Project title:	Onions: Further development and calibration of detection tests for conidia of onion downy mildew in combination with MORPH forecast model MILONCAST.
Project number:	FV 356
Project leader:	Dr Roy Kennedy
Year of project:	Annual
Date of report:	10 October 2011
Locations:	University of Worcester
Coordinator:	Andy Richardson
Project commenced:	01 October 2010
Completion:	31 August 2012
Key words:	Onions: Further development and calibration of detection tests for conidia of onion downy mildew in combination with MORPH forecast model MILONCAST.

DISCLAIMER

AHDB, operating through its HDC division seeks to ensure that the information contained within this document is accurate at the time of printing. No warranty is given in respect thereof and, to the maximum extent permitted by law the Agriculture and Horticulture Development Board accepts no liability for loss, damage or injury howsoever caused (including that caused by negligence) or suffered directly or indirectly in relation to information and opinions contained in or omitted from this document.

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.

Professor Roy Kennedy
Director and Head of Department

National Pollen and Aerobiology Research Unit
University of Worcester

Signature ..... Date .....

[Name]
[Position]
[Organisation]

Signature ..... Date .....

Report authorised by:

[Name]
[Position]
[Organisation]

Signature ..... Date .....

[Name]
[Position]
[Organisation]

Signature ..... Date .....

CONTENTS

GROWER SUMMARY

Headline

A lateral flow device (LFD) has been developed which can detect conidia of onion downy mildew in the air before symptom appearance in the crop. The use of the device has been optimised in the field in an onion crop.

Background and expected deliverables

Foliar diseases of onion crops (onion downy mildew and *Botrytis* leaf blight) can cause heavy yield losses in bulb and salad onion crops. In salad onions, yield losses can be as high as 100% with whole crops being discarded as downy mildew symptoms make them unmarketable. Actual yield losses in bulb onions of 60 to 75% have been recorded. The project will help reduce losses in onion crops resulting from this disease.

A reduction in the number of fungicide applications, while maintaining disease control, can be achieved by applying fungicides only at times when conditions are favourable for disease development. The production of large numbers of conidia of onion downy mildew are thought to be related to the times when disease spreads.

The expected deliverables from this project are:

- Better detection of downy mildew in the field before disease is visible in the crop.
- Detection tests which can be used “in field” to determine the level of risk to the onion crop posed by onion downy mildew.
- Less reliance on eradicant fungicide applications for downy mildew control.
- Lateral Flow detection tests which recognise conidia of onion downy mildew conidia.

Summary of the project and main conclusions

Using Lateral Flow Devices for detection of downy mildew conidia

This study indicates that the LFD developed in this project has potential to detect low concentrations of airborne disease inoculum that may be causing the initial disease epidemic. The field test demonstrated that the LFD device gave a positive output up to 7-9 days before symptoms were observed in the crop (Figure 1). Onion downy mildew was identified on onion plants within the crop on the 2nd June and the spores were identified in the air on the 23rd May by the lateral flow device. The use of air samplers provided information on the time of the day when onion downy mildew inoculum is present in the air. Future field trials will look to sample the airborne environment only during these periods.

Investigations so far have confirmed stability of the biological components within the developed downy mildew LFD which will ensure that the device can be stored. The prototype identified for field trials has been shown to operate over the required sensitivity of detection for airborne concentration of onion downy mildew which can initiate disease symptoms.

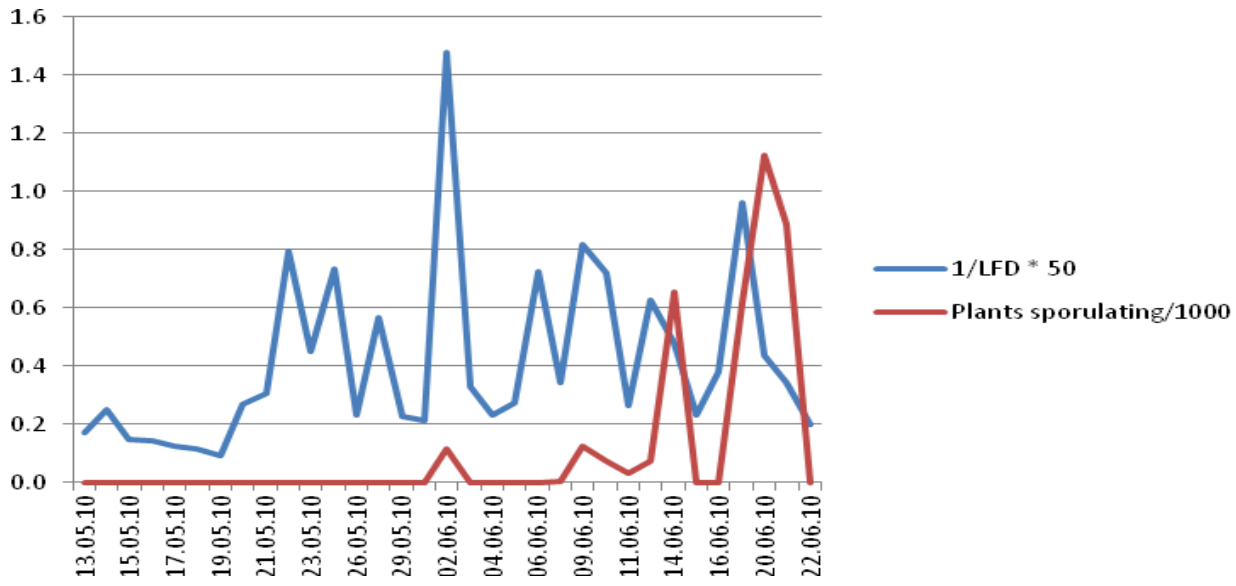

Figure 1: Monitoring onion downy mildew sporulation events in an onion field crop and available airborne disease inoculum

Financial benefit

The main financial benefits will be in the use of the device to reduce unnecessary fungicidal applications to the crop. Fungicide usage is costly and is one of the major inputs in crop production. Using the lateral flow device the grower/consultant will be able to check for the presence of onion downy mildew in the air and time the first fungicide application more. Lateral flow tests are expected to cost approximately £ 4 – 5 per test. This cost must be compared with £ 40 per hectare for fungicide treatment. In high risk years it is common to spend in excess of £300 per hectare on fungicides in a bulb onion crop. However saving will be variable between years and depend on the reductions in sprays achieved.

Anticipated practical and financial benefit

Expected financial deliverables are therefore:

- The usage of the “in field “ test for onion downy mildew will improve the timing of the first application of fungicide for controlling this pathogen in onion crops.
- There will be less reliance on metalaxyl based fungicides for onion downy mildew control (these are being withdrawn).

By using the “in field test” for onion downy mildew in conjunction with models predicting onion downy mildew infection and sporulation growers will be better able to schedule fungicide applications to crops more effectively to produce cost savings.

Action points for growers

Until the test has been developed there are currently no action points but growers are advised to monitor the project progress and keep an eye out for any tests that arise.

SCIENCE SECTION

Introduction

Downy mildew occurrence in onion crops

Onion downy mildew (*Peronospora destructor*) is geographically widespread and serious disease in bulb and salad onions and in onion seed production. Actual yield losses in bulb onions of 60 to 75% have been recorded (Cook, 1932, Cruickshank, 1958). These losses mainly result from severe infections in bulb onion crops causing early defoliation, reduced bulb sizes and poor storage quality of bulbs (Rondomanski, 1967). In salad onions, yield losses can be as high as 100% with whole crops being discarded as downy mildew symptoms on the plant make them unmarketable. Losses to seed production are frequently caused by the collapse of infected seed stalks and poor germination of seeds collected from infected stalks (Virányi, 1981). Fungicidal control of downy mildew is difficult and fungicides are only effective, if they are applied before or immediately after disease first appears in the crop (Kennedy, 1998). Fungicidal control is the only effective means of controlling the onion downy mildew and avoiding crop loss.

Biology of *Peronospora destructor* on onion crops

Large numbers of spores are produced from downy mildew lesions and this is a characteristic of downy mildew pathogens. Sporulation of *P*. *destructor* is a diurnal process and both periods of light and darkness are required. Sporulation is mainly during the night under high relative humidity of greater than 94 - 95% at temperatures of 6 - 22^{o}C provided there is no rainfall. (Yarwood, 1937, 1943). High day temperatures exceeding 24-25°C or exceeding 27, 28, 29 or 30°C for more than 8, 6, 4 or 2 h, respectively, were found to inhibit sporulation during subsequent nights (Hildebrand & Sutton, 1982). Studies by Hildebrand and Sutton suggested that a combination of night temperature, time of onset of high humidity and duration of high humidity affected the quantity of sporangia produced. Sporangial discharge is triggered when relative humidity falls below 59%. Conidia are thin walled and wind transported over considerable distances. Conidia have been detected at heights of 1500 ft. When conidia are deposited on leaf surfaces, they germinate between 1.5 and 7 h. Progress and spread of downy mildew is dependent on the survival of spore populations until conditions become favourable for germination (Sutton & Hildebrand, 1985). However viability is affected by the prevailing temperature and humidity conditions. At 10^{o}C, spore viability is unaffected by relative humidity. However, at temperatures of 30^{o}C, viability declines

rapidly at relative humidity above 55 %. The pathogen can over winter as mycelium in onion bulbs and sets and as oospores in debris from diseased foliage. The onion downy mildew has also been shown to be seed borne and when either sets, or seeds are transplanted the mycelium grows within the foliage of the plant. Downy mildew infects all the main onion types grown in the U.K. including common onion (*Allium cepa*) shallots (*A*. *cepa* var. *ascalonicum*) and Welsh onion (*A*. *fistulosum*). Welsh onion is particularly susceptible to downy mildew infection.

Methods for control of onion downy mildew

The control of downy mildew in onions relies mainly on the prophylactic application of fungicides, as frequently as every 10 days. However, to reduce the impact of fungicides on the environment, integrated pest management (IPM) systems have been developed. A reduction in the number of fungicide applications, while maintaining disease control, could be achieved by applying fungicides only at times when conditions are favourable for disease development. The times when large numbers of spores of onion downy mildew are produced are thought to be related to the times when disease spreads. Such direct relationship between airborne spore numbers and disease spread has been proposed for airborne fungal pathogens (Campbell and Madden, 1990), and have been found for *Mycosphaerella brassicicola* on Brussels sprouts (Kennedy *et al*., 2000).

The amount of spores produced during the night may vary with environmental conditions. Information on the presence or absence of critical spore threshold numbers could help growers to identify periods when disease is likely to spread. However, besides information on sporulation also further information on when conditions are favourable for infection and the latent period are required before growers can decide on the best times to apply control measures.

Methods for determining the risk of air-borne diseases in onion crops

Management systems are available for onion diseases (BOTCAST/DOWNCAST/ONION$_{SPOT}$) (Gilles, *et al*., 2004) which can predict the early development of both downy mildew and *Botrytis* in onion crops. This system incorporates the DOWNCAST model (Jesperson & Sutton, 1987), which predicts sporulation and infection events of *P*. *destructor*. DOWNCAST predicts sporulation, but cannot predict the quantity of sporangia produced. Tests of the model in Canada suggested it gave positive predictions of sporulation for 38 out of 45 nights when sporulation was observed. However, in field tests in the Netherlands, DOWNCAST gave positive predictions of sporulation for only 11 out of 24 nights when sporulation was observed (de Visser, 1998). Thus, the model often failed to predict sporulation events in a north-west European maritime climate in which the weather conditions are highly variable. Battilani and colleagues developed ONIMIL, a forecaster, which is

also based on DOWNCAST, and which gives a quantitative prediction for sporulation (Battilani, 1996).

A new model, named MILIONCAST (an acronym for 'MILdew on onION foreCAST'), was developed based on the data from controlled-environment studies investigating the effect of temperature and humidity on downy mildew sporulation. The rate of sporulation was predicted using controlled environmental data. The accuracy of MILIONCAST was compared to the accuracy of existing models based on DOWNCAST. MILIONCAST gave more correct predictions of sporulation than the DOWNCAST models and a random model. All models based on DOWNCAST were more accurate than the random model when compared on the basis of all predictions (including positive and negative predictions), but gave less correct predictions of sporulation than the random model (Gilles *et al*., 2004). Use of this system, which provides information on the timing of the first fungicide application, may result in better disease management. However inoculum can be imported into disease free crops from other localities/areas but assessments based on environmental risk alone do not take this factor into account. In order to avoid these problems new and rapid methods of detecting and quantifying pathogenic inoculum are required which can be used in conjunction with forecasting models. With this more precision approach there will be reductions in the amounts of fungicide required to control disease. Unnecessary fungicide applications, which are based on weather information alone, will be reduced especially during periods of high risk.

Using air-borne spore numbers within disease forecasting systems

It has been demonstrated that airborne inoculum plays a vital role in the development of epidemics caused by *Botrytis* leaf blight on onion crops (Carisse, 2005). Detection and quantification of airborne spore numbers can be used to predict disease accurately before it is visible in the crop. Peaks of airborne spores are always detected prior to crops becoming infected. This, results from the requirement for a threshold of inoculum to initiate disease establishment in crops and this must coincide with favourable weather conditions. The importance of airborne inoculum has been recognised in the development of many diseases. Its use in practice has been limited because of the difficulties in quantifying it.

Detecting airborne spores of fungal plant pathogens is a useful tool in crop protection if this could be done rapidly and accurately. For example it has been reported that one or two peaks in sporangial concentration in the air of the potato blight pathogen *Phytophthora infestans*

preceded the first observed symptoms of the disease in the field (Bugiani *et al*., 1998). These observations were validated in studies conducted by Philion (2003). In these studies the numbers of sprays applied to control potato blight could be successfully reduced without any impact on crop quality by monitoring the onset of thresholds of potato blight inoculum. Fungicide applications were initiated when the daytime airborne sporangial concentration reached 30 sporangia/m^3 (disease was not yet visible when this threshold was reached). By using these criteria, in combination with disease forecasts based on weather information the number of fungicide applications could be reduced with no impact on disease development. Similar results were obtained for *Botrytis* blight (*Botrytis squamosa*) on onion crops where thresholds of 15 - 20 conidia/m^3 could be used to reduce fungicide application by up to 100% (Carisse *et al*., 2003). Thresholds of inoculum required for disease establishment have also been reported for *M*. *brassicicola* which is the fungal pathogen responsible for ringspot on vegetable brassicas (Kennedy *et al*., 2000). In these studies (with the exception of ringspot) the information on spore number had to be collected manually using a microscope which was slow and time consuming. Tests which, can be conducted in the field are necessary if information on air-borne inoculum concentration is to be of more practical value. The use of air-borne spore numbers, as criteria, within forecasting systems is a new and exciting development in disease forecasting. One “in field test” which could be used in this respect is the lateral flow test.

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 (see Figure.2). However they are now commonly and widely used for detection purposes. They rely upon the specific reaction of sensitised coloured particulates. Antibodies (polyclonal or monoclonal) raised to a specific target spore, are bound by passive or covalent means to these coloured particles. These sensitised particles (latex or immunogold particulates are generally used) are then applied using an immersion procedure on to a release pad, to produce a stable particle reservoir for release on to a nitro-cellulose-based membrane. In a standard lateral flow test two lines of reagents are immobilised on to the membrane using a sophisticated reagent dispenser. The constituents of these lines will vary from test to test but commonly only two types of formats are adopted.

***The Competitive assay format*.** In a competitive assay format the test line comprises of homologous antigen (downy mildew spore components) and the other, the control, is a line of anti-species antibodies. The release pad and membrane are assembled together with an

absorbent pad into a plastic housing as illustrated below (Figure 2). The fluid sample is added to the well, releasing the specific antibody bound coloured particles, which then begin to flow across the membrane. If the target antigen (onion downy mildew spore component) is 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 (onion downy mildew spores), would induce complete inhibition of antibody attachment to the test line, a result that is indicated by a
single line of coloured particle deposition (the control line). Two lines of equal colour intensity indicate a negative result.

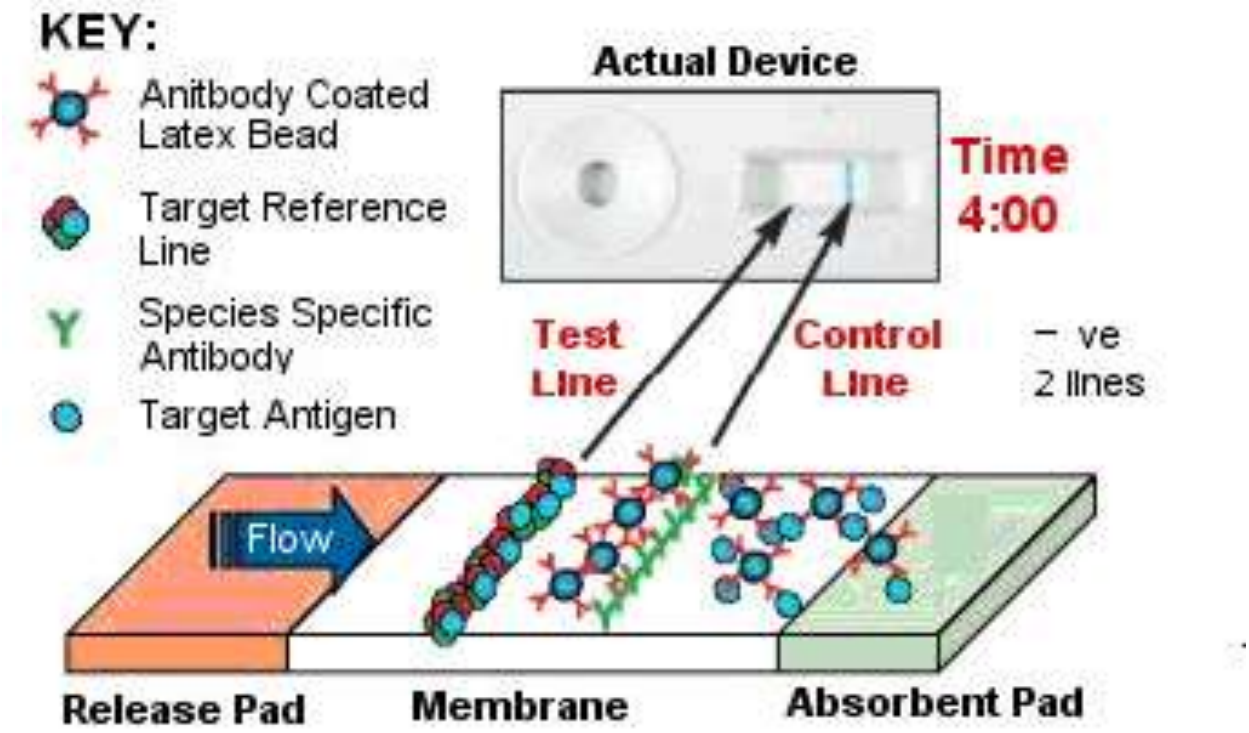

Figure 2. The Competitive lateral flow assay format.

The Non-competitive assay format. In a non-competitive assay format the test line generally comprises of an antibody complex which will bind, if present, target antigen in the test sample. The control line will generally consist of an anti-species antibody, as in the competitive format, and bind material within the test flow to indicate successful test execution. The release pad and

membrane are assembled as described above. The fluid test sample is added to the well, releasing the specific antibody bound coloured particles, which then begin to flow across the membrane. If the target antigen is present in the sample extract (onion downy mildew), antibody binding will occur to produce a coloured particulate conjugated antibody -antigen complex. As this target complex passes over the test line capture of the antigen can occur, immobilising the antibody coated coloured particulates to produce a visible line of deposited coloured particulates at the test line. Excess coloured particulate material is captured at the control line, providing a visible confirmation of the success of the test. Two lines of equal intensity indicate a positive result.

Both assay formats can produce a semi-quantifiable test. Use of reader technology allows the line intensity to be recorded, and therefore the level of particulate accumulation to be calculated using reflectance photometry. A number of readers are now available for use under field conditions. By introducing an internal control of coloured particles to the assay, a standard control line can be produced for use as a reference against the test line intensity. Variations in line intensity can be distinguished using a reader, making the test semi-quantifiable.

Developing 'in field' tests for detecting the presence or absence of onion downy mildew inoculum

If accurate 'in field' tests for inoculum are to be constructed they will require specific antibodies that can differentiate between different types of pathogenic spores recognising only onion downy mildew spores. Successful lateral flow test formats have been used to determine their accuracy. Cross-reaction of the test with spores of other pathogenic and non pathogenic species need to be ascertained. These tests would include other pathogens which are common in onion crops notably *Botrytis squamosa* and *Botrytis cinerea*. Other pathogens found in onion crops include *Cladiosporium allii cepae* Many of these pathogens survive on debris in the soil or are found on leaves on onion crops. Consequently tests which detect onion downy mildew conidia should not react with the conidia of other pathogens common in onion crops. They should also remain non reactive to other biological and non biological particles. The level of reactivity of the antibody to onion downy mildew conidia is also important as this will affect the sensitivity of the test and how it can be used to quantify the number of onion downy mildew conidia present in samples.

MILESTONE 1 : FIELD INVESTIGATIONS TO ASSESS A LATERAL FLOW DEVICE FOR THE EARLY DETECTION OF AIRBORNE DOWNY MILDEW DISEASE POTENTIAL IN AN ONION CROP.

Introduction

Studies to assess spore trapping systems for the early detection of airborne inoculum of *Peronospora destructor* and an environmental based downy mildew disease forecast model (Millioncast) were carried out in the Rushpits field trial of an overwintering onion crop at Warwick HRI in 2010. The field crop was monitored for downy mildew development and no control measures were applied during the study.

A monoclonal antibody (MAb), selected for recognition characteristics associated with the conidial stage of *P. destructor*, was evaluated for the detection of trapped airborne inoculum of onion downy mildew in a field plot. Three trapping formats were compared in this trial: An MTIST sampler (microtiter immunospore trap) and an eight day cyclone sampler were operated for 12 H periods from 06:00 to 18:00 H daily. A Burkard 24 H volumetric trap ran continuously for each 24 H period. For collection of airborne particles the MTIST trap contained 4x8 well microtitre strips. The multi-cyclone sampler was loaded weekly with eight 1.5ml microfuge tubes. By automation each tube was exposed once for a 12 H period to retain field sampled air particulates. A Burkard 24 H volumetric air sampler was used which trapped particles on a silicone coated glass slide. The glass slide and 4 x 8 well microtitre wells were changed daily and after 18:00 H. The microfuge tubes were collected weekly. All collected material was stored at -20 ^{0}C until analysed for trapped inoculum of *P. destructor* (onion downy mildew).

Air temperature, leaf wetness, relative humidity and rainfall were recorded at 30 min intervals using a Delta T data logger (Delta T Devices LTD., Cambridge,UK.). Environmental data were downloaded daily and used in a mathematical model (Millioncast) to determine sporulation risk periods for onion downy mildew.

Materials and Methods

(i) Microtitre Immunospore Trap (MTIST)

Spore trap characteristics. A detailed description of the MTIST device, which is manufactured by Burkard Manufacturing Company (Rickmansworth, Herts, UK) can be found in Kennedy *et*

al., (2000). In the outdoor version air is drawn thorough a manifold consisting of a plastic tube with a right angle bend placed over the sampler inlet. The manifold samples air through a 9cm diameter vertical circular inlet and directs it into the sampler body that is held horizontally. For field use, the sampler (including the manifold) is mounted on a wind vein so that the manifold inlet faces into the wind. Within the sampler the airflow is channelled through 32 trumpet shaped nozzles each directed at the base of the microtitre well. The sampler contains four microtitre strips each containing 8 wells. The MTIST spore trap uses a suction system to directly trap air particulates by impaction to the microtitre wells. Air is drawn through the device and particulates in the airstream are impacted on the base of each collection well of the 4 microtitre strips. The collected impacted target particulates may, if appropriate antibodies are available, be immunoquantified by PTA-ELISA.

Monitoring downy mildew conidia in collected air samples of an overwintered crop of bulb onions. The MTIST spore trap was placed in an overwintered crop of bulb onions. Held within the base plate of the machine were four coated eight well microtitre strips. Two microtitre well coating solutions were examined for use in the collection and immunoquantification of MTIST trapped *P. destructor* disease inoculum: 0.1mg ml-1 Poly-L-Lysine (Sigma P-1524) in distilled water and sodium azide. The MTIST spore trap was operated for 12H periods from 06:00 H to 18:00 daily. The coated microtitre strips were changed daily and prior to analysis stored at -20^{o}C.

Enumeration of trapped *P. destructor* spores. For each sampling period four wells of each exposed strip were viewed by microscopic examination (x200). The total number of *P. destructor* conidia deposited on the base of each microtitre well was counted using a Nikon model TMS inverted binocular microscope. The wells were then probed using a MAb raised to conidial antigen of *P. destructor* and the immunoquntification of MTIST trapped onion downy mildew inoculum was by a standard PTA-ELISA process (Kennedy *et al*., 2000).

(ii) Multi-cyclone air sampler

Spore trap characteristics. The characteristics of the cyclone sampler have been described by Ogawa & English (1995). Air is drawn through this 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. The collection efficiency of this type of trap is suitable for use with an “in field” test detection system.

Monitoring downy mildew conidia in collected air samples of an overwintered crop of bulb onions. The multi-cyclone spore trap was placed 2 m from and adjacent to the MTIST spore trap. The trap was loaded weekly with eight 1.5ml microfuge tubes. By an integrated automated mechanism each tube was exposed once for a 12 H period for collection of field air particulates. Each sampling exposure period was between 06:00 to 18:00 H daily.

Enumeration of trapped *P. destructor* spores. To each exposed microtitre tube 120µl of NPARU B2 buffer was added and agitated using a Gallenkamp Spin Mix for 5 seconds at high speed. An aliquot of 20ul was then removed and the number of *P. destructor* conidia present was determined by bright field microscopy (x 400), A Lateral flow device developed for field assessment risk of the onion powdery mildew pathogen was used to semi-quantify trapped airborne inoculum in the remaining buffer of each field exposed microtube. A 100ul aliquot of the spore suspension was applied to the sample pad (Plate 1A) and test line development was assessed.

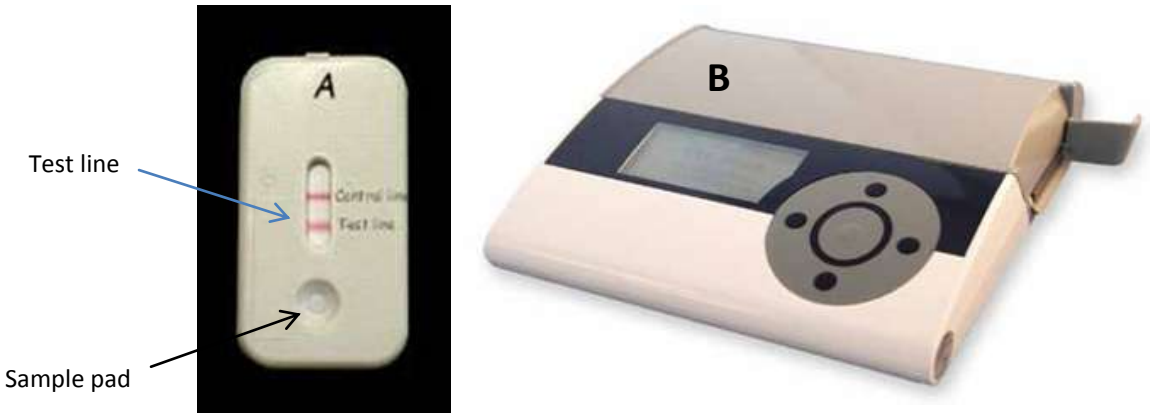

Plate 1. A lateral flow device for evaluation of field crop risk to onion downy mildew (A) and a hand held reader (B)

The development and optimisation characteristics of the Lateral flow device used are described in detail in Milestone 2 of this report.

iii) 24hr Volumetric glass slide air sampler

Spore trap characteristics.

The Burkard 24H volumetric trap used a glass slide which has been coated with silicone (BC 380S, Basildon Chemical Co, Kimber road, Abingdon, Oxon OX14 1RZ). An air output was directed onto the discreet areas of the slide which corresponded to different time intervals as the slide moved. Particulate matter from the airflow was directly impacted onto the glass slide. The glass slide was replaced daily after 18:00 H. The slide, eppendorf tube, and microtitre strips were stored at -20^{o}C after their removal from each air sampler.

Monitoring onion downy mildew conidia in air samples (from an overwintered crop of bulb onions). The Burkard 24H glass slide volumetric spore trap was placed 2 m from and adjacent to the MTIST spore trap. The trap was loaded daily with a silicone coated glass slide and air particulates were impacted directly on to the surface. The glass slide was changed daily and after 18:00 H. Prior to analysis the collected glass slides were stored at -20^{0}C.

Enumeration of trapped *P. destructor* spores. Under bright field microscopy and at a magnification of 400 x, each glass slide was examined for the presence of conidia of the onion downy mildew pathogen, *Peronospora destructor* (Plate 2). To examine diurnal periodicity the conidial counts were made in conjunction with the time of impaction on to the field exposed glass slide.

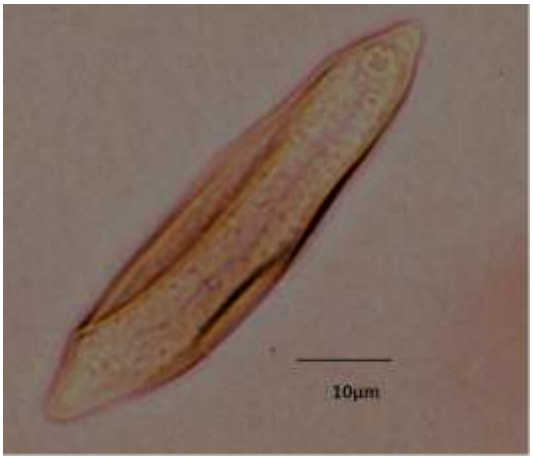

Plate 2. Bright field image of a field trapped condia of *Peronospora destructor* at 400 x magnification.

Results

Monitoring downy mildew conidia in collected air samples of an overwintered crop of bulb onions

(i) Microtiter immunospore trap (MTIST)

Daily numbers of MTIST field trapped *Peronospora destructor* conidia, as determined by bright field microscopy, are shown in Figure 3. The first airborne disease transmission event was observed on the 21st May with six successive waves of intensity observed thereafter. High *P. destructor* spore airborne levels were observed on 2nd, 9th and 18th June.

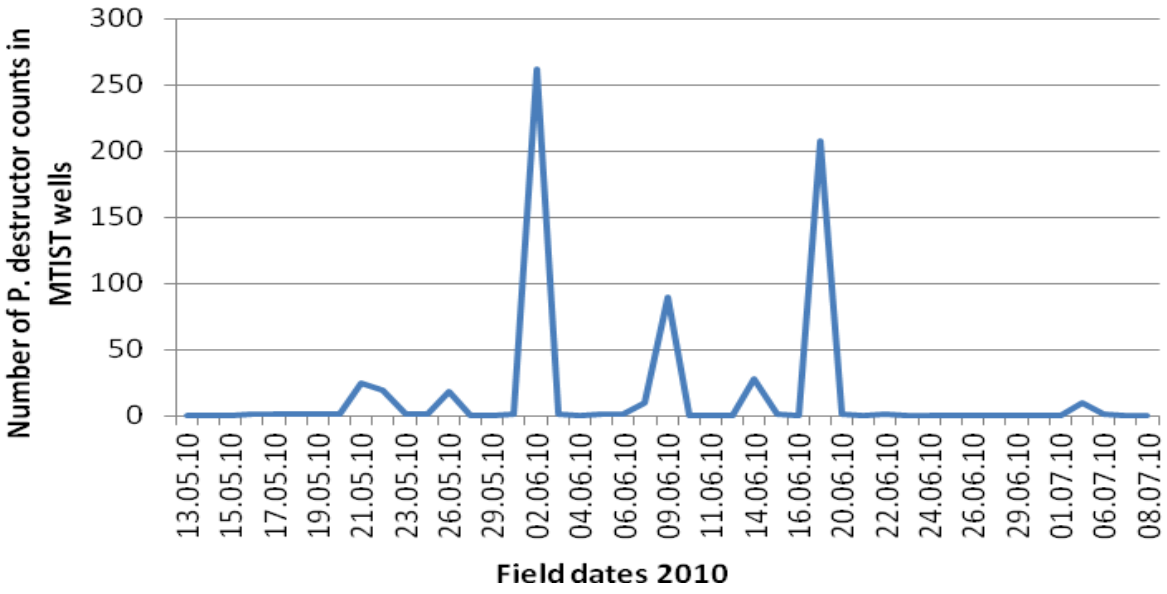

Figure 3. Monitoring downy mildew airborne disease transmission in an onion crop using an MTIST air sampler and bright field microscopy

Processing the microtitre wells by ELISA to immunoquantify MTIST trapped *P.destructor* inoculum identified a low level of disease inoculum in the crop between the 13th and 21st May. An increased spike on the 26th May occurred until 2nd June 2010. After which successive waves of downy mildew inoculum were identified throughout the monitoring period (Figure. 4).

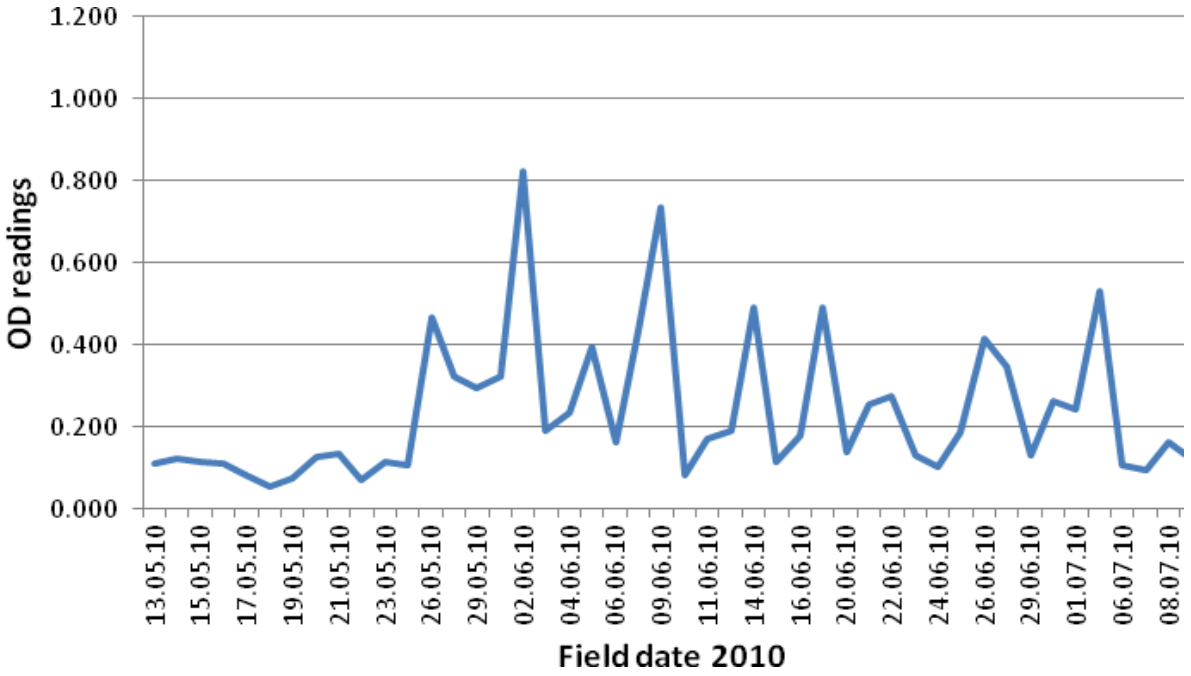

Figure 4. Monitoring downy mildew airborne disease transmission in an onion crop using an MTIST air sampler and immunoquantification by PTA ELISA

Trapping efficiency of the MTIST spore trap for *P. destructor* inoculum was not compromised by the use of different microtitre well coatings. A correlation (r^2=0.918) between the two well coatings used (Poly-L-Lysine and Sodium Azide) was observed for quantification of MTIST field trapped inoculum of onion downy mildew (Figure. 5).

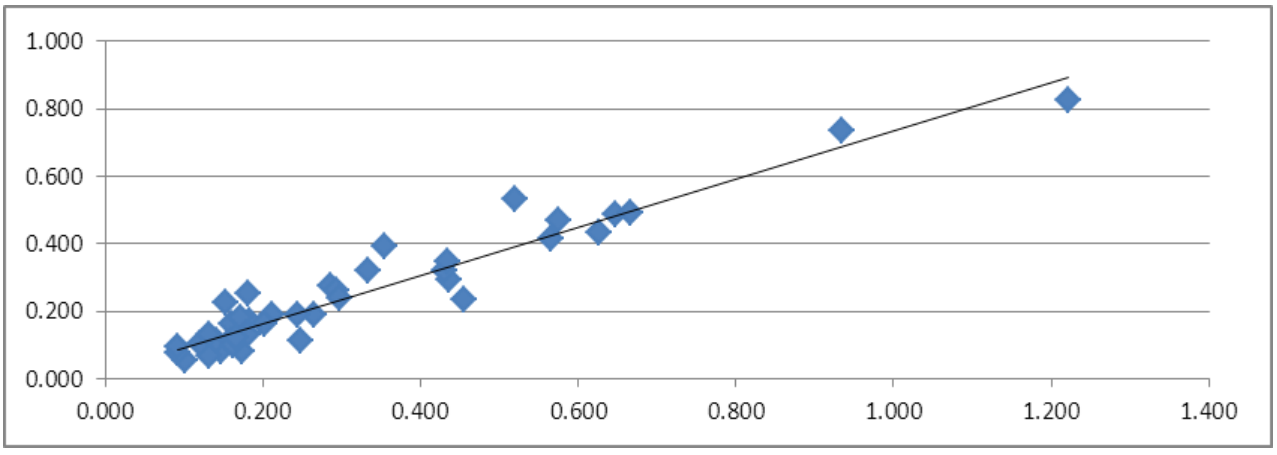

Figure 5. Relationship between two microtitre well coatings and PTA ELISA absorbance values for immunoquantification of MTIST field trapped *P. destructor* conidia**.**

(ii) Multi-cyclone sample

Airborne inoculum of *Peronospora destructor* was identified by bright field microscopy in microfuge sample collection tubes during the field air sampling period. The first airborne disease transmission event was observed on May 25th followed by 5 successive peaks (Figure 6). Analysis of the air samples by lateral flow (on-site field based test) identified the crop to be at risk from the 22nd May (ESE reading >0.4) and confirmed the dates as identified by microscopic counts. The lateral flow in addition identified the 7th June as an 'at risk day' for available *P. destructor* inoculum. A low level risk (0.2 to 0.4 lateral flow ESE reading) to the crop is identified at the end of the sampling period (27th June, 1st and 6th July).

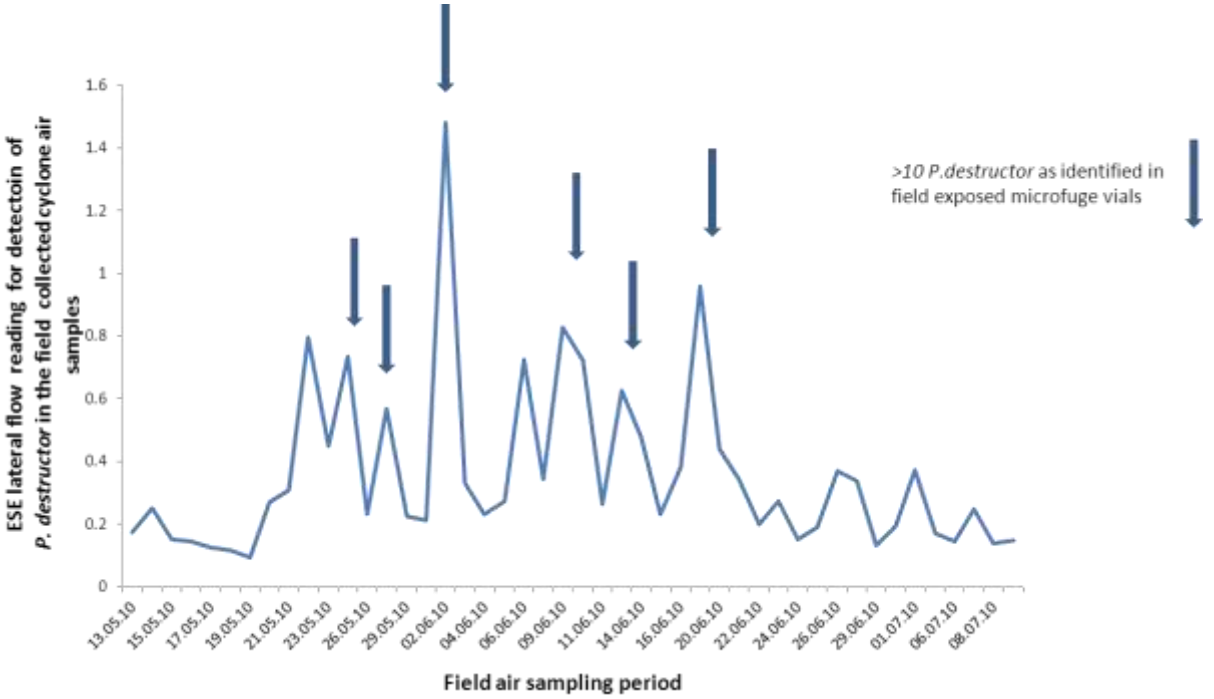

Figure 6. Monitoring downy mildew airborne disease transmission in onion crop using a multi vial (daily) air sampler with quantification by bright field microscopy ( ) and a field test (lateral flow).

With the exception of the trapping period 19nd to 26th May, a relationship is observed between the developed lateral flow field assay and the MTIST air sampler PTA ELISA assay for semi-quantitation of airborne *P. destructor* inoculum (Figure. 7). However microscopic counts of the MTIST wells identify *P. destructor* inoculum availability on the 21st to 23rd May (Figure. 4) and confirm the results of the lateral flow assay (Figure. 7).

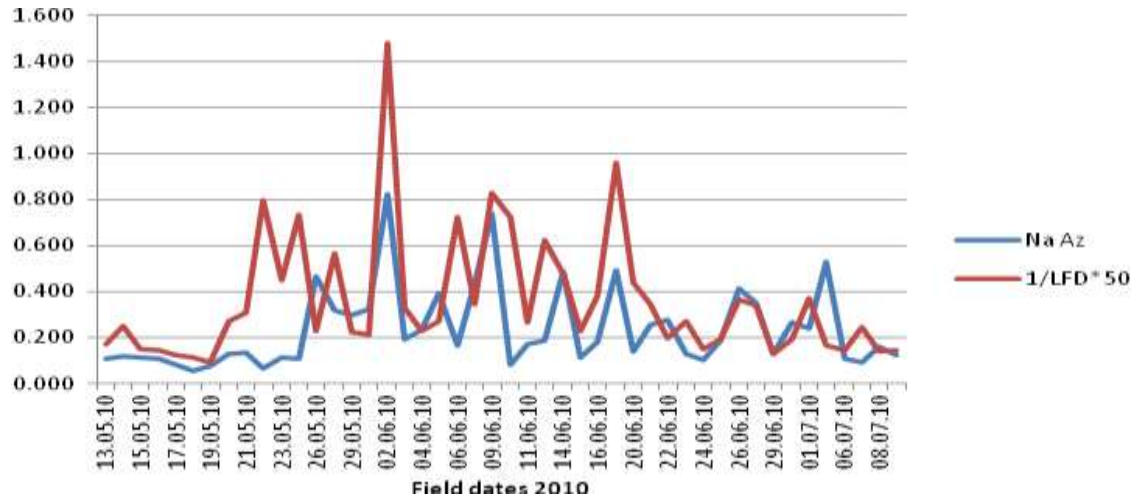

Figure 7 . Quantification of airborne inoculum of the onion downy mildew pathogen in an onion crop by PTA ELISA and lateral flow assay

(iii) 24HR Volumetric glass slide sampler

Analysis of the field exposed silicone coated glass slides identified four exposure periods when the onion crop was at significant risk to airborne onion downy mildew. These data confirm the spore count data derived from both the MTIST and the multi cyclone sampler for the same sampling period (Figure. 8).

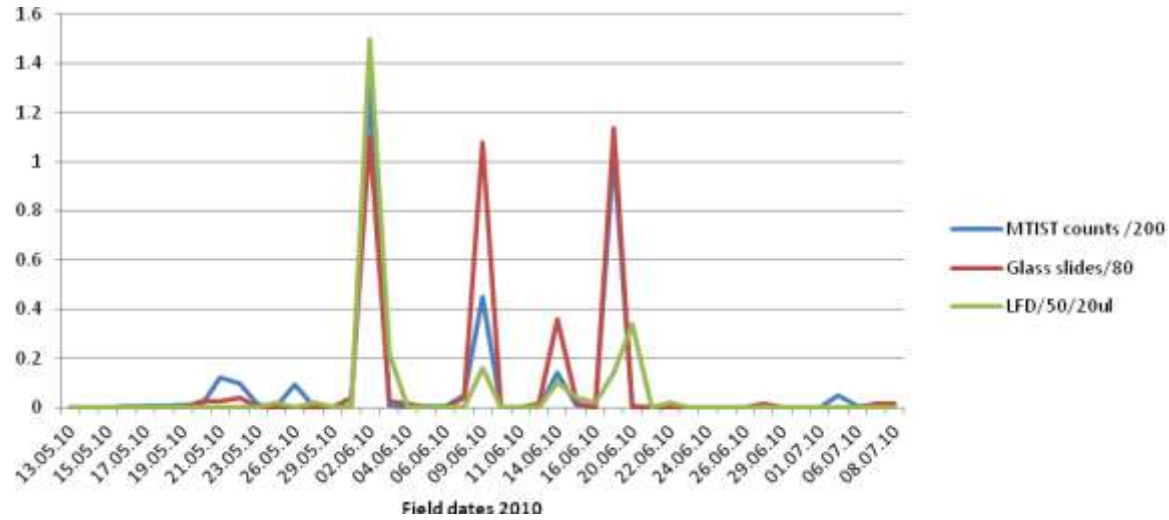

Figure 8. Relationship between the three air samplers and enumeration of trapped air spores of *P. destructor* in an overwintered onion crop

For each of these four sampling periods, *P. destructor* conidia were identified in the airborne environment of the crop between 06:00 and 16:00 hrs. Peak airborne volume was recorded between 08:00 to 12:00 hrs (Table 1).

Table 1. Volumetric glass slide air sampler: Hourly counts of *P. destructor* for four sampling dates

	06:00	07:00	08:00	09:00	10:00	11:00	12:00	13:00	14:00	15:00	16:00	17:00	Counts	CF	S/G3
02/06/10	0	1	4	15	32	28	59	25	11	1	0	0	180	0.49	88
09/06/10	2	9	37	41	40	24	8	4	6	0	2	0	173	0.49	85
14/06/10	3	3	16	8	6	4	10	2	0	0	2	0	57	0.49	28
19/06/10	0	0	31	106	24	2	0	0	0	0	0	0	163	0.49	91

Summary

A range of air sampling devices have been used to monitor airborne onion downy mildew in a field of bulb onions. Each of the air samplers, identified by conventional bright field microscopy, exhibit four distinct periods of risk to the crop. The MTIST air sampler, which samples at approximately 4 x the air flow rate of the other samplers, singularly identified an inoculum period in the environment of the crop 10 days ahead of the first established peak. Using antibody based detection systems the initial inoculum event was identified using the developed lateral flow assay.

Determining periods of downy mildew spore presence in the airborne environment of the onion cropping system will when used in conjunction with environmental based disease forecast system (Millioncast) enable growers to determine periods when the crop is at risk to infection and disease development. This study indicates that the lateral flow and sensitivity of the test has potential to detect low concentrations of airborne disease inoculum that may give rise to the initial disease epidemic. The use of the 24hr glass slide volumetric air sampler provides invaluable information on the diurnal periodicity of the onion downy mildew pathogen (time of the day when disease inoculum is present in the air) and future field trials will look to sample the airborne environment only during these periods.

MILESTONE 2: LATERAL FLOW DEVICE OPTIMISATION FOR DETECTION OF ONION DOWNY MILDEW THRESHOLD INOCULUMS IN A FIELD SETTING.

Introduction

In the development of a lateral flow for diagnostic purpose it is critical to determine the stability of the biological components used in the developed assay format (shelf life of the product). In addition, if the product is to be used for semi-quantitative use the detection threshold i.e test line depletion, should relate to disease potential. The study described below will address these two areas for the development of a competitive lateral flow format for detection of airborne inoculum of the onion downy mildew pathogen in air samples collected in an onion cropping system.

Materials and Methods

(i) Optimisation Study

Lateral flow. The lateral flow device comprised of a Millipore HF 180 HiflowTM cellulose ester membrane direct cast on to 2 mls Mylar backing (Cat no. SHF2400225, Millipore Corp, USA), an absorbent pad (Cat no. GB004, Schleicer and Schnuell, Germany), filtration section (VF2, Millipore Corp, USA) and a sample pad (Cat no. T5NM, Millipore Corp, USA). A flat bed air jet dispenser (BioDot,UK) was used to apply a test line of *Peronsopora destructor* antigen at variable rates and in different test line solutions to lateral flow construction cards. After which the membrane cards were air dried at RT for a period of 4 hours and cut into 5 mm width strips. A volume of 3 mls of a 1:40 dilution of EMA 242 Monoclonal Antibody (MAb) made up in NPARU conjugation buffer was prepared. To this three mls, 600µl of goat anti-mouse IgM 40nm gold conjugate (Code BA GAMM 40, British Biocell International, Cardiff, UK) was added and the tube was incubated on a roller incubator for 10 minutes. Each sample pad of each lateral flow device received 30µl of the antibody gold conjugate solution before air drying in an oven at 27^0C for 10 minutes. The lateral flow devices were mounted within a plastic housing device (Schleicer and Schuell, Germany) and stored in desiccated pouches prior to use and either stored at 4oC or Room temperature (RT)

Antigen stability on the test line. At monthly intervals the produced lateral flows were processed and ESE reader outputs of the lateral flow test lines obtained to determine whether stability of the biological components was retained.

Detection sensitivity of the lateral flow device. A lateral flow prototype which had been developed for detection of the downy mildew pathogen was assessed for limit of detection for the airborne stage of onion downy mildew. A tenfold dilution series ranging from $1x10^5$ to 10 conidia (whole conidia and disrupted) was applied to individual lateral flow strips. After a 10 minute development time the area of the test line for each lateral flow was read using an ESE and values recorded.

(ii) Field study.

A field plot of spring sown bulb onion *cv.* Renate FI, in 4x 10 beds of 25 metres was monitored for downy mildew sporulation over a two month period. Using a SKYE data logger (Skye Instruments Ltd, Llandrindod Wells, Powys) and attached to sensors within the crop, the temperature, humidity, leaf surface wetness and rainfall were collected at 30 min intervals and downloaded remotely by GSM portable Link. Using the generated data an onion downy mildew disease forecast model (MILIONCAST) was used to predict the occurrence of infection, sporulation and time to downy mildew symptom development. As in the reported field study, air sampling devices were used to determine the availability of airborne inoculum. A multi-vial cyclone sampler was used and the lateral flow test was assessed for sensitivity to detect target airborne inoculum and at a concentration that can elicit disease development in crop when favourable conditions exist.

Results

(i) Optimisation Study

Antigen stability on the test line. Results to date suggest that no deterioration of biological activity of the developed lateral flow assay system for quantitative measurement of *P. destructor* has occurred over a 5 month period (Figure. 9). Monthly testing of lateral flows will continue over time to determine shelf life of the product.

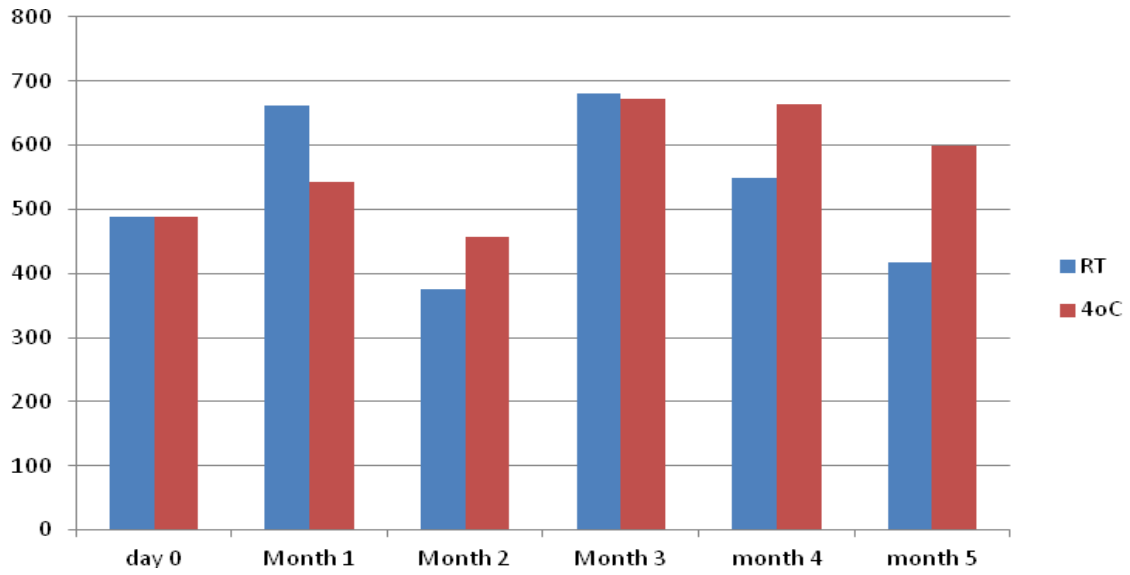

Figure 9. Shelf-life stability study of the *Peronospora destructor* lateral flow device.

Detection sensitivity of an Optimised Lateral Flow. A lateral flow device was identified which provided a quantitative measurement of *P. destructor* spores over the range of $1x10^5$ to 10 spores. Test line readings were made using an ESE portable reader (Figure. 10). Little difference in test sensitivity was observed between disrupted and whole conidia of *P. destructor*.

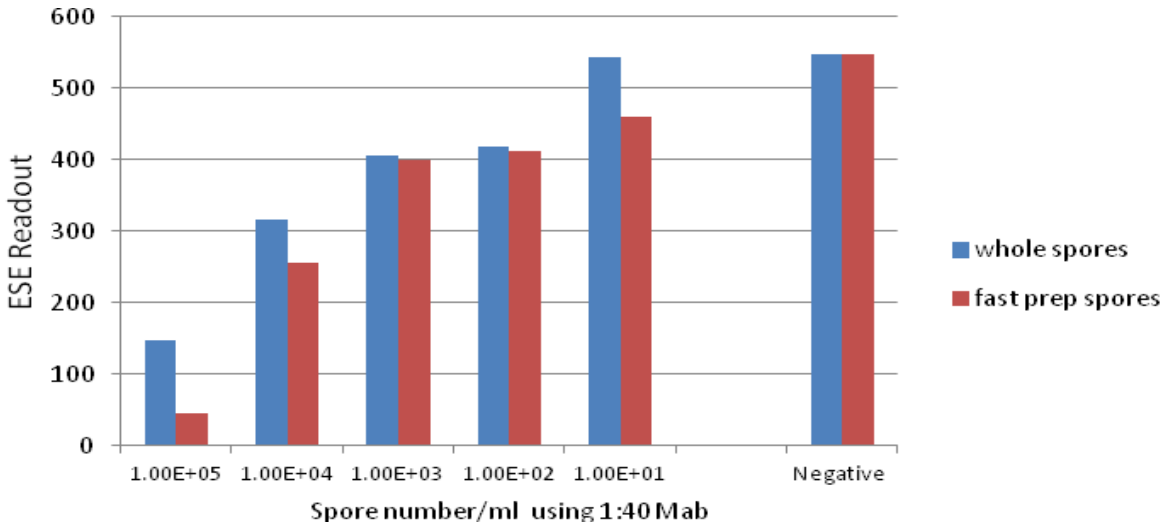

Figure 10. Dilution series of 'whole'and disrupted conidial suspension of *P. destructor* and test detection sensitivity using the developed optimised lateral flow test.

(ii) Field study

Downy mildew sporulation on plants was observed at a low level in the field crop on June 2nd (Figure. 11). Daily lateral flow tests identified a low level of disease inoculum in the crop from the 25th to the 28th May followed by high airborne concentration on the morning of the 3rd June (Figure. 11). The ESE readings of the lateral flow tests for these dates, when related to the serial dilution series of 'whole' *P. destructor* conidia (Figure. 10) determined field collected airborne inoculum of *P. destructor* concentrations in excess of $1x10^5$. Previous studies (Kennedy *et al*., 2010) have demonstrated that spore airborne inoculum occurs at relatively high concentrations in the air before crop to crop transport is possible and a requirement for a threshold to be reached before infection can occur. Results of this study would suggest a disease threshold >$1x10^5$ of *P. destructor* airborne conidia to initiate disease occurance and that the lateral flow used in this study identified this initial phase on the 23rd May (Figure. 11).

The environmental parameters recorded by the Skye data logger and the results of the daily lateral flow tests (Figure. 12) demonstrate a relationship between leaf wetness and airborne concentration of *P. destructor*.

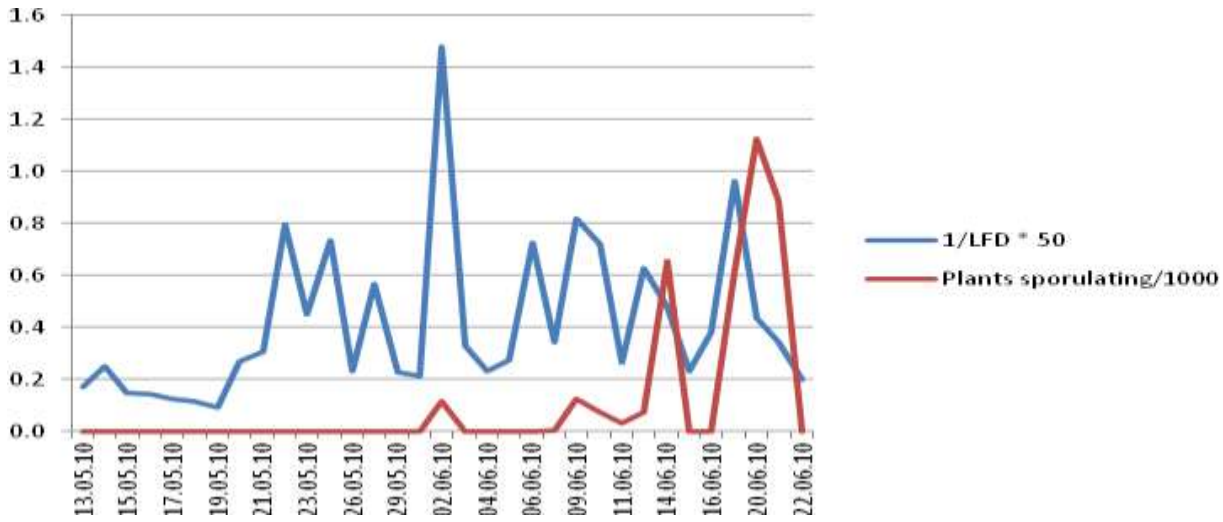

Figure 11. Monitoring onion downy mildew sporulation events in an onion field crop and available airborne disease inoculum as determined by a multi-vial cyclone air sampler and daily lateral flow tests.

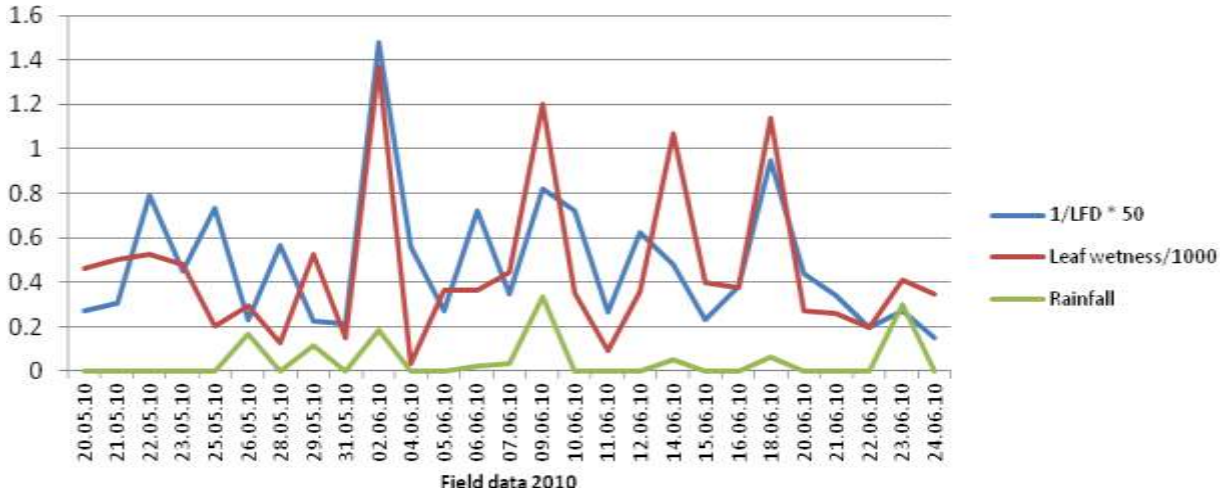

Figure 12. Relationship between crop humidity, leaf wetness and lateral flow observations of available airborne *P. destructor* inoculum

Summary

Investigations to date confirm stability of the biological components within the developed downy mildew lateral flow format. The prototype identified for field trials has been shown to operate over the required sensitivity of detection for airborne concentration of onion downy mildew which can initiate disease symptoms. In this study downy mildew was identified on onion plants within the crop on the 2nd June and the disease propagules were identified in the air on the 23rd May by lateral flow. For this period the environmental disease forecast (Millioncast) identified conditions for downy mildew infection and sporulation. The latent period of downy mildew disease (period between infection and disease symptoms) was 9 to 16 days. The lateral flow test was able to identify the initial period of infection (23rd May) ahead of symptom development on the 3rd June (*crop to crop spread*). The downy mildew sporulation observed on infected plants on the 3rd June and release of the inoculum in to the air was confirmed by lateral flow test for this period.

MILESTONE 3: TEST OPTIMISED ONION DOWNY MILDEW LATERAL FLOW DEVICES IN THE FIELD IN OVER WINTERED COMMERCIAL ONION CROPS

Introduction

A comparison of trapping formats and a weather based disease forecast for onion downy mildew was carried out at Moor Farm, Leasingham, Sleaford, Lincolnshire from early June through to August in 2011. A monoclonal antibody (MAb) exhibiting specificity for *P.destructor*, was evaluated for detection of airborne onion downy mildew inoculum in a field plot. The tests were carried out at a site where onion downy mildew is commonly found in the crop. Three trapping formats were compared in this trial: a) An MTIST sampler, b) a single cyclone sampler and c) a multi cyclone air sampler. Each were operated for 12 H periods from 06:00 H to 18:00 H daily. The MTIST trap containing 4x8 well microtitre strips was changed daily after 18:00 H. The multi cyclone air sampler, containing eight microfuge tubes, provided 24hr sampling periods for each microfuge tube over an eight day period. Sample tubes were then changed at weekly intervals. The single cyclone sampler contained a single microfuge tube and this was changed weekly. Following removal from the field air samplers the collected sample tubes and microtitre strips were stored at -20^{o}C prior to analysis.

Materials and Methods

(i) Microtitre Immunospore Trap (MTIST)

Monitoring downy mildew conidia in collected air samples of a commercial field crop of onions. The MTIST spore trap was placed in a commercial onion crop and operated for 12 H periods from 06:00 H to 18:00 H daily. Within the base plate of the machine were four coated eight well microtitre strips which were changed once weekly. Prior to analysis the strips were stored at -20º C.

Enumeration of trapped *P. destructor* spores. For each sampling period four wells of each exposed strip were viewed by microscopic examination (x200). The total number of *P. destructor* conidia deposited on the base of each microtitre well was counted using a Nikon

model TMS inverted binocular microscope. The wells were then probed using a MAb raised to conidial antigen of *P. destructor* and the immunoquntification of MTIST trapped onion downy mildew inoculum was by a standard PTA-ELISA process (Kennedy *et al*., 2000).

(ii) Multi-cyclone air sampler

Monitoring onion downy mildew conidia in a commercial field crop of onions in 2011. The multi-cyclone spore trap was placed 2 m from and adjacent to the MTIST spore trap. The trap was loaded weekly with eight 1.5ml microfuge tubes. By an integrated automated mechanism each tube was exposed once for a 12 H period for collection of field air particulates. Each sampling exposure period was between 06:00 to 18:00 H daily. Microfuge tubes were collected once weekly and stored prior to analysis at -20ºC.

Enumeration of trapped *P. destructor* spores. Each exposed microfuge tube had 120µl of NPARU B2 buffer was added and agitated using a Gallenkamp Spin Mix for 5 seconds at high speed. A Lateral flow device developed for field assessment risk of the onion downy mildew pathogen was used to semi-quantify trapped airborne inoculum in the remaining buffer of each field exposed microtube. The sample pad of the lateral flow device had 100ul aliquot of the spore suspension applied to it and test line development was analysed using an ESE QUANT hand held reader as previously described.

(iii) Single cyclone sampler

The sampling characteristics of the single cyclone sampler are as described for the multi-vial sampler however only one microfuge tube is held within the sampling mechanism. The spore trap was placed 2 m from and adjacent to the MTIST spore trap. The trap was loaded weekly with a single 1.5ml microfuge tube. The air sampler operated between 06:00 to 18:00 H daily. The microfuge tube was collected once weekly and prior to analysis was stored at -20ºC.

Enumeration of trapped *P. destructor* spores. Exposed microfuge tubes had 120µl of NPARU B2 buffer added and agitated using a Gallenkamp Spin Mix for 5 seconds at high speed. A Lateral flow device developed for field assessment risk of the onion downy mildew pathogen was used to semi-quantify trapped airborne inoculum in the remaining buffer of each field exposed microtube. The sample pad of the lateral flow device had 100ul aliquot of the

spore suspension applied and test line development was analysed using an ESE QUANT hand held reader as previously described.

Results

(i) Microtitre Immunospore Trap (MTIST)

Field exposed microtitre wells strips were examined by microscopic examination for presence of *P. destructor* trapped airborne inoculum. On each of the exposure days and for the whole sampling period no conidia of *P. destructor* were observed. Analysis of the weekly field exposed microtitre wells by PTA ELISA using a MAb raised to *P. destructor* provided low Optical Density readings and indicated low or no risk to the crop (Figure. 13). In an earlier study (2010) field exposed microtitre wells gave rise to absorbance values between 0.2 and 1.4 for the two month sampling period. Only on days with readings above 0.4 was the crop found to be at risk to onion downy mildew inoculum. The 2011 field trial involved MTIST sampling times over a seven day period (cumulative sampling period) whereas field aerosols were collected daily in the 2010 trial.

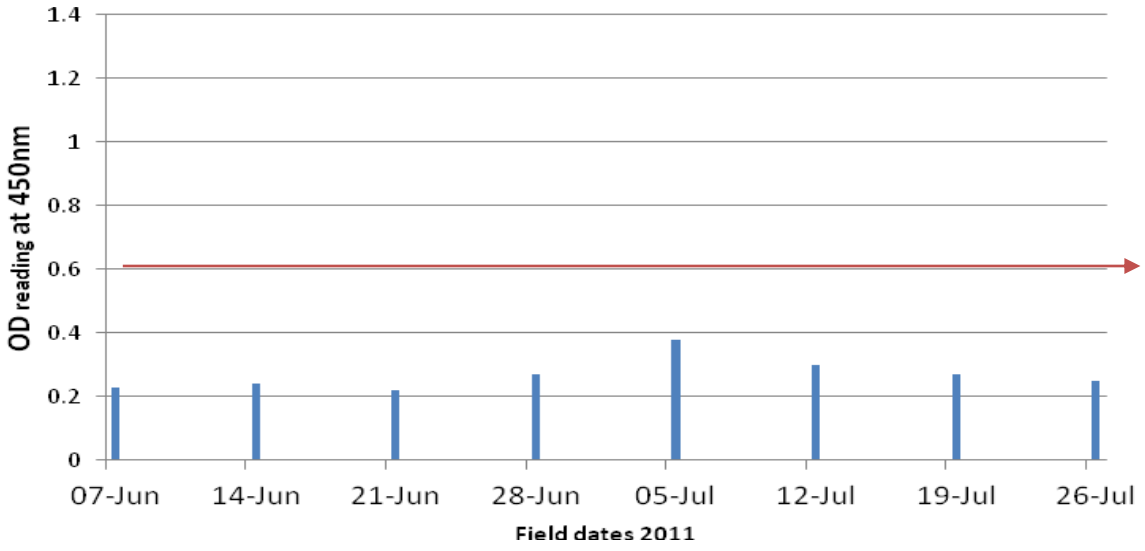

Figure 13. Weekly monitoring for *P. destructor* airborne disease inoculum in a commercial onion crop using an MTIST spore trap.

(ii) Multi-cyclone air sampler

Daily field exposed microfuge tubes were examined by microscopic examination for presence of onion downy mildew trapped airborne inoculum. On each of the exposure days and for the whole sampling period no conidia of *P. destructor* were observed. Analysis by lateral flow provided ESE readings below 0.4 and with the exception of three sampling periods were below the baseline control figure of 0.2 (Figure. 14). This indicated that the crop was exposed to low risk of onion downy mildew at the beginning of the season on the 14th June and thereafter no risk periods were identified.

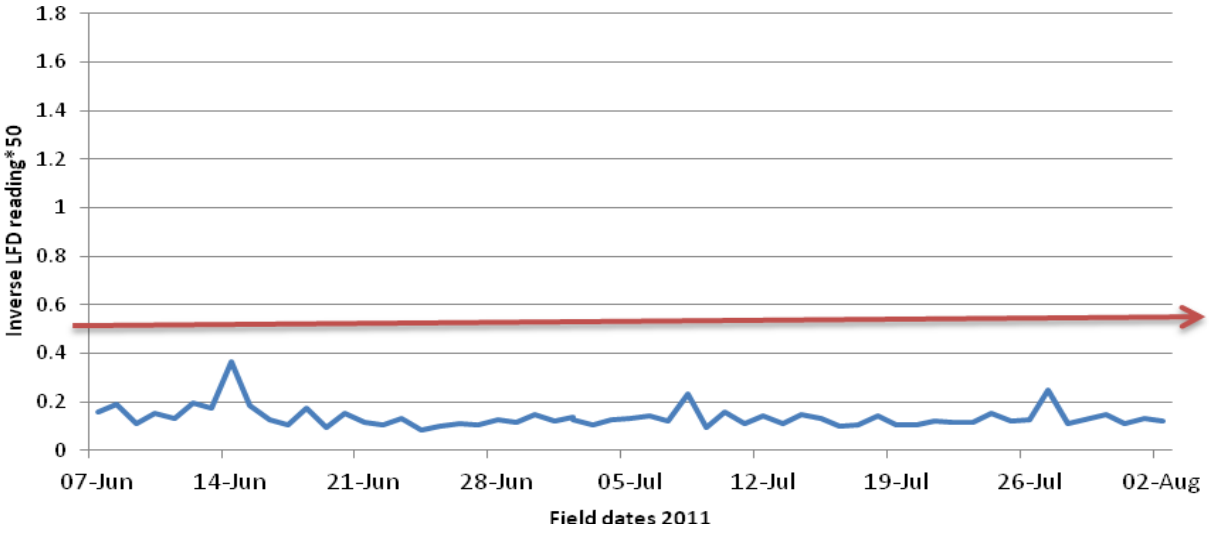

Figure 14. Monitoring downy mildew airborne disease concentration in an onion crop using a multi vial (daily) air sampler and an on-site lateral flow test.

(iii) Single cyclone sampler

Weekly field exposed microfuge tubes were examined by microscopic examination for presence of *P. destructor* trapped airborne inoculum. For each seven day sampling period no onion downy mildew disease inoculum in samples was observed. Analysis by lateral flow however identified two single week periods where the crop was predicted to be at risk to downy mildew inoculum (Figure. 15). For the sampling period 14th June a high level of rain was recorded and the microfuge tube was affected by water.

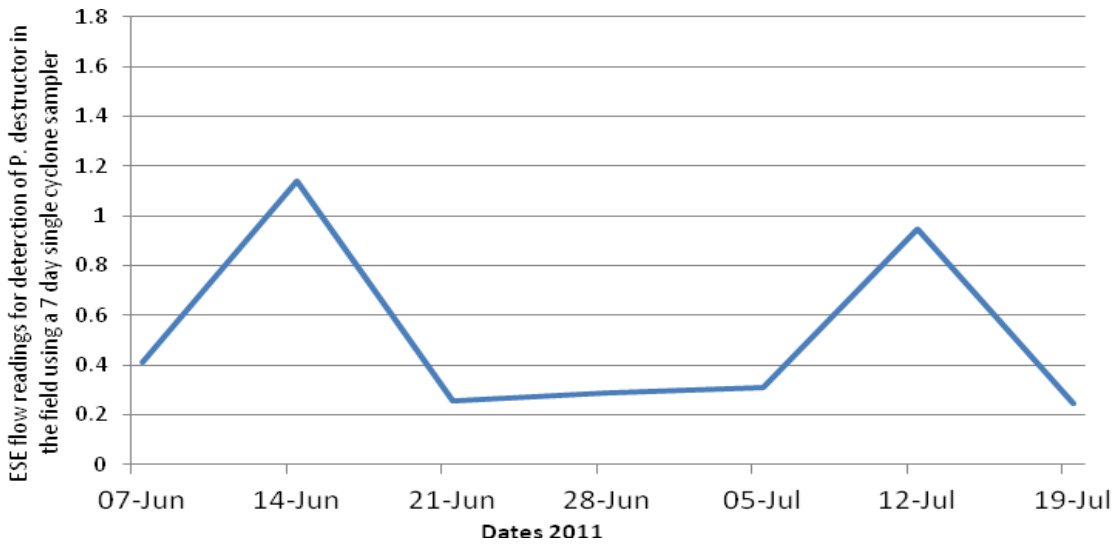

Figure.15. Monitoring weekly downy mildew airborne disease concentration in an onion crop using a single tube cylone air sampler and an on-site lateral flow test

Summary

The field results using daily air samples to predict airborne inoculum of the downy mildew pathogen *Peronospora destructor* compare with the previous field study carried out in 2010. The developed lateral flow provided negative test results for most days and ESE readings confirmed for each that there was very low (two dates identified) or no disease inoculum present in the airborne environment of the crop. Visual assessment of the crop confirmed this and no disease symptoms were observed in the onion crop for the period tested. This was in contrast to air samples collected on a weekly basis where consecutive daily air samples were held in one microfuge tube prior to testing. The identification of water within tubes on weeks identified with high ESE readings would suggest release of soluble antigen or growth of trapped aerobiological particles. This may have resulted in the production of a non or specific interaction of the developed lateral flow assay system. The collection of water within microfuge tubes was not observed when air samples were collected daily using the multi-vial sampler. Conversely the MTIST air sampler, which operated on a weekly basis, was not affected by water collection during the extended sampling period. The microtitre wells of the MTIST air sampler were however coated with sodium azide to inhibit microbial growth. This may have proved critical in preventing growth of trapped airborne microflora over the seven day exposure period and the

soluble release of material which could have reacted with the immunoassay process of the lateral flow.

General Conclusions

The critical date for applying fungicide applications to the crop can now be identified using a Lateral Flow device. Disease development can be detected in the absence of visible symptoms. This is a critical point in considerations of disease control since if early applications of fungicide can be targeted to when onion downy mildew conidia are present the activity of control methods will be enhanced. Establishing a network of traps (3- 4 traps) which could be applied to localities could be used as an early warning system for onion downy mildew. With high sampling rates these traps if positioned to reflect prevailing wind patterns could be used to designate the onset of disease risk in different areas and pinpoint specific transmission events affecting different crops and areas. Many transmission events are due to the effect of rain acting as, the agent for deposition of spores from the air on to crops. Other studies with other pathogens have shown that pathogenic inoculum builds up in the air before transmission is possible. Protective applications of fungicide can therefore be precisely timed at different locations. This will help the onion industry meet any short fall in fungicide types in the future by improving the efficiency of existing approved products.

The use of weekly inoculum estimates did not work in 2011 studies. The 'trapping duration' is one area of study which is yet to be confirmed and further studies in year three of the project will address this requirement.

References

P. Battilani, V. Rossi, P. Racca, and S. Giosuè (1996). ONIMIL, a forecaster for primary infection of downy mildew of onion. Eur. Mediterr. Plant Prot. Organ. Bulletin 26:567-576.

R. Bugiani, P. Govoni, & L. Cobelli. (1998). First large scale application of IPI model for potato late blight prediction in the Po valley, p. 188–199. *In* H. Schepers and E. Bouma (ed.), Proceedings of the Workshop on the Euro-pean Network for the Development of an Integrated Control Strategy of Potato Late Blight. PAV, Carlow, Ireland .

C. L. Campbell and L.V. Madden (1990). Introduction to plant disease epidemiology. John Wiley & Sons, New York

O. Carisse, H.A. McCartney, J.A. Gagnon, and L. Brodeur (2005). Quantification of air-borne inoculum as an aid in the management of leaf blight of onion caused by *Botrytis squamosa*. Plant Disease 89, 726 – 733.

O. Carisse, D, Rolland, A. Lefebvre, and B. Talbot (2003). Using aerobiology data to manage onion blight caused by *Botrytis squamosa*. Proceedings of the 8th International Congress of Plant Pathology. 1 p 5

H. T. Cook (1932). Studies on the downy mildew of onion and the causal organism, *Peronospora destructor* (Berk.) Caspary. New York Agr. Exp. Station, Ithaca, Mem. 143:1-40.

I.A. M. Cruickshank (1958). Environment and sporulation of phytopathogenic fungi. IV. The effect of light on the formation of conidia of *Peronospora tabacina* Adam. Aust. J. Biol. Sci. 16:87-98.

C. L. M. de Visser, (1998). Development of a downy mildew advisory model based on downcast European Journal of Plant Pathology 104, 933 -943.

T. Gilles, J.P. Clarkson, K Phelps, R. Kennedy (2004). Development of MILIONCAST, an improved model for predicting downy mildew sporulation on onions. Plant Disease, 88, 695-702.

P. D. Hildebrand, and J. C. Sutton (1982). Weather variables in relation to an epidemic of onion downy mildew. Phytopathology 72:219-224.

G. D. Jesperson, and J.C. Sutton (1987). Evaluation of a forecaster for downy mildew of onion (*Allium cepa* L.). Crop Prot. 6:95-103.

R. Kennedy, A. J. Wakeham,, K.G. Byrne, U. M. Meyer & F. M. Dewey (2000). A mew method to monitor airborne inoculum of the fungal plant pathogens *Mycosphaerella brassicicola* and *Botrytis cinerea*. Applied and Environmental Microbiology 66 pp 297 - 307

R. Kennedy (1998). Bulb onions: evaluation of fungicides for control of downy mildew (*Peronospora destructor*). Horticultural Development Council. Annual Report (Year 1) FV 189 *pp* 10.

V. Phillon (2003). Timing of sprays against potato late blight, based on daytime average airborne spore concentration. Proceedings of the 8 th International Congress of Plant Pathology. 1 p 6

W. Rondomanski (1967). Studies on the epidemiology of onion downy mildew, *Peronospora destructor* (Berk.) Fries. Technical Report for 1962-67. Research Institute for Vegetable Crops, Skierniewice, Poland. 23.

J. C. Sutton, and P. D. Hildebrand, (1985). Environmental water in relation to Pe*ronospora destructor* and related pathogens. Canadian Journal of Plant Pathology **7**, 323 - 330.

F. Virányi, (1981). Downy mildew of onion. Pages 461-472 in: The downy mildews. D. M. Spencer, ed. Academic Press, New York. 27.

C.E. Yarwood, (1937). Relation of light to the diurnal periodicity of sporulation of certain downy mildews. Journal of Agricultural Research 54 , 365 - 373 8.

C.E. Yarwood, (1937). Relation of light to the diurnal periodicity of sporulation of certain downy mildews. J. Agr. Res. 54:365-373.

C. E. Yarwood, (1943). Onion downy mildew. Hilgardia 14:595-691

