Project title:	Diagnostics: Validation of the lateral flow detection devices for the light leaf spot and powdery mildew vegetable Brassica pathogens and testing of white blister detection test prototypes
Project number:	CP 099
Project leader:	A. Wakeham, University of Worcester
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Previous report:	None
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Location of project:	University of Worcester
Industry Representative:	A Richardson, Allium and Brassica Centre
Date project commenced:	1 August 2012
Date project completed (or expected completion date):	31 July 2015

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

AUTHENTICATION

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

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GROWER SUMMARY

Headline

Large batches of lateral flows for field diagnosis of airborne diseases transmission events of light leaf spot, Brassica powdery mildew and white blister spores in collected aerosols have been prepared and, with a diagnostic range suitable for use in field studies. A disease threshold for light leaf spores has provisionally been identified at 200 spores per cubic metre and the on-site test will be supplied to an Agronomist for trials in 2013.

Background

Background and expected deliverables

In the airborne environment many plant diseases are able to spread between and within cropping systems. In the UK, using either laboratory based analysis or a field based pregnancy style test, HDC funded work has provided the development of systems to monitor field aerosols for target disease inoculum either on a daily or weekly basis. Air sampling systems and tests are available for the following vegetable plant pathogens: *Peronospora destructor* (onion downy mildew), *Mycosphaerella brassicicola* (ringspot), *Alternaria brassicae* (dark leaf spot), *Pyrenopeziza brassicae* (light leaf spot), *Erysiphe cruciferarum* (Brassica powdery mildew) and *Albugo candida* (white blister). By identifying disease (spores) in field air samples growers can time sprays more effectively and make informed decisions on which type of fungicide application to make.

Studies measuring *M. brassicicola* (ringspot) in airborne spore samples has shown that under ideal environmental conditions, high concentrations of spores are required in the air for infection to occur (2000 spores per cubic metre). The current study aims to identify field spore concentrations of light leaf spot and powdery mildew that are required to cause disease symptoms on crops at a commercial scale. The developed diagnostic test formats for each of these diseases will be adjusted to reflect this. Results from previous studies show that light leaf spot ascospores appear in the air in large enough levels to be a problem only during discrete periods. Light leaf spot inoculum may be present at other times but occurs at too low concentrations to become a problem in developing sprout crops. Fungicides applications can provide good control of Light leaf spot in Brussels sprout crops if applied at times when the disease is in the air. Where routine ‘blanket’ crop spray programmes have been applied, control can be ineffective. Light leaf spot is endemic in Scotland and becoming common in Brassica production areas of Northern England. Targeted application of effective fungicides in response to spore concentrations can play a

vital role in controlling the disease. Inappropriate or unnecessary fungicide applications are not only costly but will increase the pressure for development and selection of pathotypes able to resist previously effective control measures.

Powdery mildew as, light leaf spot can infect any above ground plant part reducing plant growth and yield. Most horticultural brassicas are susceptible to infection and these include Brussels sprouts, cabbage, Chinese cabbage, kohlrabi, broccoli, kale, mustard, collards, cauliflower, radish, and horse radish. Powdery mildew disease is highly airborne and small numbers of conidia (spores) can be wind dispersed over large distances. To date there is little information on the environmental requirements for Brassica powdery mildew development although it appears to be favoured by dry conditions and, in the UK, these usually only occur during early summer. Infection of vegetable Brassica crops is unaffected by the powdery mildew occurring on oilseed rape crops. The occurrence of older tissues where powdery mildew development is more favoured, during autumn and winter, may act as a bridge for the pathogen to occur on Brussels sprouts buttons. The application of fungicidal sprays (Nativo) is approved for control of the disease, however as for light leaf spot, information about the availability of powdery mildew inoculum would be useful in control regimes. The airborne concentration of powdery mildew required for disease development in the crop is currently unknown but it is thought to play a vital role in the initial development of powdery mildew in Brassica crops.

White blister is caused by the Oomycete pathogen *Albugo candida* and is a common disease of many economically important cruciferous vegetables and oilseed crops. Significant yield losses from this disease have been reported on the oilseeds *B. rapa* and *B. juncea* and, to a lesser extent, on susceptible lines of *B. napus.* Affected vegetables include broccoli, Brussels sprouts, cauliflower, radish, mustard, Chinese cabbage and turnip. The impact of disease in these crops is of a cosmetic nature and can render crops unmarketable. To date, more than 10 distinct biological races of *A. candida* have been identified and classified based on host specificity. Race 9 infects *B. oleracea* and a management strategy to control the disease in these crops has included the development of an environmental model (Brassica spot^{TM} – White Blister model). The present study aims to improve the white blister disease risk forecast by including information on availability of *A. candida* airborne disease. Monoclonal antibodies, with recognition sites to *A. candida* (Race 9) spores and with reactivity to UK commercial isolates will be used in an immunological chromatographic test strip (lateral flow) to provide ‘in-field’ information on *A. candida* in collected field air samples. Similarly, lateral flow tests and laboratory diagnostic

tests developed in HDC FV33 for identification of airborne disease of light leaf spot and Brassica powdery mildew will be adjusted for commercial field usage. Enabling provision of information for requirement of fungicide spray applications in response to peaks in airborne spore numbers .

The expected deliverables from this project are:

- Assess target disease spore concentrations in field aerosols and evaluate the effect on infection and symptom development in commercial Brassica cropping systems
- To provide tests which can be used directly by UK growers or consultant to identify presence of light leaf spot, Brassica powdery mildew or white blister spores in the air at concentrations likely to cause disease at a commercial scale.
- Ability to detect white blister, Brassica powdery mildew and light leaf spot in the field before disease is visible in the crop.
- Improved use of fungicide applications within vegetable Brassica production systems and the reduced likelihood of tebuconazole resistance within light leaf spot populations (already been reported).
- Assess the potential to develop a multiplex test to identify risk of multiple pathogens on a single test device

Summary

Batches of lateral flows for field diagnosis of light leaf spot ascospores and Brassica powdery mildew in collected aerosols have been prepared with a diagnostic range suitable for use in field studies. Using a field portable electronic reader, light leaf spot ascospores of between 200 and two million can be estimated. For Brassica white blister, spore concentrations of between 30 and three hundred thousand can be differentiated using an ESE reader. For field trial usage the white blister lateral flow indicates a shelf life of upwards of one year when stored at room temperature. The light leaf spot recorded good test line stability over an 18 month period but the gold antibody conjugate is stable for a three month period only. Studies in Year 2 of the project would look to improve this.

Preliminary investigations in to the development of a multiplex test (several tests on one lateral flow test) have proved promising. At high contaminating spore concentrations, test

line sensitivity or specificity for either target pathogen remained unaffected when two spore types were used in one test sample.

The present investigation has assessed the use of monoclonal antibodies, developed previously in an Australian study, to identify a suitable probe that could be used in a lateral flow to quantify airborne spores of *Albugo candida* within UK *Brassica oleracea* cropping systems. A monoclonal cell line (UW 256) showed a level of specificity which was able to discriminate between A. candida (race 4, white blister on Shepherds purse) and *A. candida* (race 9, white blister on B. oleracea). The lateral flow ('in field' test) developed for use in Australian commercial cropping systems and, which also used UW 256 as the specific probe , provided a detection sensitivity of 100 white blister race 4 spores. Using *A. candida* isolates from UK B. oleracea plants the lateral flow prototype has a detection sensitivity of 1000 white blister spores. Depending on the disease threshold (under optimal environmental conditions the number of spores in the air required to initiate uniform disease expression on susceptible plants) the sensitivity of the white blister lateral flow may require alteration for field usage.

At a commercial site in Scotland a crop of Brussels sprout *c.v*. Petrus was monitored for light leaf spot disease from the 13th August 2012 to February 2013. Three types of air sampler were used to monitor light leaf spot disease transmission events. Light leaf spot spore counts, derived from the volumetric spore count and identified by immunofluorescence, indicate that a spore concentration of 200 per cubic metre would under appropriate environmental conditions, lead to infection at low level in semi-resistant B. sprout varieties (*c.v.* Petrus) and significant infection in susceptible cultivars. Throughout this period and, to inform of potential light leaf spot disease risk periods, results derived from weekly laboratory analysis of the field exposed MTIST air samples were made available for use in crop protection decision management strategies. An 'in field' lateral flow was also used in processing weekly collected field aerosols and correlated with the MTIST value. In year 2 of the project, lateral flow batches will be available for use and compared with the weekly laboratory test for monitoring light leaf spot disease transmission events in a commercial crop. The disease threshold of each test will be set at 200 spores per cubic metre of air sampled.

Trials to monitor airborne disease transmission events for Brassica powdery mildew and White blister remain in progress and will continue throughout 2012 to December (Brussels sprout trials) and January 2013 (Cabbage crop). All three air sampling devices are monitored weekly either by a consultant or University of Worcester staff member and to date little disease has been observed using by air samples. The crops remain visually clean

at present. Once disease has established within the crop the fungicide resistance interaction trials should prove useful in determining the disease threshold required to set the MTIST ELISA and in field lateral flow devices to identify disease risk periods.

Financial benefit

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

- The Light leaf spot in field test has a detection threshold set at 200 spores per cubic metre air sampled for provisional timing for application of Signum to vegetable Brassica crops. This is likely to improve the efficacy of this chemical especially in production of vegetable brassicas in Northern Britain.
- Tests can also be used in conjunction with disease forecasts

Action Points

- Consultants and Growers can assist validation with the 'in field' test to determine when light leaf spot, Brassica powdery mildew and white blister spores are present in their crop.

SCIENCE SECTION

Introduction

Antibody probes developed to disease propagules of *Erysiphe cruciferarum* (Brassica powdery mildew) *Pyrenopeziza brassicae* (Light leaf spot) and *Albugo candida* have been incorporated in to lateral flow format for field testing. The lateral flow devices have been developed to detect disease aerosols of Brassica powdery mildew, light leaf spores and Albugo in collected field aerosols The tests are semi-quantitative and, based upon test line depletion (visual or by electronic measurement), spore concentrations in the air can be estimated. A control line remains constant to show that the test has worked. The test is counter intuitive in that as spore concentration increases the test line decreases in colour intensity. At high spore concentrations no test line is visible (Fig. 1) .

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

In a field setting a cyclone air sampler is used to sample daily aerosols The air sample can be collected in to a single tube over multiple days (i.e. a week) or as daily aerosols using a multi-vial sampler (Fig. 2). The air sampler can be powered from tractor batteries / solar power units and requires once weekly attention for changeover / collection of the tube(s). After sampling the tube is removed, liquid added and transferred to a field lateral flow device. After approximately 10 minutes the lateral flow is assessed for test line development and an estimation made as to whether the target spore type is present and at what concentration. An electronic reader can be used to provide a digital print out or the assessment can be made by eye.

Figure 2. Automatic Multivial field cyclone sampler (www.burkard.co.uk)

The airborne concentration of each of the spore types required for disease development in the crop is currently unknown. A previous study (Kennedy *et al.,* 2000) demonstrated that under optimal environmental conditions upwards of 2000 *Mycosphaerella brassicicola* ascospores per cubic metre air sampled were required for significant ringspot disease development on exposed susceptible Brussels sprout plants. Lateral flow tests developed for the measurement of ringspot inoculum in the air have been calibrated to reflect this information. At concentrations close to those required for crop disease establishment (2000 spores per cubic metre) the test line of the lateral flow is depleted (not visible). This study looks to determine the airborne inoculum concentrations required of the light leaf spot, powdery mildew and white blister pathogens for disease development on commercial cropping systems.

Materials and Methods

Diagnostic Tests: Evaluation of light leaf spot, powdery mildew and albugo

Batches of of the light leaf spot and powdery mildew lateral flows, as developed and reported in HDC FV333 Final Report, were prepared to include test and control lines. Test sensitivity for each spore type was determined and the stability of the incorporated reagents for each lot was determined over time (shelf life) as described below. Preliminary studies to assess development of a multiplex lateral flow for multi disease targets are described. The construction of a lateral flow for measurement of UK *A. candida* race 9 was assessed

Lateral Flow Sensitivity to Target Spore Type

Lateral flow assembly: For each test (light leaf spot and powdery mildew) lateral flows comprised of a Millipore 240 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), a filtration and sample pad (Cat No. T5NM, Millipore Corp., USA). Using a flatbed air jet dispenser (Biodot Ltd, The Kingley Centre, West Sussex, UK), control lines of an IgG purified polyclonal antiserum was sprayed directly on to the membrane surface of each lateral flow. The lateral flows were then divided in two groups: Group A received a test line application of collected *Pyrenopeziza brassicae* soluble ascospore suspension, adjusted to a protein concentration of 500µg ml-1 in in a solution of sucrose, trehalose, isopropanol and sodium azide and applied at a rate of 10mm sec^{-1}. Group B, received a test line application of *Erysiphe cruciferarum* soluble spore antigen, adjusted to a protein concentration of 500µg ml^{-1} in a solution of sucrose, trehalose, isopropanol and sodium azide and applied at a rate of 10mm sec^{-1}. The lateral flows were air dried at 37ºC for a period of 4 hours and cut in to 5 mm strips.

A volume of 500 µl purified IgM monoclonal antibody which had been raised to to *P. brassicae* (coded UW 268) was mixed with a goat anti-mouse IgM 40nm gold conjugate (Code BA GAMM 40, British Biocell International, Cardiff, UK). After which was made up to 2ml with protein A 20nm gold conjugated beads (British Biocell International) in 25% PBS. Sample pads, taken from each *P.brassicae* lateral flow device, each received 60µl of the antibody gold conjugate solution before air drying at 27ºC. After which the lateral flows devices were re-assembled with the UW268 gold conjugate and mounted within a plastic housing device

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

Lateral Flow sensitivity: An 'invitro' *P. brassicae* tenfold ascospore serial dilution series ranging from 20,000,000 to 200 spores/ml was prepared in NPARU lateral flow extraction buffer (HDC FV33 Final Report). To microfuge tubes 3 x 100µl of each ascospore dilution was then aliquoted. From a light leaf spot lateral flow device, an antibody (UW 268) gold conjugate sample pad was removed and inserted into the extraction buffer of a microfuge tube. This process was repeated for each ascospore tube dilution series. The pads were gently agitated to release the gold conjugate in to solution and allow binding to the ascosporic material present. After a 3 min. incubation period the antibody gold conjugate/ extraction buffer was removed and transferred to a light leaf spot lateral flow device. Determination of lateral flow test and control line development was then made by visual assessment and using an ESE QUANT hand held reader at 20 mins. Each device was scanned on two occasions using the ESE QUANT hand held reader.

Conidia of *E. cruciferarum* (Brassica powdery mildew) were produced as described in HDC FV33 Final Report Section 3.2.1, and a doubling dilution series (3,000,000 to 30 spores ml^{-1}) was prepared in NPARU extraction buffer. To sample pads of the prepared Brassica powdery mildew lateral flow devices, 100µl aliquots of the *E. cruciferarum* spore dilutions were then applied. The lateral flows were read 20 minutes after sample application for test and control line development by visual assessment and using an ESE lateral flow reader (Figure 4a, b).

Figure 4a. Lateral flow read in ESE reader

Assessment of the lateral flow batches for shelf life stability

Control and Test line stability: On a monthly basis a 100µl aliquot of lateral flow extraction buffer was applied to each of six stored lateral flow devices. The lateral flow devices were prepared as described

above, and identified as Batch 001LL/13 (Light leaf spot) and 001BPMD (Brassica powdery mildew). Storage was in individually sealed foil pouches at either room temperature (18 - 20^{0}C) or at 4^{0}C. Testing was at monthly intervals and the lateral flow results read 20 min. after sample application to the lateral flow device. For the purpose of this study gold antibody conjugate pads were prepared on the day of testing

Gold antibody conjugate pads: On a monthly basis a 100µl aliquot of lateral flow extraction buffer was applied to each of six freshly prepared lateral flow devices (six light leaf spot and six Brassica powdery mildew). The lateral flow devices were prepared as described above. Gold antibody conjugate pads for each lateral flow disease type, prepared as described above and identified as Batch 001LL/13gc and 001BPMDgc were stored in sealed pouches at either room temperature (18 - 20^{0}C) or at 4^{0}C.Testing was at monthly intervals and the lateral flow results read 20 min. after sample application to the lateral flow device.

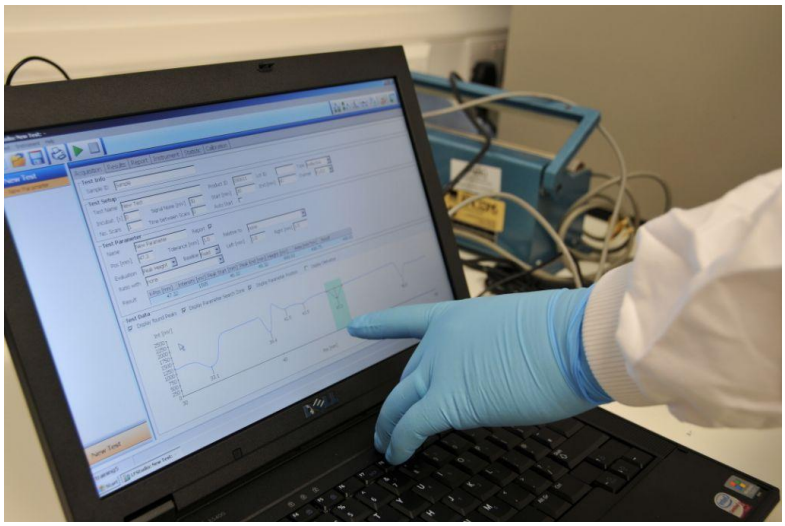

Figure 4b. Lateral flow test and control line digital output as displayed on a portable lap top screen

Preliminary studies to investigate the potential of a multiplex lateral flow

Lateral flow membrane strips were set up as previously described however test line strips of both Light leaf spot and Brassica powdery mildew antigen were applied to the same lateral flow but at different application points. Spore suspensions of the two disease types were applied in isolation or mixed to the lateral flows and the test line readings recorded using an ESE lateral flow reader.

Preliminary studies to develop a lateral flow to albugo candida (white blister) race 9

A batch of 500 lateral flows (Code No: 001AC) was prepared and optimized as previously described but this time using UW 256 MAb to identify and quantify UK race 9 isolates of *A. candida*. This MAb had previously been used in a study to monitor airborne disease of A. candida race 9 isolates found in Australasia.

Field trials: Light leaf spot, powdery mildew and albugo candida

Air samplers (Burkard Manufacturing, www.burkard.co.uk) were positioned within commercial Brassica cropping systems (Brussels sprouts and Cabbage) and the air monitored for disease propagules of light leaf spot in Scotland, powdery mildew and white blister in Lincolnshire. The air samplers were placed at 2m distance from each other. To collect spores in the air, three types of air sampler were used: a volumetric sampler to identify target spores by microscopic analysis, a microtitre air sampler (MTIST) for antibody based laboratory analysis and a cyclone sampler to provide a '10 minute ' on-site field reading (the lab on a stick, lateral flow assay). Agricultural batteries 12V were used to power the air samplers and where possible solar panels were attached to reduce the labour of battery changes. A detailed description of the air samplers, operation and spore assessment is described below:

Air sampling equipment and target spore analysis

Microtitre Immunospore Trap (MTIST). A detailed description of the MTIST device can be found in Kennedy *et al.,* 2000. In the outdoor 'field' version air is drawn thorough a manifold consisting of a plastic tube with a right angle bend placed over the sampler inlet (Fig. 5). The sampler contains four microtitre strips each containing 8 wells. The MTIST air sampler uses a suction system and particulates in the airstream are impacted on the base of each collection well of the four microtitre strips. The four coated eight well microtitre strips were coated with a combination of 2 x strips at 0.1mg ml-1 Poly-L-Lysine (Sigma P-1524) in distilled water and 0.05% sodium azide (Sigma P-1524) and 2 x strips 5:1 mixture of petroleum jelly and paraffin wax (Wakeham *et al.,* 2004). The MTIST spore trap was operated for 12H periods from 06:00 H to 18:00 daily.

Figure 5. MTIST air sampler positioned within a commercial Brussels sprout cropping system and operated from an environmental data logger

The 4x 8 well coated microtitre strips were changed weekly. For trials in Scotland the field exposed microtitre wells were posted to NPARU and processed immediately on receipt by PTA ELISA (plate trapped antigen enzyme-linked immunosorbent assay (Kennedy *et al.,* 2000)) to provide weekly light leaf spot disease alerts. Field exposed microtitre strips at trial sites in Lincolnshire were collected at weekly intervals, stored at -20^{o}C prior to quantification of aiborne inoculum of powdery mildew and white blister by PTA ELISA.

Burkard 24hr Volumetric glass slide air sampler. A Burkard volumetric air sampler which contained a Melinex tape coated with silicone (BC 380S, Basildon Chemical Co, Kimber road, Abingdon, Oxon, UK) operated at an air flow of 10 L of air per minute over a full 7 day period. Field sampled air particulates were impacted directly on to an area of the tape which corresponded to time intervals by movement of the tape over an hourly period. Following weekly field exposure the Melinex tape was removed and posted to NPARU. On receipt the tape was sectioned in to daily segments, mounted and by bright field microscopy assessed for *A. candida* and *E. cruciferarum* (white blister and powdery mildew) spores between the hours of 06:00 H to 18:00 daily. The Melinex tapes received at NPARU from the Scottish site were processed over time by immunofluorescence (Kennedy *et al.,* 1999). Ascospores of light leaf spot were identified on the tape using MAb UW 277 attached to an anti-species Fluorescein conjugate (Figure 6) and the numbers calculated to spores / cubic metre present in the crop between the hours of 06:00 H to 18:00 daily.

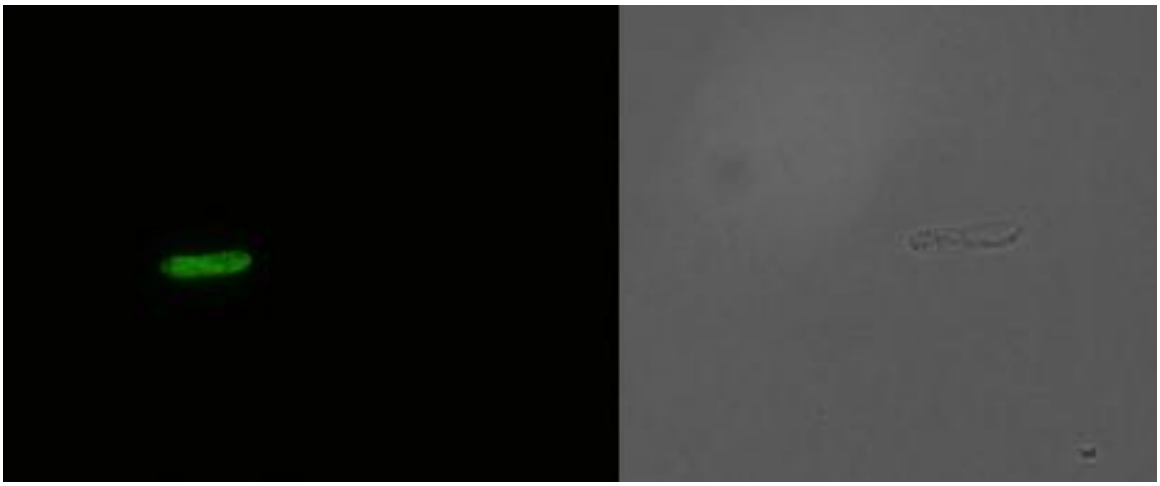

Figure. 6. Light leaf spot ascospore (airborne disease) as visualised by immunofluorescence and bright field light microscopy.

Burkard cyclone air sampler. The characteristics of a cyclone air sampler are described by Ogawa & English (1995). Air is drawn through the sampler using a vacuum pump in the form of a cyclone. The height of the cyclone and air inlet, along with the width of the air inlet, air exhaust diameter and the diameter of the cyclone within 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 (Burkard Manufacturing Co.). The cyclone air sampler operates at an air flow rate of 10 to 15 L air / min, is adapted for field usage and air particulates trapped in a 1.5ml microfuge tube (Fig. 2) . At each of the sites the field exposed tube was changed weekly and prior to assessment was stored at -20^{0}C. The tubes were assessed for the target spore types at the end of each trial period by lateral flow.

Lateral flow process: To each field exposed microfuge tube 200µl of NPARU B2 buffer was added, swirled and incubated at room temperature for five minutes. A lateral flow device developed for field assessment risk of one of the target spore type was identified. A 100ul aliquot of the field spore suspension (taken from the tube) was then applied to the sample pad of the lateral flow device (Fig. 7) and test line development was assessed at 10 and 20 minutes using an ESE Quant reader (Fig. 8).

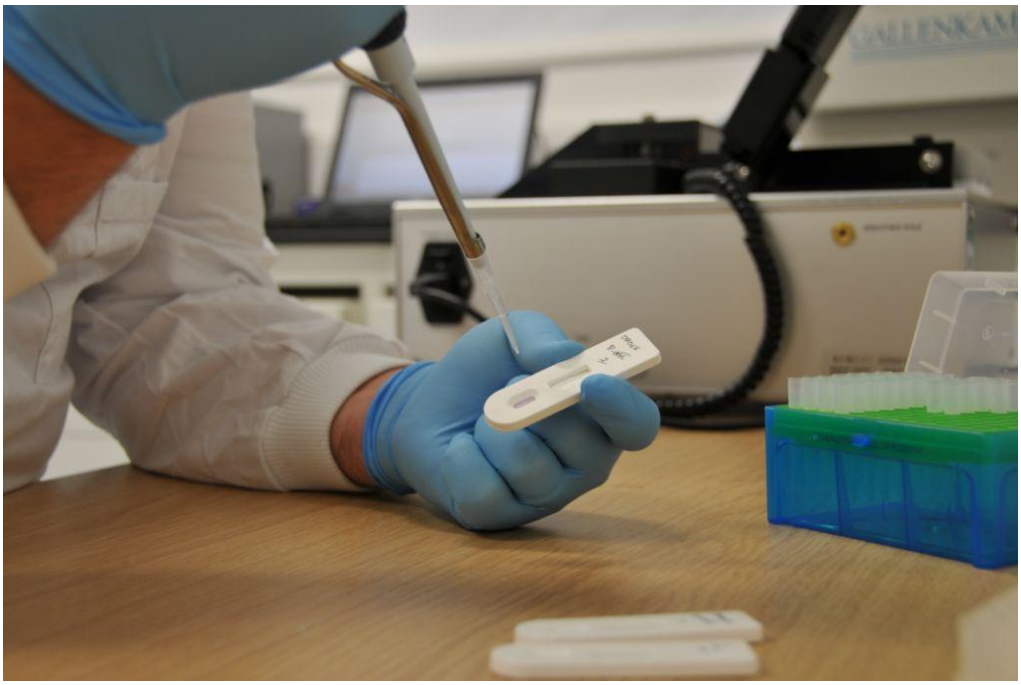

Figure. 7 A lateral flow device for evaluation of field crop risk to white blister

Figure 8. An ESE lateral flow reader

Field trials

Light leaf spot. At a commercial site in Scotland (Boarhills, Fife OS ref. NO 582 137) a crop of Brussels sprout *c.v.* Petrus was monitored for light leaf spot disease from the 13th August 2012 to February 2013. Throughout this period and, to inform of potential light leaf spot disease risk periods, results derived from weekly laboratory analysis of the field exposed MTIST air samples (as processed by the National Pollen and Aerobiology Research Unit (NPARU) were made available for use in crop protection decision management strategies. As a result of repeated mechanical failure, the volumetric air

sampler failed to operate fully over the monitoring period. Nevertheless for seven of the weekly assessment periods (August and September 2012) the collected field aerosols were processed by immunofluorescence for light leaf spot presence. The 'in field' tests, using the cyclone air sampler, provided 'quick' weekly visual assessment of the light leaf spot disease risk. These readings (both visual and optical density readings) were compared with results observed using the volumetric and the MTIST laboratory processed air samples.

Plant assessments were carried out for light leaf spot presence on twenty leaves of ten tagged Brussels spout plants on the 18th August and 10th December, 2012. Prior to crop harvest (January 2013), 16 Brussels sprout shanks *c.v.* Petrus were removed from the crop and the buttons assessed for light leaf spot (Fig. 9). At a location aside the commercial crop a Brussels sprout breeding trial (58 breeding lines) had been in progress for the same period. The breeding trial received the same crop protection spray programme as the commercial Brussels sprout *c.v.* Petrus crop. Having gained permission from the trials breeding company (Syngenta UK Limited, Capital Park, Fulbourn, Cambridge, CB21 5XE, United Kingdom) button assessments were made on a selection of five cultivars which exhibited susceptibility to light leaf spot infection. A final assessment was made on January 16th, 2013 prior to crop harvest.

Figure 9. Light leaf spot on a Brussels sprout button

White blister and Powdery Mildew. Two trial sites of Brussels sprouts were transplanted between the 10th and 15th May, 2013 at Dotams Lane, Butterwick (OS ref. TF 376 458) and Wigtoft, Kit Cat Lane. (OS ref. TF267337 Google maps). On the 17th July, 2013 savoy

cabbage transplants were prepared at Church Road, Frieston (OS ref. TF358436). Transplants of Green Cabbare are to be prepared at Fosdyke in August 2013 (awaiting date). At each of the four sites a commercial variety was grown alongside a genetically identical variety but incorporating intermediate resistance to *Mycosphaerella brassicicola* (Cabbage and Brussels sprout), *Albugo candida* and *Plasmodiophora brassicae* (Brussels sprout only). At each site, 2000 plants of each variety were positioned and used within a commercial fungicide interaction trial. With the exception of the Savoy cabbage, a further 1000 plants of each variety were planted as a separate block for HDC CP99 disease monitoring studies. Within this block (HDC CP99) and, for each variety, 500 plants remained untreated with no fungicide applications. Air sampling equipment (MTIST, volumetric and cyclone) was set up as described above in the untreated areas at Butterwick (Brussels sprouts) and at Fosdyke (Green cabbage). The Melinex tape of the volumetric air sampler, the four MTIST strips and the microfuge tube of the cyclone sampler were at each site changed on a weekly basis and processed at NPARU for presence of *A. candida* (white blister) and *E. cruciferarum* (powdery mildew). At each of the four trial sites twenty leaves of ten tagged plants were identified in each cultivar of the sprayed and non-fungicide treated area of HDC CP99 trial plots. A total of 40 plants at each site were assessed at two to three week intervals for disease presence of powdery mildew and white blister.

Air monitoring for spores of *E. cruciferarum* and *A. candida* will remain in progress until December 2013 (Brussels sprouts) and February 2014 (Green Cabbage). Two to three weekly plant assessments remain throughout the trial period.

Results

Diagnostic tests: Evaluation of light leaf spot, powdery mildew and albugo candida

Lateral Flow Sensitivity to Target Spore Type

Light leaf spot. A correlation of r^2=0.9661 was recorded for concentrations of *Pyrenopeziza brassicae spores* and the the Lateral flow batch test devices of 001LL. A detection limit of > 200 ascospores was recorded using the ESE reader (Figure 10). Similar results were previously reported in HDC FV33 final report where a visible test line formation was observed when ascosporic numbers were at or below 1000 ascospores ml $^{-1}$.

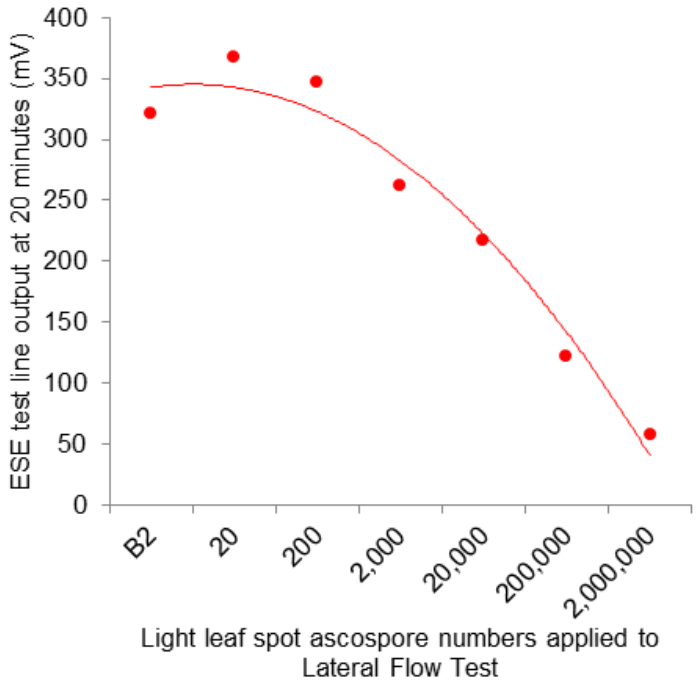

Figure 10. Light leaf spot ascospore (airborne 'spore' disease stage) concentration series and quantitative measurement by lateral flow device (in field test) using an ESE reader. B2 is the control and contains no spores.

Brassica Powdery Mildew. A correlation of r^2=0.9799 was recorded when dilutions of powdery mildew spores were tested using Lateral flow batch 001BPM (Fig. 11). A detection limit of 30 spores was observed and this confers with the results previously reported in HDC FV33 final report.

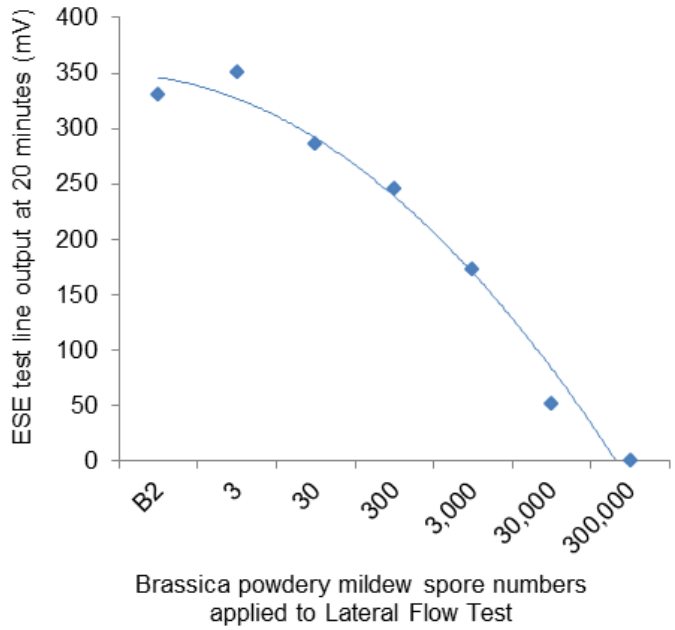

Figure 11. Brassica powdery mildew spore concentration series and quantitative measurement by lateral flow device (in field test) using an ESE reader. B2 is the control and contains no spores.

White blister.

A correlation of r^2= 0.9718 was recorded when dilutions of *A. candia* spores were tested using Lateral flow batch 001AC (Fig.12). A detection limit of 1000 spores (UK Race 9) was observed.

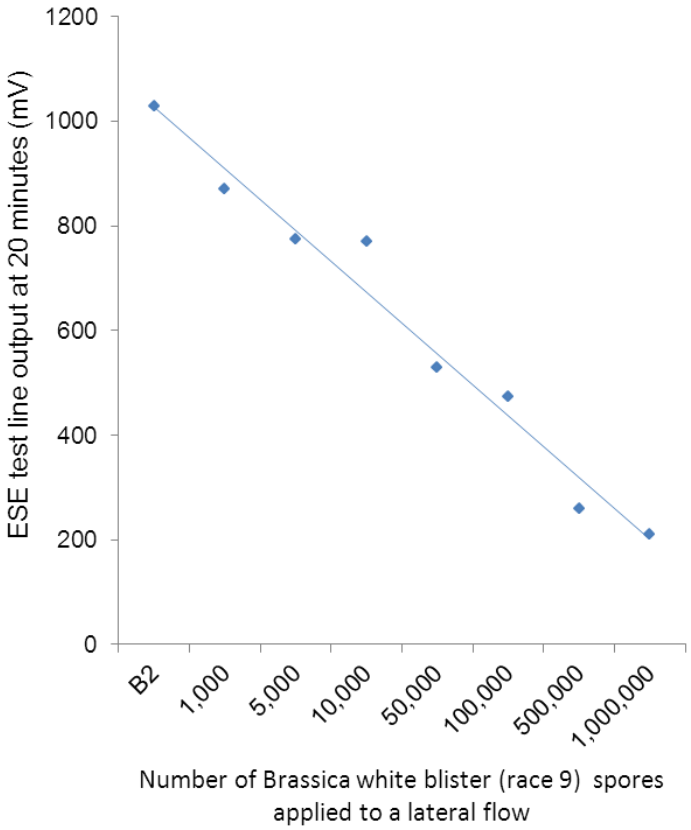

Figure 12. Brassica powdery mildew spore concentration series and quantitative measurement by lateral flow device (in field test) using an ESE reader. B2 is the control and contains no spores.

Assessment of the light leaf spot and powdery mildew lateral flow batches for shelf life stability

Test line: The test line antigen of both light leaf spot and Brassica powdery mildew remained stable and biologically active on the lateral flow membrane over an 18 month period.

Gold antibody conjugate pad: Conversely the same was not observed for either of the IgM antibody specific probes bound to the gold conjugate. For each test the gold conjugate was observed to lose activity within the first few weeks of application. However by incorporating Sucrose and Mannitol within the conjugate application buffer stability was significantly improved for the Brassica powdery mildew IgM monoclonal antibody which retained biological activity to 10 months (Fig 13.). Testing remains on-going. The addition of sugars to the buffer improved the light leaf spot lateral flow stability to three months thereafter

longevity was problematical. Tests and studies relating to the conjugate application buffer are ongoing and continue in to Year 2 of the project.

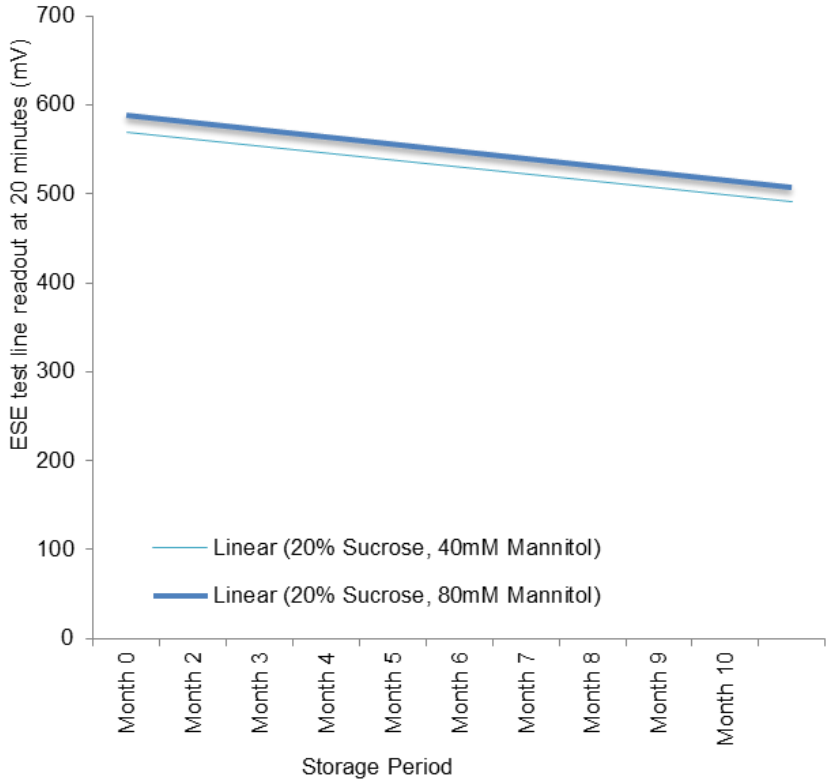

Figure 13. Brassica powdery mildew specific antibody gold conjugate lateral flow stability over a 10 month period.

Preliminary studies to investigate the potential of a multiplex lateral flow

Preliminary results indicate that the test line of each multiplex lateral flow was unaffected by the addition of a dual spore suspension (Brassica powdery mildew and Light leaf spot spores) over a spore concentration range (Fig. 14a, b). For each test, control lines were recorded using the E0 SE reader and visualized by eye.

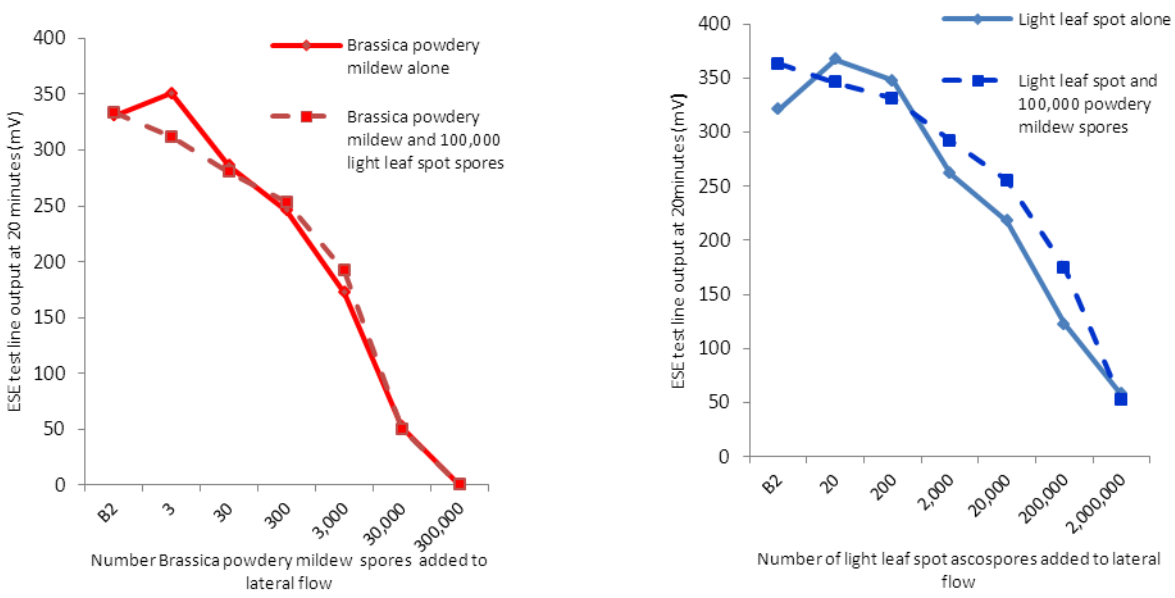

Figure 14. Effect of multiple spore types applied to a multiplex lateral flow and homologous test line development over a spore concentration range.

Field trials: Light leaf spot, powdery mildew and albugo candida

Light leaf spot

Location: Boarhills, Fife. OS ref. NO 582 137, Crop Brussels sprouts

Air Samplers: From the 13th August 2012, light leaf spot ascospores were identified in the crop. Ascospore concentrations of light leaf spot were visually identified by immunofluorescence, when weekly collected Melinex tapes of the Burkard volumetric spore were viewed by UV microscopy. Light leaf spot spore concentrations varied over the monitoring period. An excess of 10000 spores per cubic metre were recorded for the weeks beginning the 28th August and 25th September (Figure 15). From the 5th September 2013 numbers declined sharply and for the week commencing 12th September spore numbers were recorded at concentrations below 100 per cubic metre air sampled. No readings could be made between the 4th October to 27th November as the air sampling machine broke. Similarly, the MTIST spore trap recorded a light leaf spot inoculum peak for the weeks commencing 13th , 21st and 28th August and then with a significant decline in numbers by the week commencing 12th September (Figure 15). Additional spore peaks (PTA ELISA ≥ 0.1) were identified for weeks commencing 25th September, 17th October, 8th November and the 10th December 2012 and 'an at risk' warning issued (Fig 15). A relationship was observed between the 'in field' test (lateral flow) and the weekly MTIST laboratory analysis with related peaks and troughs of light leaf spot inoculum identified over the monitoring

period (Fig 16). At the start of the monitoring period the 'in field test' identified light leaf spot disease presence however for the following three weeks the tubes filled with rain water and no readings could be made. Thereafter the cyclone machine had periods where it was non-operable due to mechanical failure and / or the weekly tube was affected by rain water and the sampling efficiency affected. For these periods no readings by lateral flow were made and not shown in Fig 16.

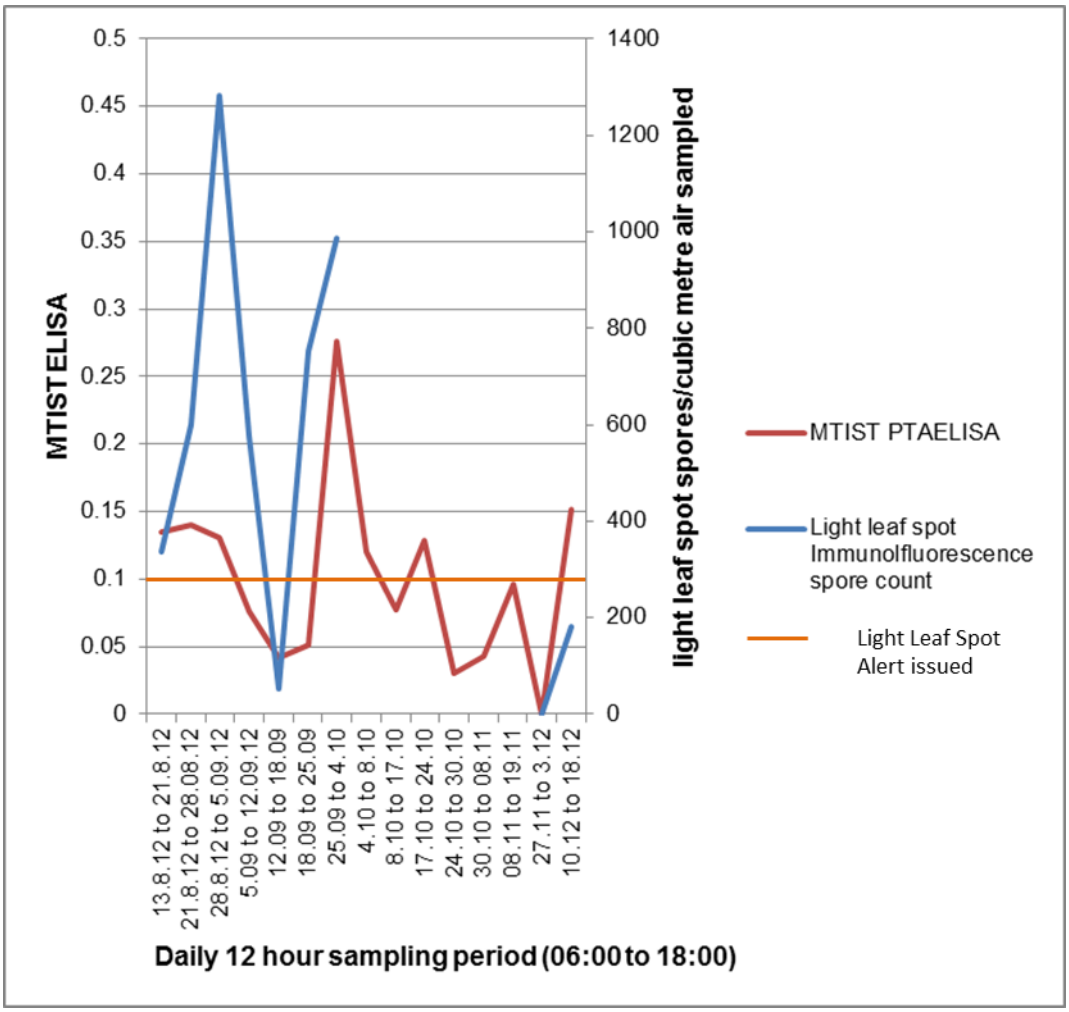

Figure 15. Monitoring light leaf spot airborne disease inoculum in a commercial cropping system over a five month period

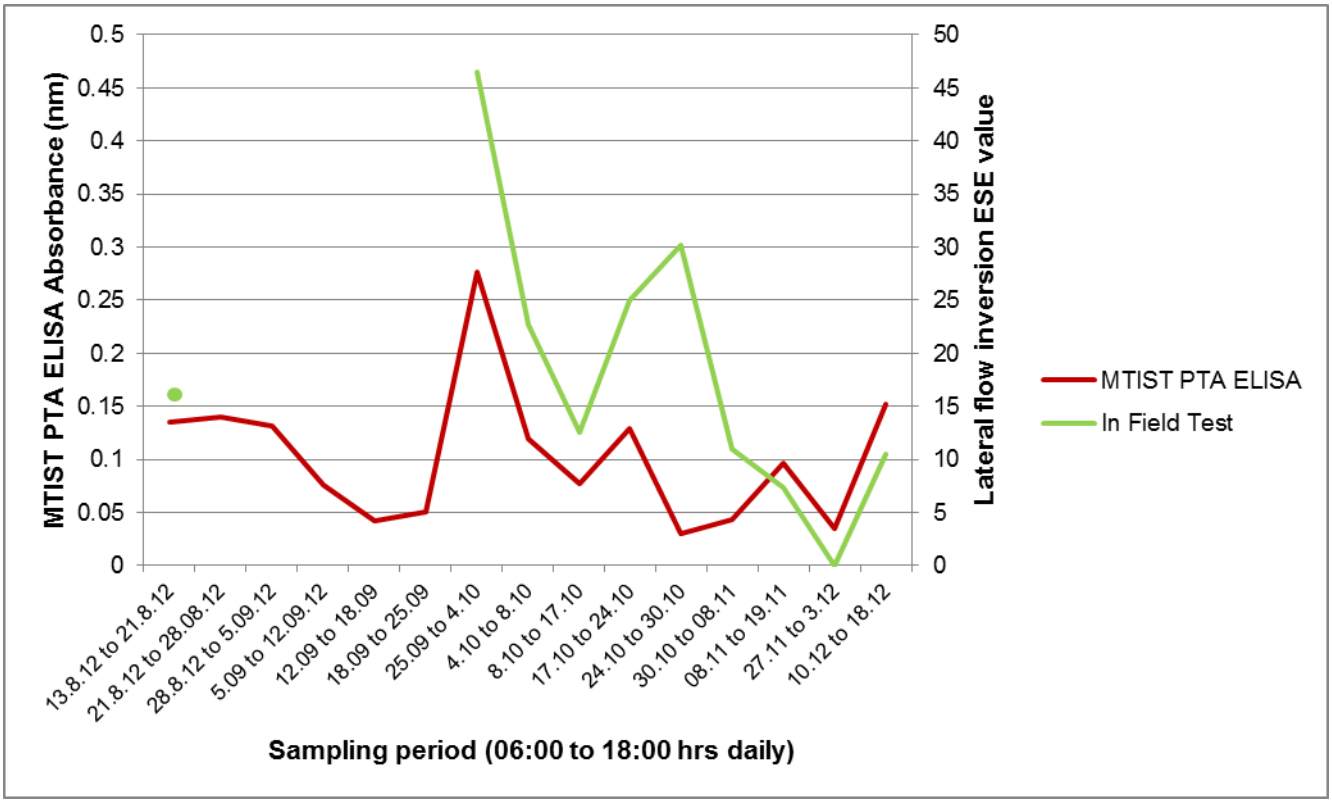

Fig. 16. Monitoring weekly light leaf spot airborne disease inoculum in a commercial cropping system over a five month period by MTIST PTA ELISA (laboratory test) and lateral flow (in field test)

Fungicide applications (full details of commercial name and active withheld due to confidentiality restriction) were made throughout the trial and actives for (light leaf spot) were applied as described in Table 1. At risk periods were based upon weekly MTIST ELISA results and issued when values reached in excess of 0.1 (Fig. 15) .

Table 1. Schedule and Application of Fungicides to assist control of light leaf spot

Planned *Pyrenopeziza brassicae* Fungicide Active Application	Applied	MTIST Warning for increasing light leaf spot disease inoculum
6/8	14/8	13/8 to 05/09
30/8	2/9	to 05/09
21/9	27/9	25/9 to 04/10
22/10	26/10	17/10 to 24/10
28/11	29/11	08/11 to 19.11
No application	Non Applied	10/12 to 18/12

Plant Assessments : At the commercial Brassica site in Scotland (Boarhills, Fife OS ref. NO 582 137) the crop of Brussels sprout *c.v.* Petrus was visually monitored for the development of light leaf spot disease on the 18th September, 2012. All twenty leaves of each of the ten plants assessed were clean and no disease was recorded. Minor slug damage was observed. An assessment on the 10th December 2012, reported leaf and sprout buttons free of light leaf spot disease and clean. Some slug damage was noted. Of the five experimental breeding lines selected for disease presence, light leaf spot damage was observed on most of the buttons assessed (Table 2).

Table 2. Assessment of Brussels sprout buttons for light leaf spot presence at Boarhills, Fife on the 10th December, 2012

Cultivar or assigned Breeding number	**Mean Number of light leaf spot lesions / button at <1cm**	**Mean number of light leaf spot lesions / button >1cm**
Petrus	0	0
30	0.3	0.7
31	0.3	1.3
32	0.5	1
34	0.5	1
36	0.3	1.7

By mid-January 2013, a low level of disease was observed on Brussels sprout buttons of c.v. Petrus (1% of 1065 buttons assessed exhibited light leaf spot infection). An experimental breeding line (32) recorded 11% of the 258 buttons to be heavily infected with the disease. Fungicide spray applications ceased on the 29th November 2011.

Light leaf spot and Powdery Mildew Trials

Location: Butterwick OS ref. TF 376 458, Crop Brussels sprouts

Weekly exposed Melinex tape (microscopic analysis), microfuge tube (cyclone sampler) and 4 x microtitre wells (MTIST sampler) have been received by post on a weekly basis since the 7th June 2013. To date few disease propagules of either pathogen have been observed (Fig.17) and no disease has been observed on the plants assessed. The trial remains ongoing to December 2013.

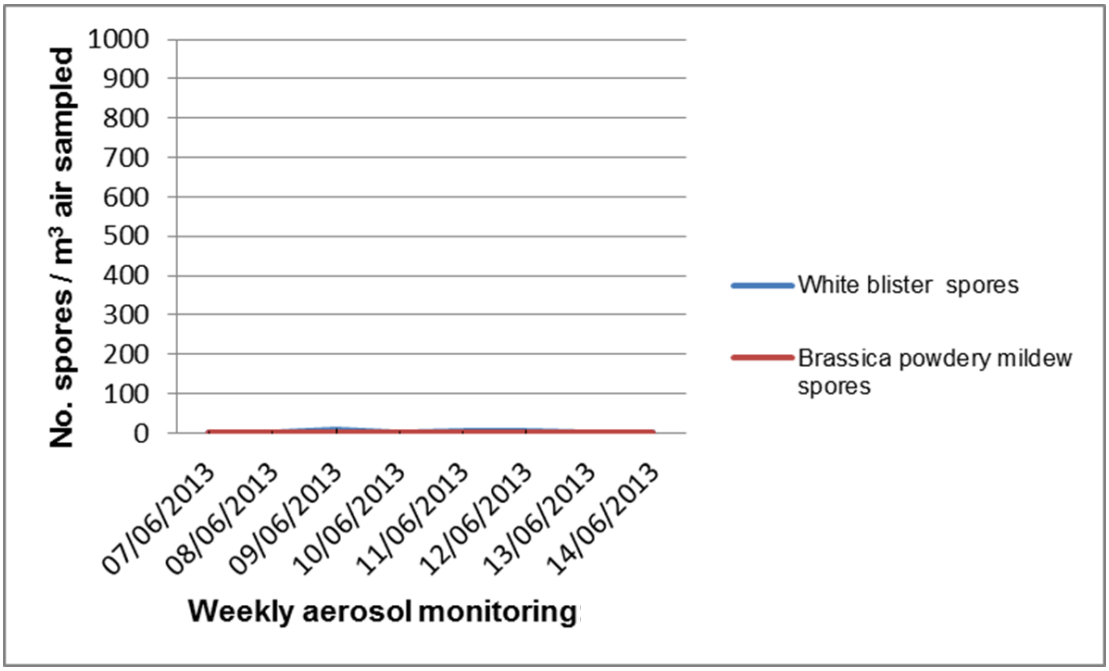

Fig. 17 Monitoring weekly collected field aerosols at Butterwick for White blister (*Albugo candida*) and Brassica Powdery Mildew (*Erysiphe cruciferarum*) spores.

Kit Cat Lane, Wigtoft. TF267337, Crop Brussels sprouts

No air samplers are sited at this location. Plant assessments to date record no disease of either pathogen present on leaf material. The trial remains ongoing to December 2013.

TBC, Fosdyke. *To be supplied* , **Crop Green cabbage**

Weekly exposed Melinex tape (microscopic analysis), microfuge tube (cyclone sampler) and 4 x microtitre wells (MTIST sampler) are to be received by post on a weekly basis from August , 2013. The trial remains ongoing to February 2014.

Church Road, Frieston. TF358437, Crop Savoy cabbage

No air samplers are sited at this location. Transplanting took place on the 17th July and assessments to date record no disease of either pathogen present on leaf material. The trial remains ongoing to February 2014.

Discussion

Diagnostic Tests

Light leaf spot and Powdery Mildew. Batches of lateral flows for field diagnosis of light leaf spot ascospores and Brassica powdery mildew in collected aerosols have been prepared with a diagnostic range suitable for use in field studies. Using a field portable electronic reader, light leaf spot ascospores of between 200 and two million can be estimated. For Brassica white blister, spore concentrations of between 30 and three hundred thousand can be differentiated using an ESE reader. For field trial usage the white blister lateral flow indicates a shelf life of upwards of one year when stored at room temperature. The light leaf spot recorded good test line stability over an 18 month period but the gold antibody conjugate is stable for a three month period only. Studies in Year 2 of the project would look to improve this.

Preliminary investigations in to the development of a multiplex test (several tests on one lateral flow test) have proved promising. At high contaminating spore concentrations, test line sensitivity or specificity for either target pathogen remained unaffected when two spore types were used in one test sample.

White Blister. The present investigation has assessed the use of monoclonal antibodies, developed previously in an Australian study, to identify a suitable probe that could be used in a lateral flow to quantify airborne spores of *Albugo candida* within UK *Brassica oleracea* cropping systems. A monoclonal cell line (UW 256) showed a level of specificity which was able to discriminate between *A. candida* (race 4, white blister on Shepherds purse) and *A. candida* (race 9, white blister on *B. oleracea*). The lateral flow ('in field' test) developed for use in Australian commercial cropping systems and, which also used UW 256 as the specific probe , provided a detection sensitivity of 100 white blister race 4 spores. Using *A. candida* isolates from UK *B. oleracea* plants the lateral flow prototype has a detection sensitivity of 1000 white blister spores. Depending on the disease threshold (under optimal environmental conditions the number of spores in the air required to initiate uniform disease expression on susceptible plants) the sensitivity of the white blister lateral flow may require alteration for field usage.

Field trials

Light leaf spot. Over a six month period (August – January) a commercial Brussels sprout crop *c.v.* Petrus was monitored for light leaf spot disease incidence. A fungicide spray programme was operated throughout the crop and disease assessments at the site reported no disease present to the 10th December, 2012. The final fungicide spray was applied on the 29th November 2012. A crop assessment was then made on the 16th January 2013 and a low level of disease incidence was reported (1% buttons). An experimental breeding line grown aside the Petrus crop recorded at this time 11% of buttons to be heavily infected. Air samples taken in the crop by the three air samplers all report for the period 10th through to the 18th December a rise in light leaf ascospores. The Burkard volumetric air sampler, processed in the laboratory and *Pyrenopeziza brassicae* ascospores identified by immunofluorescence, estimated for this period 200 spores present per cubic metre of field air sampled. The latent period (the time period from infection to visible disease symptoms) will vary depending on the environmental conditions. Published works (Gilles *et al.,*2000) report on average between 10 days (temperature at 16ºC) and 26 days (temperature at 4ºC) for visible symptom development. A thirty day period was available from the light leaf spot risk period identified by the three air sampling systems and the final plant assessment. The crop for this period was unprotected as no further fungicide applications were made. No data was available on light leaf spore numbers after the 18th December as the air samplers remained for several weeks unchanged. An alert of disease risk was made on the 18th December as the MTIST PTA ELISA (3 hour laboratory test) had identified the start / peak of an epidemic. This rise in light leaf spot spore activity was observed using the 'in field' lateral flow test and followed a similar pattern to the ELISA throughout the field trials. Initial data from the 13th to the 25th September for the Lateral flow 'in field test' is not available' either due to mechanical failure or the tubes filling with water. For weekly sampling this is a problem that would need the manufacturer of the air sampling system to address as sampling efficiency will be significantly compromised when the tube is near / full of water. Although the tube is coated with a germination inhibitor the addition of rain water would prove optimal for spore growth and potentially affect the quantitative measurement of the assay system.

Trials to monitor airborne disease transmission events for Brassica powdery mildew and White blister remain in progress and will continue throughout 2012 to December (Brussels sprout trials) and January 2013 (Cabbage crop). All three air sampling devices are monitored weekly either by a consultant or University of Worcester staff member and to date little disease has been observed using by air samples. The crops remain visually clean

at present. Once disease has established within the crop the fungicide resistance interaction trials should prove useful in determining the disease threshold required to set the MTIST ELISA and in field lateral flow devices to identify disease risk periods.

Conclusions

Control of plant pathogens could be improved if inoculum could be detected quickly in the field directly by the grower. Airborne inoculum plays a vital role in the development of epidemics caused by Botrytis leaf blight on onion crops (Carisse *et al.,* 2005). In this work, a linear relationship was found between number of lesions on plants and the air-borne Botrytis conidial concentration. Airborne conidial concentrations of 25 to 35 conidia m^{-3} of air were associated with 2.5 lesions per leaf. When detection of Botrytis inoculum was used as a control criterion under field conditions it led to a reduction in fungicide usage of 75 and 56% in 2002 and 2003. A similar relationship between spore number and disease intensity has been reported for *Cercospora apii* on celery. In both these studies, microscopes were used to determine spore numbers from air samples. In vegetable Brassica crops, detecting pathogenic spores before they can infect crops has also been shown to be a useful approach in controlling airborne diseases (Kennedy *et.al*. 2006, Wakeham & Kennedy, 2010, Wakeham & Kennedy, 2010). This study details the development of air sampling systems (laboratory) and 'in field' tests for monitoring disease transmission events in commercial crops of Light leaf spot , Brassica powdery mildew and White blister. If the technique is to be of value practically *i.e.* targeted and effective usage of crop protectants, the method of detecting spores in the field should be combined with information derived from forecasts based on environmental risk of infection (BrassicaTM Spot). The current study assesses the use of weekly estimates of target disease in air samples as this has been reported for other diseases of field crops (Wakeham and Kennedy, 2010). Where daily environmental risk forecasts are available it may be of use to consider daily air sampling regimes. This process is being used to predict ringspot disease risk alerts at sites in Lincolnshire (http://www.syngenta-crop.co.uk/brassica-alert) and Lancashire. The field air samplers are based on the single cyclone sampler but using an automated system can capture daily air samples in to different tubes over a week period (www.burkard.com). In developing a daily or weekly system it is necessary to determine the inoculum concentration required for infection in conjunction with the effect of environmental parameters on this process. The current study assesses the use of weekly sampling but daily air sampling microscopic counts have been recorded using the volumetric air sampling device.

The development and use of 'in field' diagnostics described in this report has the advantage of detecting the very earliest possibility of disease occurrence. The grower or consultant is able to estimate real disease risks which he can control using fungicides. The system could enhance the activity of protectant fungicides or even biological control agents. By applying fungicides quickly the grower will be in a situation where he can use protectants to control

disease inoculum before it is established within the crop. Use of protectant fungicides could help reduce residues in the crop at harvest. The requirement for zero or low fungicide residues is of increasing importance in crop production. In addition, using crop protectant measures in a timely, efficient and effective way should assist in the prevention of the breakdown of plant resistant varieties and the potential for increase of virulent pathotypes. A reduction of chemical usage with an informed targeted approach towards disease control will prove popular not only with the producer user on a cost / benefit case but also with with the supermarkets and the end user i.e the consumer. A win win situation.

Control of light leaf spot on Brussels sprout crops also varies with cultivar. In Scotland very susceptible cultivars such as cv. Millennium could not be used successfully in control regimes. This has major implications in the control of light leaf spot in Brussels sprouts where a range of cultivars are grown with differing maturity dates during the season. Often cultivars are grown side by side in the same locality or field. Use of cultivars with high susceptibility to light leaf spot increases the risk of light leaf spot epidemics as the season progresses even when effective chemicals are used to control infection and these are applied when light leaf spot inoculum is present. The use of partially resistant cultivars such as *cv*. Petrus has been successful in producing disease free crops late in the growing season until final harvest in the following year. Consideration should be given to separating cultivars into different areas to reduce short range disease transmission events. This risk was demonstrated in 2012 /2013 at the field trial in Fife, Scotland where infected plants (fungicide interaction trial of susceptible and resistant crop varieties) were in close proximity to the commercial crop and, provided an inoculum source for disease transmission to occur in December 2012. Results from this period indicate that light leaf spot airborne disease was at 200 spores per cubic metre of air sampled. After which low level infection was then observed in mid-January on the partially resistant cultivar (*c.v.* Petrus) and at an increased level on less resistant cultivars. The threshold for low level disease risk will be set at 200 spores per cubic metre for the 2013 - 2014 trials. It has been reported that under optimal conditions the disease threshold for significant ringspot disease was at 2000 spores per cubic metre (Wakeham & Kennedy, 2010).

The present study also aims to determine the relationship of airborne powdery mildew and white blister disease transmission events and subsequent crop disease development. ‘In field’ diagnostic tests have been developed to each of these two pathogens and are currently being trialed at commercial sites in the UK. The tests have been set to identify spore concentrations that are likely to cause disease risk and measurement quantified using a digital reader. To date however there is little information on the requirements for powdery

mildew development in vegetable brassica crops. The pathogen can infect and develop over a wide range of environmental conditions found in the field within crops. The epidemiology of the disease in the crop is poorly understood. It is possible that serious epidemics result only from the influx of substantial amounts of inoculum into the crop. Powdery mildew on vegetable Brassicas appears to be favoured by dry conditions and these usually only occur in vegetable Brassica crops during early summer. It's likely that development of disease in the crop above threshold levels during June, July and August is key in the degree of damage that this pathogen causes. The current field trials will look to examine this and will be fully reported in Year 2 of the project. The occurrence of older tissues where powdery mildew development is more favoured, during autumn and winter, may act as a bridge for the pathogen to occur on Brussels sprouts buttons. However it is also possible that powdery mildew penetration into axillary buds may also lead to button infection. This is more likely if large amounts of conidia are present within crops.

For each of these diseases work will be required on how to use these air sampling devices to schedule fungicide usage. For example will sprays be applied to a threshold detection level or will they be applied when each diseases is first detected using the device. For light leaf spot sprays of Tebuconazole could be applied in response to peaks in airborne numbers of light leaf spot. Tebuconazole is one of the few active ingredients available to vegetable Brassica growers which have activity against light leaf spot. For this reason Tebuconazole (Nativo) is widely used by vegetable Brassica growers to combat the potential for light leaf spot development within their crop. However, tests on isolates, taken from vegetable Brassica crops has shown the prevalence of isolates that could grow in the presence of 10ppm Tebuconazole. Fungicides with the active ingredients of Boscalid and Pyraclostrobin (Signum) have relatively recently been given approval for use on vegetable Brassica crops. Additionally the fungicide Rudis (which contains triazolinthione) has also given good control of light leaf spot. Signum gave good control of light leaf spot infection in Brussels sprout crops provided that it is applied at the time when light leaf spot ascospores were present in the air. The development of diagnostic devices that can identify inoculum in the air at a concentration that is able to initiate disease will prove critical if fungicides are to be targeted for minimum input with maximum effect and to prevent disease resistance for long term and widespread usage. Trials at Fife in the summer and autumn of 2013 will have available for use an 'in field' lateral flow test which will be set to a disease threshold of 200 spores per cubic metre. This should provide the opportunity to effectively target fungicides appropriately and at times when light leaf spot spores are in the air at concentrations that could lead to symptom development on commercial Brussels sprout cropping systems. The results may influence the choice of fungicide active used at key time points. Trials currently

running in Lincolnshire will look to identify spore populations of white blister and Brassica powdery mildew to determine disease threshold requirements in a commercial setting and using different *B. oleracea* varieties. Lateral flow tests for each of these two pathogens are currently being assessed and will be made available for trial to the agronomist overseeing the sites as the current trial progresses in to Year 2 of the project.

Knowledge and Technology Transfer

Roy Kennedy and Alison Wakeham (2013). Use of diagnostics in disease control in Horticulture BPOA technical seminar. 6th February, The Belfry, Oxfordshire

Roy Kennedy (2012). Detection, forecasting and control of vegetable diseases. Presentation: Vegetable, Salad & Herb Growers Technical Update meeting, 7th Feb-2012, Pershore College. UK.

Roy Kennedy Vegetable (2012) , Salad & Herb Growers Technical Update meeting, 7th Feb 2012, Pershore College. UK.

Roy Kennedy (2012). Detection and control of clubroot and other diseases. Presentation and Discussion group, Grower mini conference, Lancashire 14th February 2012

Alison Wakeham (2012). Detection and Control of airborne/ soilborne disease of brassicas. Presentation: Getting to the Heart of Horticulture 13th January 2012 NFU and Wychavon Council

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