop. There could have been several possible sources of the dark leaf spot. One source could have been oilseed rape harvesting although the spores would have had to come from a long way as there is little or no oilseed rape in the Hesketh Bank area. Another more likely source could have been a nearby vegetable brassica crop which was being harvested. There were very few positive

 

tests observed by the Allium and Brassica centre in Lincolnshire for dark leaf spot using the device (Carl Sharp, Personnal Communications). In limited observations on the crops no ringspot or dark leaf spot was observed until October 2006. At this time only small numbers of mainly ringspot lesions were observed. The dark leaf spot lateral flow assay proved very sensitive in its reaction to low numbers of dark leaf spot conidia in test samples. The lateral flow device when used on these samples could detect between 47 and 23 dark leaf spot conidia per sample.

**Sensitivity testing the ringspot lateral flow device at Warwick HRI**

The ringspot competitive lateral flow tests was assembled and used in tests at Warwick HRI. Again the environmental conditions did not make these tests easy to perform. Sensitivity testing of the device had to be limited due to the limit number of test tubes where ringspot ascospores were present. Naturally produced ascospores in the field had to be used for sensitivity testing as laboratory ringspot cultures have lost their ability to produce ascospores. Ringspot ascospores are sticky and once trapped in the sample vessel they cannot be removed. They are also difficult to distinguish from other ascospores produced by other fungi. For this reason the number of ascospores present in each collection vessel had to be estimated using infected trap plants placed beside the spore trap over the sampling period before removal and misting to ensure that all ascospores present on the leaves could infect. In this way the reactions of the ringspot lateral flow device could be directly compared to the number of lesions on trap plants as a means of identifying samples with high, moderate or low levels or ringspot ascospores. This system of sensitivity testing was useful as it allowed the device to be calibrated to detect the appropriate amount of ringspot ascospores which resulted in lesion formation given favourable environmental conditions. However in other respects it was not satisfactory because the number of lesions on trap plants may have over estimated the number of ringspot ascospores in the sampling vessel. This was due to the unequal exposure periods for collection of air samples in comparison to exposure times for trap plants in the field. However other tests using different immunological tests (Kennedy & Wakeham, 1999) might in future overcome these problems.

**Development of the two step lateral flow test for estimation of ringspot ascospores and dark leaf spot conidia from the same vial**

It is a requirement that both the ringspot and dark leaf spot lateral flow lateral flow tests are carried out using the same tube. As the ringspot ascospore is sticky it will be possible to carry out the dark leaf spot test first but the ringspot lateral flow test must carry out part of the reaction in the sample tube. The ringspot test can therefore be carried out independently of the dark leaf spot test or after the dark leaf spot test has been carried out. The initial reaction in the tube using a conjugate pad containing ringspot conjugated antibody means that when the buffer containing these components is added to the sample vessel only remaining unbound antibody/conjugate is transferred to the ringspot lateral flow device. The presence or absence of this component when transferred to the ringspot lateral flow device will give the reaction which results in the presence or absence of a test line. The dark leaf spot test cannot be used after the ringspot test because the dark leaf spot conidia may have

been transferred to the ringspot lateral flow device as dark leaf spot conidia do not stick to the collection vessel.

**Practical usage of the dark leaf spot and ringspot lateral flow tests under field conditions in commercial crops.**

Validation of the tests developed within project Fv 233a in the field requires environmental data from a weather station and a spore trap which, could be integrated with the weather station. At each test location both the environmental data and the air-borne spore risk can be assessed (the latter using the lateral flow device). For each test period the vial would be replaced with a fresh one and the sample in the removed vial tested. The results of the test would be visible as lines on the lateral flow device or could be assessed using a lateral flow reader device. Practical usage of the system has shown that the procedure can lose accuracy if water collects in the sampling vial. To reduce this occurrence a cowl was fitted to the spore trap which has stopped water collection in the test vials during rainfall. Using wire mesh over the spore trap orifice has also reduced insect contamination of the test vial.

By using the existing sensitivity, the system has the advantage of detecting the very earliest possibility of disease transmission. The grower or consultant will be able to estimate real disease risks which he can control using fungicides. It is hoped that the system will enhance the activity of protectant fungicides or even biological control agents. By applying fungicides quickly the grower will be in a situation where he can use protectants to control disease. Use of protectant fungicides could help reduce residues in the crop at harvest. The requirement for zero or low fungicide residues is of increasing importance in crop production.

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