ANNUAL REPORT

To:
Horticultural Development Council
Bradbourne House
Stable Block
East Malling
Kent, ME19 6DZ

ONIONS: DEVELOPMENT OF DETECTION SYSTEMS FOR CONIDIA OF *PERONOSPORA DESTRUCTOR* (DOWNY MILDEW) IN ONION CROPS

HDC PROJECT FV 189a

R Kennedy and A J Wakeham

Warwick HRI
Wellesbourne, Warwick, CV35 9EF

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Grower Summary

FV 189a

DEVELOPMENT OF DETECTION SYSTEMS FOR CONIDIA OF *PERONOSPORA DESTRUCTOR* (DOWNY MILDEW) IN ONION CROPS

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

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1. GROWER SUMMARY

1.1 Headline

Detection systems for spores of onion downy mildew have been developed which can be used in the field to detect onion downy mildew in the air. Use of detection tests in onion crops has shown that onion downy mildew can be detected at high levels in the air before disease is visible on the crop. Use of this information will improved onion downy mildew control in bulb and salad onion crops.

1.2 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. Onion downy mildew (*Peronospora destructor*) is geographically widespread and serious disease in bulb and salad onions and in onion seed production. 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. These losses mainly result from severe infections in bulb onion crops causing early defoliation, reduced bulb sizes and poor storage quality of bulbs. Losses to seed production are frequently caused by the collapse of infected seed stalks and poor germination of seeds collected from infected stalks. 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. Fungicidal control is the only effective means of controlling the disease and avoiding crop loss.

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. Production of large numbers of conidia of onion downy mildew 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). An understanding of how the environment affects sporulation could be used to predict sporulation. However, the effects of environmental factors on sporulation by *P. destructor* on onion plants are complex. The amount of spores produced during a 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.

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 predicted 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. For this reason tests which, can be conducted in the field are necessary if information on air-borne inoculum concentration is to be of more practical value. The use of air-borne spore numbers, as criteria, within forecasting systems is a new and exciting development in disease forecasting. One "in field test" which could be used in this respect is the lateral flow test for downy mildew inoculum.

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 downy mildew.
- Less reliance on eradicant fungicide applications for downy mildew control. More effective use of fungicides with protectant modes of activity.
- Monoclonal antibodies which recognise conidia of onion downy mildew conidia.

1.3 Summary of first year work on FV189a

In year one of the work, specific monoclonal antibodies which recognise downy mildew were developed using downy mildew spore washings. In addition a polyclonal antibody was raised in rabbit against onion downy mildew conidial washings. However high background reactions were observed when this polyclonal antibody was tested for its specificity. A level of cross reactivity was observed to the spores of a number of fungal species using this polyclonal antibody. No reactivity was observed to *Hyaloperonospora parasitica w*hich causes downy mildew on vegetable brassicas. The polyclonal antibody was weakly cross reactive with *Botrytis cinerea* but did not react with *Botrytis allii*. Both species of *Botrytis* are commonly found in onion crops. Tests with *Botrytis squamosa* another pathogen present in onion crops have still to be carried out. The antibody reacted strongly with *Ascochyta rabiei* (blight of chickpea) and *Pyrenophora teres* (net blotch of barley).

Spore trapping studies were used to ascertain environmental factors required for spore dispersal and to assess spore trapping formats for detecting onion downy mildew conidia. These experiments were conducted in a seeding crop of bulb onions which was extremely susceptible downy mildew infection and sporulation. Despite the crop being heavily infected

with downy mildew at the beginning of the spore trapping period there were few days where further downy mildew sporulation was predicted and observed. The results of the trial show that the predictions of onion downy mildew sporulation were accurate particularly those from the MILIONCAST system in comparison to the DACOM onion downy mildew forecasting system. The environmental requirements for downy mildew spore dispersal were particularly difficult to ascertain. The results indicated that when downy mildew conidia were produced windspeeds of 5 km (over 5 sec bursts) are required for their dispersal. There is also potentially a requirement for increasing humidity. Results indicated that conidia of onion downy mildew could be trapped within the vials in cyclone traps to give accurate estimates of conidial number when assessed by immunological methods (ELISA).

1.4 Summary of second year work on FV189a

In year two of the project two further monoclonal antibodies which recognised onion downy mildew conidia were raised using disrupted *P. destructor* soluble spore antigens. Both antibodies (designated as EMA 242 and EMA 243) did not react when tested with the downy mildew species notably *Bremia lactucae* (downy mildew on lettuce) and *Hyaloperonospora parasitica* (downy mildew on Brassicas). A third monoclonal, identified as EMA 240 reacted at a genus level with other downy mildews. Monoclonal antibodies EMA's 242 and 243, had specificity to onion downy mildew conidia when tested by PTA ELISA. In immunofluorescence tests EMA 242 and 243 reacted with the spore wall of *P. destructor* whilst EMA 240 showed recognition only to soluble elements associated with the downy mildew isolates tested. Both EMA 242 and 243 did not react against a range of fungal organisms commonly found under field conditions in soil and air (notably *Botrytis* and *Alternaria* sp. Both potentially important in onion crops).

A competitive lateral flow (LFD) format was developed using these antibodies. The competitive lateral flow device detected onion downy mildew conidia in test samples. Another LFD format tested (double antibody sandwich DAS LFD format) was unsuccessful in detecting onion downy mildew conidia. Many LFD fungal assays are based on the competitive format. The development of a successful LFD depended on the antigen concentration at the test line and the activity of the specific antibody used. The type and capillary flow rate of the membrane was also important. For the current test results indicated that using a Millipore 135 HiFlow™ cellulose ester membrane direct cast on to 2ml Mylar backing and, an antigen test line concentration (onion downy mildew conidia) between the range of 125 to 250µg protein ml^{-1} is optimal for the detection of onion downy mildew conidia when a gold conjugated antibody dilution range of between 1 : 160 to 1 : 320 is used.

Tests conducted under field conditions showed that coatings (used in the trapping vessel within the air sampler) comprising of paraffin wax or silicone gave consistently higher collection /retention of downy mildew conidia in comparison to an untreated control wells and other wells coated with poly-lysine. Neither of these well coatings affected the ELISA. Correlations between PTA absorbance values and numbers of onion downy mildew conidia in microtitre well stripes varied significantly between paraffin wax (r^2 = 0.5493) and untreated wells (r^2 = 0.3455). User of poly-lysine disrupted the ELISA. The results suggest that well coatings can be used to significantly enhance the number of downy mildew conidia trapped within air samplers.

In other field tests onion downy mildew conidia detection systems have been used within an over-wintered crop of bulb onions (seeding crop). Over-wintered onion crops frequently carry onion downy mildew infection as latent infections which can occur within bulbs as oospores or infected tissues. The results of this trial demonstrate that onion downy mildew conidia could be detected within the crop for many weeks before symptoms were visible. Several peaks in onion downy mildew conidial numbers occurred during late April and early May well in advance of visible symptoms which were present at the end of May and early June 2005. Results show that peaks of several hundred onion downy mildew conidia were necessary to initiate infection in the crop. This suggests that onion downy mildew exists within developing crops as either atypical symptoms or at level of disease which is very difficult to detect visually. This also suggests that disease could also be initiated in many onion crops by threshold levels of inoculum from outside the crop. The results of the trial also confirmed the accuracy of the Warwick HRI onion downy mildew forecasting system. Cyclone samples collected during this trial will be used in conjunction with the onion downy mildew lateral flow device to determine suitable conidial thresholds for detection of onion downy mildew in the field.

1.5 Action points for growers

There are no specific action points for growers at this stage in the project. However in the future

- Growers can use the in field test to determine when onion downy mildew is present in their crop.
- The system will be useable by both salad and bulb onion producers.

1.6 Anticipated practical and financial benefit

- 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 need for and reliance on metalaxyl based fungicides which should reduce the costs associated with onion downy mildew control in bulb and salad onion crops.

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.

SCIENCE SECTION

2. INTRODUCTION

2.1 Downy mildew occurrence in onion crops

Foliar diseases of onion crops (onion downy mildew and *Botrytis* leaf blight) can cause heavy yield losses in bulb and salad 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 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 disease and avoiding crop loss.

2.2 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 humidities 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 30ºC, viability declines rapidly at relative humidities above 55 %.

The pathogen can over winter as mycelium in onion bulbs and sets and as oospores in debris from diseased foliage. The disease 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.

2.3 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 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). An understanding of how the environment affects sporulation could be used to predict sporulation. However, the effects of environmental factors on sporulation by *P. destructor* on onion plants are complex. The amount of spores produced during a 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.

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

Management systems are available for both 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. This model is not yet available within ONION*SPOT* but it is hoped that this model can be added to the system at some future date. 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 by eliminating unnecessary fungicide applications, which are based on weather information alone.

2.5 Using air-borne spore numbers within disease forecasting systems

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

Detecting airborne spores of fungal plant pathogens is a useful tool in crop protection if this could be done rapidly and accurately. For example it has been reported that one or two peaks

in sporangial concentration in the air of the potato blight pathogen *Phytophthora infestans* preceded the first observed symptoms of the disease in the field (Bugiani *et al*., 1998). These observations were validated in studies conducted by Philion (2003). In these studies the numbers of sprays applied to control potato blight could be successfully reduced without any impact on crop quality by monitoring the onset of thresholds of potato blight inoculum. Fungicide applications were initiated when the daytime airborne sporangial concentration reached 30 sporangia/m^3 (disease was not yet visible when this threshold was reached). By using this criteria, in combination with disease forecasts based on weather information the number of fungicide applications could be reduced with no impact on disease development. Given that potato blight is a difficult pathogen to control great scope exists in applying this approach in the control of other less aggressive pathogens and in different localities. This means that disease can be predicted accurately before it is visible in the crop. Similar results were obtained for *Botrytis* blight (*Botrytis squamosa*) on onion crops where thresholds of 15 - 20 conidia/m^3 could be used 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 for downy mildew inoculum.

2.6 Immunological tests (Lateral Flow Devices)

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

The Competitive assay format

In a competitive assay format the test line comprises of homologous antigen (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 1). The fluid sample is added to the well, releasing the specific antibody bound coloured particles, which then begins to flow across the membrane. If the target antigen (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 (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.

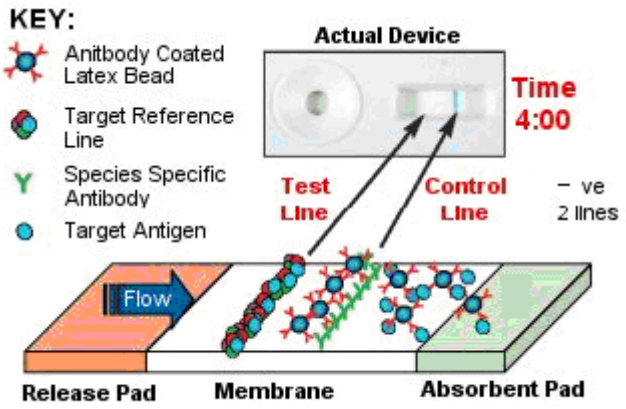


Figure 1. The Competitive lateral flow assay format.

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

2.7 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 downy mildew spores. Some specific antibodies have been raised and characterised for onion downy mildew in year one of this project. This report details the raising of further specific antibodies to onion downy mildew and their incorporation within lateral flow tests which detect conidia of the onion downy mildew pathogen. With the developed lateral flow test format its use in the field can then be investigated. Cross-reaction of the test with spores of other pathogenic and non pathogenic species can 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 downy mildew conidia on onions should not react with the conidia of other pathogens common in onion crops. 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 downy mildew conidia present in samples. The second year of the work details the development of the “in field” test for onion downy mildew conidia and the comparison of air sampling systems for detecting onion downy mildew in crops of onions.

3. PRODUCTION OF ANTISERA

3.1 Production of monoclonal antisera to *Peronospora destructor*

3.1.1 Introduction

Leaf material infected by the downy mildew pathogen (*Peronospora destructor*) is required if antibodies are to be raised against downy mildew spores. Onion downy mildew is an obligate parasite which means that it cannot be cultured on agar and will only grow and develop on onion plants. For this reason the downy mildew pathogen must be isolated and routinely maintained on susceptible plants for there to be a ready supply of downy mildew conidia. The pathogen infects more mature tissue and does not infect and develop readily on young onion tissues.

3.1.2 Materials and Methods

3.1.2.1 Production of *P. destructor* immunogen for antibody production

Infected leaf material was obtained from an infected crop of salad onions Hampton Lucy, Bomfords) which had not been sprayed with fungicide. The infected material was maintained in the glasshouse and misted (100 % rh at 16 C for 24 H) to induce fresh sporulation. Infection was maintained in the glasshouse over the winter months. Prior to inoculation with *Peronospora destructor* leaf surface wax material of ten onion sets (*Allium cepa* c.v. White Lisbon) was removed by gentle agitation with sheep's wool. After which, twenty five 20µl droplets of *Peronospora destructor* ($1x10^4$ conidia ml^{-1} H_2O) was applied to each sheep's wool treated leaf. To induce infection inoculated plants were incubated in high humidity for 2 days after which plants were removed and placed in a temperature controlled glasshouse (18ºC) for a further 2 weeks. To induce *P .destructor* sporulation the inoculated plants were returned to a high humidity environment for a period of 24hrs. Leaves were then examined for the production of freshly sporulating material of *Peronospora destructor*.

3.1.2.2 Collection of *P. destructor* spores from leaf surfaces

Spores of onion downy mildew were collected from leaves using a hand held Burkard cyclone sampler (Burkard Manufacturing Co., Rickmansworth, Herts, UK. The collected spore material was suspended in 10 ml of chilled Phosphate buffered saline, pH 7.4 (PBS) and held at 0- 4ºC while a conidial count was taken using a haemocytometer (the suspension was adjusted to $1x10^5$ *P. destructor* ml^{-1} using PBS). To remove soluble plant related material the spore suspension was filtered through a 10µm spectra mesh membrane. The retained spores of *P. destructor* were then re-suspended in 3 ml of chilled sterile distilled water. Using a Fast

Prep instrument (QBiogene, UK) *P. destructor* spores were disrupted and the soluble phase of the sample was collected using a microfuge (MSE Microcentaur), at 13 r.p.m for 5 minutes. The collected soluble fraction of the *P. destructor* spore sample was concentrated at first by freeze-drying (Modulyo 4k, Edwards) and then rehydrating to a final volume of 1ml PBS.

3.1.2.3 Immunization of mice with *P. destructor* resting spores

The collected soluble *P. destructor* material was sent directly to Harlan Sera Lab where two Balb C female mice (coded PAS 8209 and PAS 8210) were immunized (by intraperitoneal injection) each with 100µl of the concentrated soluble *P. destructor* spore preparation mixed with an equal volume of Freunds incomplete adjuvant (immunogen preparation). At day 14 and 28 each mouse received a further immunogen boost and, these were administered as described above using the same antigen preparation techniques. At day 38 a test bleed of whole blood sample was taken from each mouse, shipped to Warwick HRI and screened by plate trapped antigen enzyme-linked immunosorbent assay (PTA-ELISA) and immunofluorescence assay (IF) to determine whether an immune response to *P. destructor* had been produced. Each mouse received a further two immunization boosts, administered at days 42 and 56 after the initial injection. At day 60 a terminal bleed from each mouse was produced and the spleen was removed. The terminal bleed and spleen were shipped directly to Warwick HRI where an antibody fusion (antibody producing B cells are isolated from the spleen and fused *in vitro* with a lymphoid tumour cell (myeloma)) was carried out. Hybridoma tissue culture supernatants were screened both by immunofluorescence (IF) and by PTA ELISA (plate-trapped antigen enzyme-linked immunosorbent assay) 14 days after cell fusion for the presence of antibodies which recognised components associated with conidial spores of *P*. destructor (downy mildew).

3.1.2.4 Monoclonal Antibody Screening

Plate trapped antigen ELISA (PTA ELISA)

Using polysorp microtitre well strips (Nunc, Roskilde, Denmark; Cat. No. 469957), 100µl of *P. destructor* soluble conidial washings in 0.01M Phosphate buffered saline, pH 7.4, were aliquoted in to each of 96 wells. The strips were then incubated overnight in an enclosed chamber at 18ºC. Unbound material was removed and the microtitre wells were washed once with 200 µl PBS. The microtitre wells were blocked with 200 µl of 1 % Casein buffer (1 % (w/v) casein PBS) and incubated at 37 º C for 45 min. Residual blocking buffer was removed and wells were washed four times for one min each with 200 µl PBS, 0.05 % Tween 20 and 0.1% Casein (PBSTw C). After which each well received 100 µl per of fusion hybridoma tissue culture supernatant mixed with PBS, 0.05 % Tween 20 and 0.1% Casein. Following

incubation in a Wellwarm shaker incubater (30° C) for a period of 45 mins as above, wells were washed three times for one min each with 200 µl PBSTincTw. After which a DAKO duet amplification system was used (DAKO Ltd, Angel Drive, Ely, Cambridge,UK; Cat no. K0492) to amplify the signal generated by bound tissue culture supernatant antibodies. Wells were washed as described above and 100µl of 3,3',5,5'- tetramethylbenzidene substrate (Sigma, Poole, Dorset UK; Cat. No. T-3405 and P-4922) was then added to each well. The reaction was stopped by adding 25µl of a 20% 1M H_2S0_4 solution to each well. Absorbance at 450nm was determined with a Biohit BP800 ELISA plate reader (Alpha Laboratories, 40 Parham Drive, Eastleigh, Hampshire, UK).

Immunofluorescence

Twenty µl of a $1x10^3$ spores ml $^{-1}$ *P. destructor* conidial spore suspension was aliquoted to individual multiwell glass slides (Cel-Line/eries Scientific Corp, USA; Cat No. 10-3404). Following air drying any unbound spore material was removed with a PBSTwC wash. Material remaining bound to the multiwell glass slides was incubated with 20µl of hybridoma tissue culture supernatant antibodes (TCS) for a period of 30 minutes at room temperature. A counterstain of Evans blue and Eriochrome black was incorporated within the TCS antibody suspension to quench *P. destructor* spore autoflourescence. The multiwell received a wash as described above and following air drying were incubated with an anti-mouse antibody which had been conjugated to fluoroscein isothanyacyte dye. A counter-stain was again included to ensure quenching of conidial spore autoflourescence. Incubation was carried out at room temperature in darkness to prevent photo-bleaching of the conjugated antibody. The processed microwells received a final wash of PBSTwC and after air drying were mounted and viewed by episcopic fluorescence for the presence of antibody / flourescein tagged resting spores of *P.destructor*.

Hybridoma antibody tissue culture supernatants, which were identified as positive to *P.destructor* conidial spore material using either PTA ELISA and IF were selected. Following a preliminary reactivity screen to other fungal pathogens selectected hybridomas were cloned twice to achieve monoclonal antibody status.

Selection of specific P. destructor monoclonals

To determine specificity of the selected *P. destructor* monoclonal cell lines an expanded range of fungal species were screened by PTA-ELISA and IF. Tests were carried out on *Bremia lactucae*, *Peronospora parasitica*, *Botrytis cinerea*, *B. squamosa*, *Stemphyllium sp*. *Aureobasidium pullulans*, *Phoma betae*, *Ascochyta rabei*, *Fusarium culmorum*, *Penicillium roquefortii*, *Pyrenophora terres* and conidia of *Peronospora destructor*. With the exception of *P. destructor*, *P. parasticata*, *B. lactucae* and *Aschocyta* (all of which were grown on infected plant material) the fungal species used in the reactivity tests had been grown on a synthetic medium covered with a sterile Supor membrane filter prior to inoculation. Fourteen

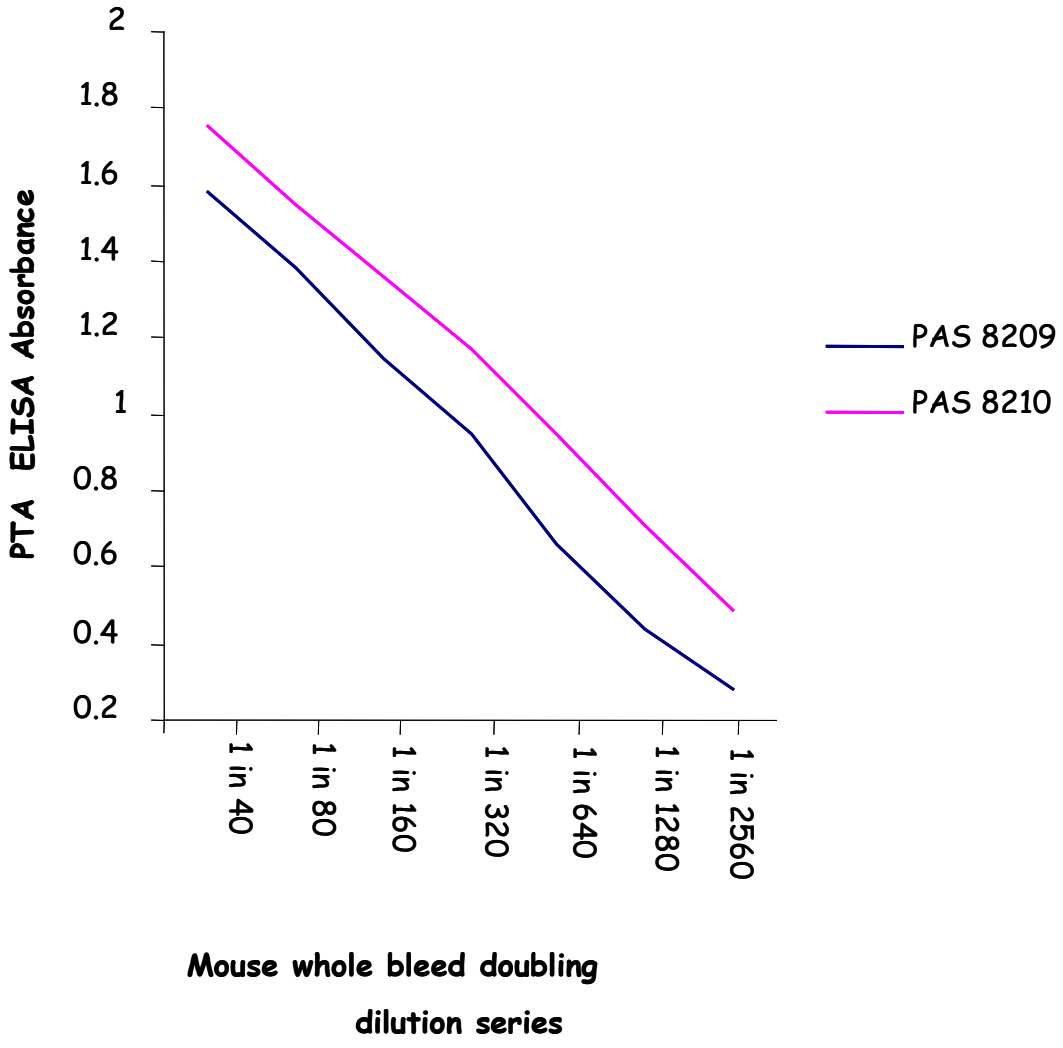
days after inoculation mycelial growth 5ml of phosphate buffered saline (pH 7.5) solution was applied to the culture surface. Using a glass spreader surface washings were taken by gently stroking the culture surface with a glass spreader. The solution was transferred to a chilled container and the collected spore numbers were determined by bright field microscopy (x 200). After which all spore concentrations were adjusted to a final concentration of $1x10^5$ spores ml^{-1} PBS. The spore solutions were individually aliquoted in to each micro wells (100µl per well) of a polysorp microtiter strip. The wells were covered and incubated overnight at 4ºC. After which unbound material was removed and the microtitre wells were washed once with with 200 µl PBS Tinc (PBS mixed with 0.05% Tincture of Merthiolate (1 mg ml^{-1} thimerosal, 1 mg ml^{-1} pararosanoline in ethanol) per well. The microtiter wells were blocked with 200 µl of 1 % casein buffer (1 % (w/v) casein PBS) and incubated at 37 º C for 45 min. Residual blocking buffer was removed and wells were washed four times for one min each with 200 µl PBS Tinc 0.05 % Tween 20 (PBSTincTw). Following this procedure wells received 100 µl per well of each selected monoclonal antibody (diluted in PBST TincTw). Following incubation as above, wells were washed three times for one min each with 200 µl PBSTincTw. After which a Protein-A Horseradish peroxidase conjugate was applied to each well (100µl) and the microtiter stips were incubated for 1 hour at 37ºC. Wells were washed as described above and 100µl of 3,3', 5,5'-tetramethylbenzidene substrate (catalogue no. T-3405 and P-Sigma 4922 Sigma) was added to each well. The reaction was stopped by adding 25µl of a 20% 1M H_2S0_4 solution to each well. Absorbance at 450nm was determined with a Biohit BP800 ELISA plate reader (Alpha Laboratories, 40 Parham Drive, Eastleigh, Hampshire, UK).

3.1.3 Results

3.1.3.1 Immunization

An immune response from each mouse terminal bleed was observed when a concentrated soluble fraction of freshly collected *P. destructor* spore material from field induced sporulating downy mildew on bulb onions was used as an immunogen and tested by PTA-ELISA (Figure 2). The Immunofluorescence test determined that antibodies were present in the initial test bleed of each mouse to components of *P. destructor* conidia (Plate 1).

Figure 2 Response of whole blood tail bleeds of 2 Balb C mice immunised with disrupted conidial spore soluble fraction of *P. destructor* to homologous antigen by PTA ELISA.



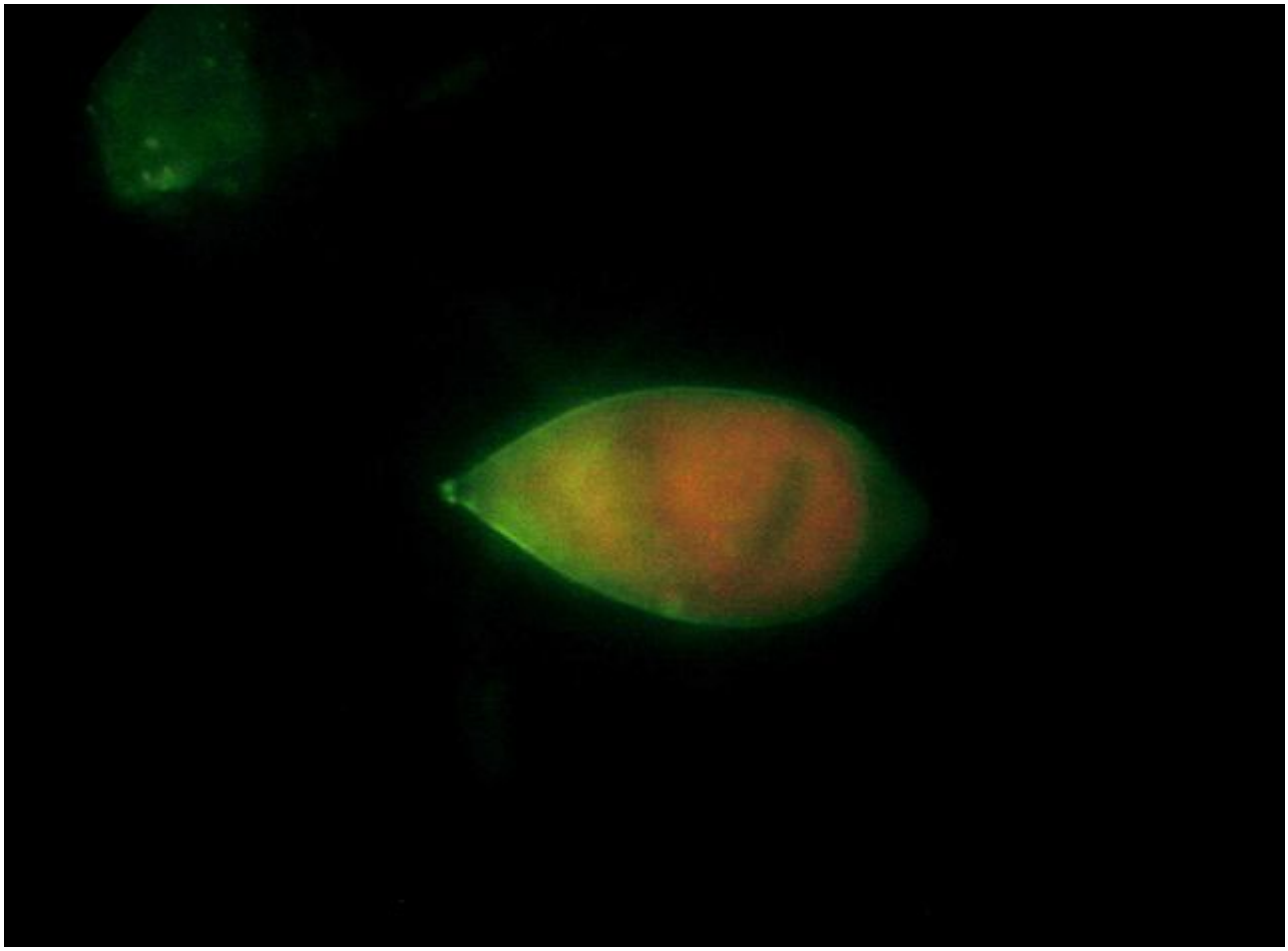


Plate 1. Immunofluorescence of onion downy mildew conidia using fluorescein tagged monoclonal antibodies .

3.1.3.2 Monoclonal Antibody Screening

Plate trapped antigen ELISA (PTA ELISA)

Eleven hybridoma cell lines were identified (using PTA ELISA) as producing antibodies which recognised components associated with conidial material of *P. destructor*. A preliminary screen against a range of plant fungal pathogens identified 3 tissue culture supernatants for expansion to monoclonal. These were selected, cloned to monoclonal antibody status and were coded EMA 240, 242, and 243. Monoclonal antibody cell line EMA 240 was not used in extended reactivity tests as it was observed to react with other downy mildew species when tested by ELISA (data not shown). In extended reactivity tests monoclonal antibodies EMA 242, and 243 demonstrated a high level of specificity to their homologous antigen (*P. destructor* conidia) (Figure 3).

Immunofluorescence

Only eight cell lines were identified as producing antibodies which recognised components directly associated with the conidial spore of *P. destructor* when visualised by

immunofluorescence. Of these, six were excluded following preliminary reactivity studies. Those selected for further testing by IF were EMA 242 ad 243. EMA 240 did not react with material directly associated with *P. destructor* however an area of diffuse speckling was noted surrounding the spore.

Selection of specific** P. destructor **monoclonals

An initial specificity screen employing two additional downy mildew fungal species: *Bremia lactucae* (downy mildew on lettuce) and *Peronospora parasitica* (downy mildew on Brassicas), identified that EMA 240 reacted at a genus level. However EMA's 242 and 243, exhibited specificity, at the species level, when tested by PTA ELISA. In immunofluorescence studies both EMA 242 and 243 reacted with the spore wall of *P. destructor* (Plate 1) and retained a high level of specificity when tested against other fungal species. EMA 240 showed no reactivity to the conidal wall of *P. destructor* but recognised an area of 'light' diffuse speckling surrounding the spore. As a result of the high level of specificity and sensitivity exhibited to *Peronospora destructor* ,monoclonal antibody cell lines EMA 242 and 243 were taken forward to lateral flow developmental studies for detection of onion downy mildew.

Figure 3 Reactivity of monoclonal antibodies EMA 242 and 243 to a range of airborne fungal species as tested by PTA ELISA.

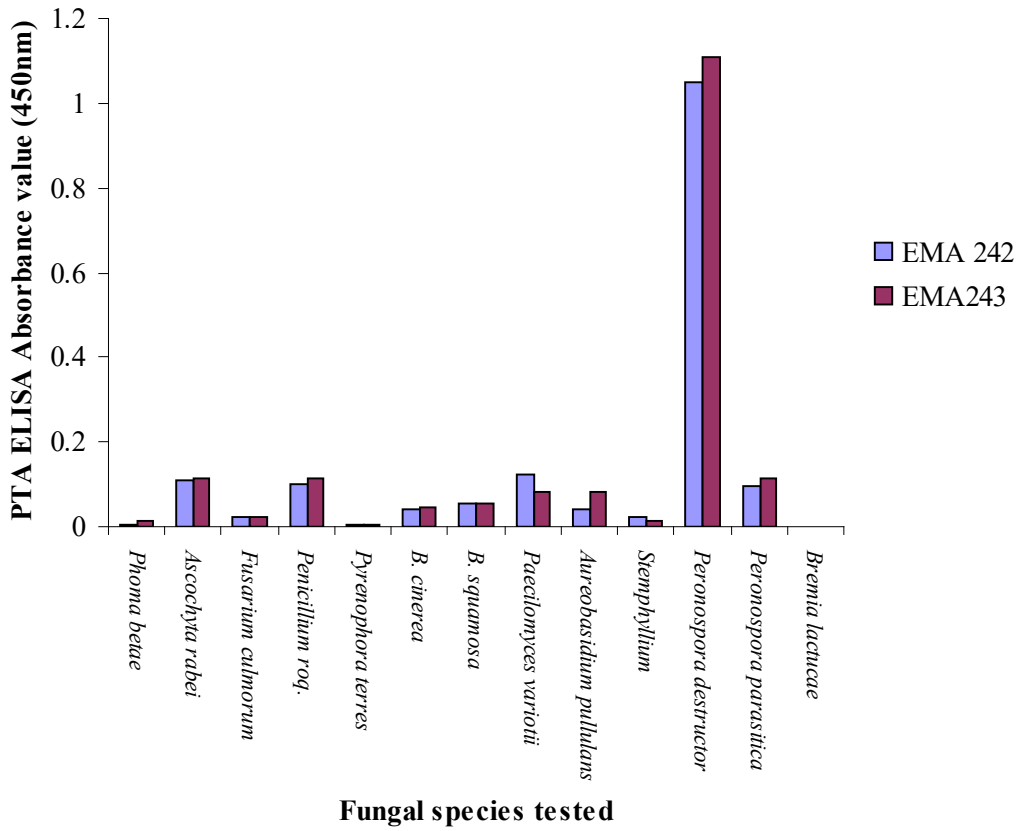


Table 1. Reactivity of monoclonal antibodies EMA 242 and 243 to a range of airborne fungal species as tested by Immunofluorescence.

Fungal species tested	EMA 242		EMA 243	
	Mycelium	**Spores**	**Mycelium**	**Spores**
Phoma	☒	☒	☒	☒
Aschochyta rabei	Not tested	☒	Not tested	☒
Fusarium culmorum	☒	Not Tested	☒	Not Tested
Penicillium roqueforti	Not tested	☒	Not tested	☒
Botrytis cinerea	☒	☒	☒	☒
Botrytis squamosa	☒	☒	☒	☒
Paecilomyces variotii	Not tested	☒	Not tested	☒
Aureobasidium pullulans	Not tested	☒	Not tested	☒
Stemphyllium	☒	☒	☒	☒
Peronospora destructor	Not tested	☑	Not tested	☑
Peronospora parasitica	Not tested	☒	Not tested	☒
Bremia lactucae	Not tested	☒	Not tested	☒

a. No fluorescence observe

☑ Fluorescence observed denoting reactivity

3.1.4 Conclusion

In Year 1 Annual report (FV189a) we reported that when five Balb C mice were immunized with *P. destructor* spores collected from glasshouse cultivated onion sets, a poor immune response was observed to their homologous immunogen (*P. destructor* whole conidia) when tested by PTA-ELISA. This was also confirmed by immunofluorescence. A good immune response however was observed when a concentrated soluble fraction of freshly collected *P. destructor* spore material from field induced sporulating downy mildew on bulb onions was used as an immunogen (Mice 7996, 7997) and tested by PTA-ELISA (FV189A Figure 1). However the positive hybridoma cell lines selected from the post fusion screening of mice numbers 7996 and 7997 lacked the required sensitivity and specificity required for further evaluation and inclusion within rapid assay detection formats for *P. destructor* (downy mildew). Nevertheless the immunizations using disrupted *P. destructor* soluble spore antigens have proved optimal in producing two monoclonal antibody cell lines with the required specificity and sensitivity for further evaluation within rapid assay detection formats for trapped

airborne inoculum of *P. destructor*. It is possible that the disruption process of the *P.destructor* spore prior to immunization may have facililated the release of additional antigenic material which induced a more specific and sustained immune response in the immunized mice. The increased intensity of the immunization schedule may also have proved beneficial in this matter.

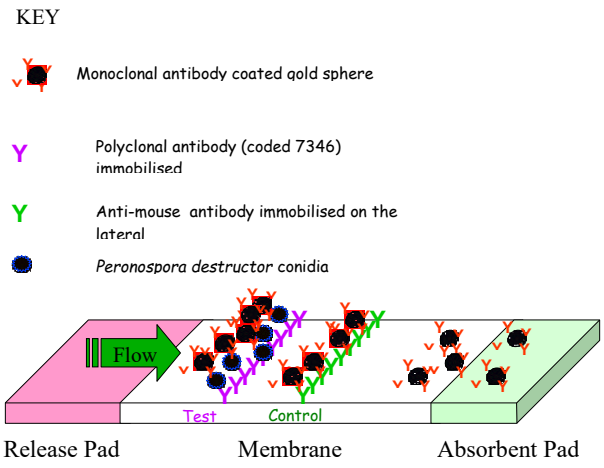
4. DEVELOPMENT AND OPTIMISATION OF LATERAL FLOW FOR DETECTION OF *PERONOSPORA DESTRUCTOR* (DOWNY MILDEW ON ONIONS)

Lateral flow devices (LFD) require the development of a test format which can give rise to adequate visualisation of the test line to establish target antigen prescence (downy mildew conidia). Several elements within the lateral flow must also be optimised once a working format has been ascertained. These include the membrane, the concentration and type of antibody and the conjugation with a carrier molecule (gold/latex). This section of the report details the development of a working LFD format which can detect onion downy mildew conidia and its optimisation.

4.1 Development and optimisation of lateral flow device for detection of conidial of *P. destructor*

4.1.1 Double Antibody Sandwich (DAS) test format (non competitive lfd)

Two sources of antibody are required within the double antibody sandwich test format, which can comprise either a pair of the same or two different antibody types. One antibody type is bound to the nitrocellulose membrane (test line) the other is labelled with a visual marker (in all tests listed below gold spheres are used) and held within a release pad (Figure 4). To ascertain successful test operation a control line was prepared on the same membrane as the test line to capture additional latex particles



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

Figure 4. Schematic drawing of a Double Antibody Sandwich (DAS) lateral flow device *(Positive result shown)*

4.1.1.1 DAS lateral flow test procedure

When a few drops of the test sample containing the target spore are placed on the lateral flow release pad the gold sphere conjugated monoclonal antibodies are released in to solution and flow with the sample laterally towards the antibody test line. If the target antigen (conidia of *Peronospora destructor*) are present within the sample the specific antibody conjugated gold spheres bind to the target antigen (*P. destructor*) and, as this complex flows over the test line, it is captured by the immobilised test line antibody. This reaction is visualised by the formation of a red line (Plate 2). If no target antigen (*ie*. no *P. destructor* conidia) is present within the sample the antibody conjugated gold spheres are not captured on the test line and no line is visible. In either situation, excess antibody conjugated latex spheres will become immobilised at the control line and a clearly visible red line will form showing that the test has operated satisfactorily.

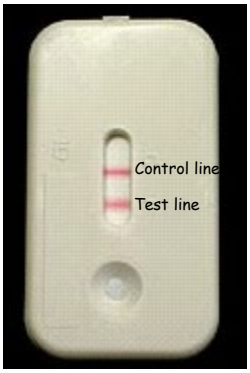


Plate 2. DAS lateral flow test exhibiting a positive result with visualisation of test and control lines.

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

To determine the applicability of the DAS lateral flow format, for the detection of conidial material of *Peronospora destructor* selected antibody combinations were examined (Table 2).

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

Gold sphere conjugated antibody	Test line antibody
PAS 7346	EMA 242
PAS 7346	EMA 243
PAS 7346	PAS 7346
EMA 242	EMA 242
EMA 242	EMA 243
EMA 242	PAS 7346
EMA 243	EMA 242
EMA 243	EMA 243
EMA 243	PAS 7346

4.1.1.3 Membranes and buffers used

All tests were carried out using lateral flows comprised of a Millipore 135 HiFlow™ cellulose ester membrane direct cast on to 2ml Mylar backing (Cat No. SHF2400225, Millipore Corp, USA.), absorbent pad (Cat No. GBOO4, Schleicer and Schuell, Germany) and a sample pad (Cat No. T5NM, Millipore Corp., USA). The specified test line antibodies (Table 2) were applied directly to the membrane in Phosphate buffered saline solution, pH 7.5 (PBS) employing a flat bed air jet dispenser (Biodot Ltd, The Kingley Centre, West Sussex, UK). Afterwhich the membranes were air-dried at 35°C for a period of 4 hours. The antibody labelled lateral flow membranes were cut in to 4 mm strips and labelled. The gold conjugated antibody complex and the test antigen (*P. destructor* spores $1x10^5$ spores / ml) were mixed and then applied to the sample pad. The lateral flow device strips were viewed 5 minutes post sample application.

4.1.1.4 Results

For all antibody combinations results were negative i.e. no test line development was observed. However control line development was noted to confirm that the test had run with antibody / gold flow (Plate 3) .

Plate 3 DAS lateral flow format showing a negative result.

4.1.1.5 Conclusions

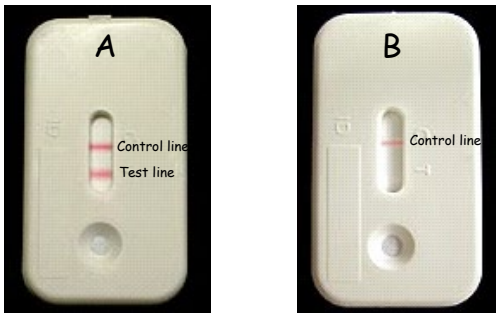
The double antibody sandwich lateral flow test, in the format used, was unsuccessful for the detection of *P. destructor* whole spores. From earlier immunoflourescence studies it was determined that both the monoclonal and polyclonal antibodes raised bound to components associated with the conidial spore wall. The *P. destructor* sample contained whole spores. As a result of their size this spore type would be unable to travel across the lateral flow membrane and would remain in the sample pad only allowing flow of soluble *P. destructor* components.. As a result it is probable that a large percentage of the gold conjugated specific antibodies bound to the whole spores of *P. destructor* would be retained within the sample pad. The formation of a control line would suggest however that a number of gold conjugated specific antibodies travelled across the lateral flow membrane. The dynamics of the initial sample mix will likely result in a number of specific antibodies not coming in to contact with the homologous antigen (*P. destructor*). These antibodies would be free to travel across the membrane and bind, at the control line, to the anti-species antibody. In addition, ELISA results indicate that the target epitope (site antibody binds to) present on the *P. destructor* conidial wall is repeated, although at a reduced level, in the soluble fraction of a spore suspension. As such it is possible that an antibody bound antigen complex was able to travel the membrane but capture of this product did not occur and / or was not at a level that provided a visible test line.

The specific retention of an antibody / antigen complex by a capture antibody is multifasceted and can prove problematical. Both the monoclonal antibodies used were isotyped as being of the IgM subclass of which these are generally considered to be of low affinity to their target antigen. Consequently antibody / antigen capture would prove problematical with continuous and rapid travel occurence. Polyclonal antibodies, as a result of hydrophobic

bonding, can prove problematical in a DAS format and for use as test line antibodies, tend to be avoided.

4.1.2 Competitive assay test format and procedure

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



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

Plate 4. Competitve lateral flow assay showing a positive sample result (A) and (B) a negative sample result.

Test operation is as described for the DAS lateral flow format. The sample extract is applied to the release pad, facilitating the 'immobilised' specific antibody bound gold spheres to flow across into the nitrocellulose membrane. As the sample extract and the specific antibody bound latex gold spheres flow laterally though the membrane there is potential for binding between the target antigen (*P. destructor*) and specific gold labelled antibodies. The formation of a target antigen / gold conjugated antibody complex this time inhibits capture at the test line. With sufficient target antigen present (*P. destructor*) complete inhibition occurs and no test line is observed. In a negative sample (target *P. destructor* antigen absent) the antibody conjugated gold spheres remain unbound and are captured at the test line to produce a visible line of gold deposit. In either situation, excess antibody conjugated gold spheres will become immobilised at the control line. The control line is composed of an anti-species antibody which

will react with the gold/antibody complex flowing from the sample pad. A clear visible red control band will form showing that the test has been completed satisfactorily.

4.1.2.1 Assessment of competitive lateral flow assay format for the detection of *Peronospora destructor*

Preliminary tests were carried out using lateral flows comprised of a Millipore 135 HiFlow™ cellulose ester membrane direct cast on to 2ml Mylar backing (Cat No. SHF2400225, Millipore Corp, USA.), an absorbent pad (Cat No. GBOO4, Schleicer and Schuell, Germany) and a sample pad (Cat No. T5NM, Millipore Corp., USA). Following lateral flow construction (Fig. 5) control lines of an anti-mouse serum were sprayed directly on to the membrane surface using a flat bed air jet dispenser (Biodot Ltd, The Kingley Centre, West Sussex, UK). A collected soluble fraction of a *P. destructor* spore sample, prepared as described above (Section 3.1.2.1), was adjusted to a protein concentration of 500µg ml^{-1} , 250 µg ml^{-1} and 125µg ml^{-1} in PBS and applied as a test line again employing a flat bed air jet dispenser. After which embranes were air dried at 35ºC for a period of 4 hours. The test and control line labelled lateral flows were cut in to 4 mm strips and each strip housed within a plastic case (Schleicer and Schuell, Germany). The test antigen (60µl spore suspension (3 x 10^{3} *P. destructor* spores)) was then mixed with MAb EMA 242 gold conjugated spheres. Following application to the sample pad, the competitive lateral flow devices (*c lfd*) were viewed 5 minutes post sample application. For each test a spore free suspension mixed with MAb EMA 242 gold conjugated spheres was employed to act as a negative control. Variable antibody dilutions of EMA 242 were used in these tests (Table 3).

Table 3

Antibody type and dilution factor	**Peronospora destructor protein concentration (µg/ml) at test line**		
EMA 242	**500**	**250**	**125**
1 in 160	**500**	**250**	**125**
1 in 320	**500**	**250**	**125**
1 in 640	**500**	**250**	**125**

4.1.2.2 Results

At a test line application was at a 500ug ml^{-1} spore deposition, test line inhibition (ie no test line development) was only observed when a *P. destructor* spore sample was mixed with gold conjugated EMA 242 at an antibody dilution greater than 1 : 160. For all negative control samples (ie no *P. destructor* spore present) control and test line development was observed for each *c lfd*. However at an antibody dilution of 1: 640 line development was barely visible.

At a test line application of 250ug ml^{-1} spore deposition , strong test and control line development was observed at antibody (Ab) dilutions of 1 : 160 and 1 : 320 when a spore free suspension was applied *ie* test predicts no spores present in sample. Testing a positive sample of *P. destructor* and, using EMA 242 at a dilution of 1 : 160 gave rise to a barely visible test line and, strong control line. At an antibody dilution of 1 : 320 test line depletion was complete *ie* conclusive prediction of spore presence in sample. Employing an Ab dilution of 1 : 640 gave no test or control lines for either spore positive or spore negative samples.

At a test line of 125 ug ml^{-1} spore deposition and, testing a *P. destructor* spore free suspension, test and control line development was observed when an antibody dilution of 1 : 160 was used. Using a positive *P .destructor* spore sample gave rise to a clear control line but no test line development (*ie* test predicts *P. destructor* in sample). At all other antibody dilutions control lines were barely visible and no test line development was noted for any of the samples tested.

4.1.2.3 Conclusions

Employing a competitive lateral flow format (*c lfd*) enabled the rapid detection of *P. destructor* spores in the sample tested. This was in contrast to the double antibody sandwich lfd format where test development proved unsuccessful. The competitive assay is used most often when testing for small molecules with single antigenic determinants, which can-not bind two antibodies simulateneously. As a result many fungal assays are based on antigen-trapped assays where antibodies are used to label antigen rather than attach it to a solid phase.

In the development of a successful *c lfd* the antigen concentration at the test line and activity of the specific Ab used is critical to optimal development of the test. The type and capillary flow rate of the membrane is also of importance. For this current test results indicate that employing a Millipore 135 HiFlow™ cellulose ester membrane direct cast on to 2ml Mylar backing and, striping test *P. destructor* antigen between the range of 125 to 250µg protein ml^{-1} is optimal for the detection of *P. destructor* spores when a gold conjugated antibody dilution range of between 1 in 160 to 1 in 320 is used.

4.1.2.4 Detection threshold of *clfd* employing two membrane types for *P. destructor* spores

Studies were carried out using a competitive LFD (*c lfd*) format for the detection of known concentrations of *P. destructor* spores. However two different membrane types were examined (Table 4a and b) a Millipore 135 HiFlow™ cellulose ester membrane direct cast on to 2ml Mylar backing and a Milipore 240 HiFlow™ cellulose ester membrane. The LFD devices were prepared as described above and a test line of 250µg ml^{-1} *P. destructor* soluble antigen in PBS was applied. The membranes were air dried at 37ºC and cut in to 4 mm strips and each strip housed within a plastic case as previously described.

Test procedure

A known spore concentration of *P. destructor* spores (60µl) was mixed with EMA 242 conjugated gold spheres (5µl) to produce a final antibody dilution of either 1 : 150 or 1 : 400, 1 : 600 (Table 4a and 4b). The mixture was applied to the *c lfd* sample pad of each *c lfd* and results viewed 5 minutes post sample application. For each membrane type a 'spore free suspension' was mixed with MAb EMA 242 gold conjugated spheres to act as a negative control.

4.1.2.5 Results

Using a Millipore HiFlow™ 135 Membrane and, the detection antibody (EMA 242 conjugated to gold spheres) at a dilution of 1 : 150, test line formation was observed for all spore samples tested. This denoted that the detection sensitivity of the test was poor and unable to detect spore numbers when present at a concentration of 2000. However by diluting the activity of the detector antibody to 1 : 400 test sensitivity was improved and only when spore numbers fell to 250 could they no longer be detected by the *c lfd* (Plate 5). At a detector antibody dilution of 1 in 600 the test became void with no test line formation for any of the samples tested.

Using a Millipore HiFlow™ 240 membrane *c lfd* and, the detector antibody (EMA 242 conjugated to gold spheres) at a dilution of 1 in 150, test line formation was again observed for all spore samples tested (ie test does not detect *P. destructor* spore presence). As previously noted, by diluting the activity of the detector antibody to 1 : 400 sensitivity of the test was improved (Table). However this was not to the same level as the 135 membrane. For this test a detector antibody dilution of 1 : 600 was required to achieve the same level of detection test sensitivity as that of the 135 membrane (Plate 5).

Table 4a Millipore HiFlow™ Membrane 135

No *P. destructor* spores in sample

EMA 242 Ab dilution	0	62	125	250	500	1000	2000
1 in 150	✓	✓	✓	✓	✓	✓	✓
1 in 400	✓	✓	✓	✓	×	×	×
1 in 600	×	×	×	×	×	×	×

Table 4b Millipore HiFlow™ Membrane 240

No *P. destructor* spores in sample

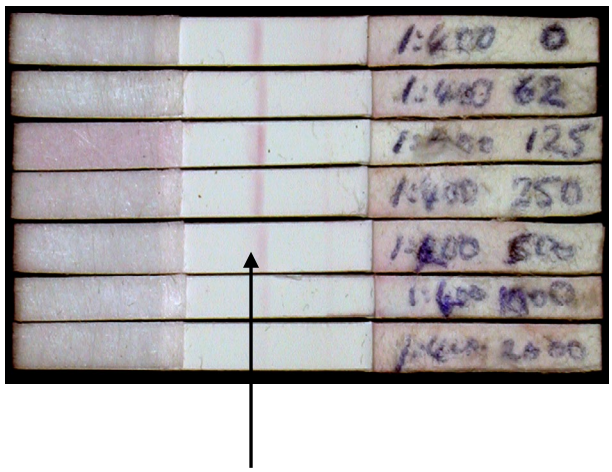
EMA 242 Ab dilution	0	62	125	250	500	1000	2000
1 in 150	✓	✓	✓	✓	✓	✓	✓
1 in 400	✓	✓	✓	✓	✓	×	×
1 in 600	✓	✓	✓	✓	×	×	×

✓ Clear test line (*P. destructor* not detected in sample)

× No test line development (*P. destructor* presence detected by *c lfd*)

×/✓ Weak test line development

(a)



Limit of detection

(b)

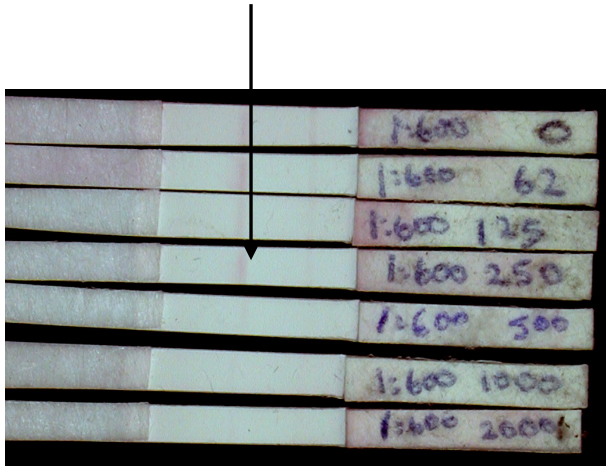


Plate 5 Development of a competitive lateral flow for onion downy mildew conidia (a) competitive lateral flow test employing detection antibody concentration of 1: 400 (b) competitive lateral flow test employing detection antibody concentration of 1 : 600

4.1.2.6 Conclusions

In the tests carried out above two membrane types were examined for their use in a *c lfd* test for onion downy mildew conidia. The HiFlow 240 membrane has a nominal capillary flow rate of 240 s / 4cm travel, whilst the 135 HiFlow membrane is at a flow rate of 135 seconds /4cm travel. The 135 membrane will result in a test which completes in a faster run time than one using a 240 membrane. A faster run time is considered commercially desirable and to be less prone to background streaking. Nevertheless test line reaction kinetics are key to development

of a successful lateral flow device. A membrane with a slower travel time across the test line can prove more sensitive. The reaction rate at the test line capture point decreases with the square of the increase in flow rate. As a result test sensitivity decreases with the square of the increase in flow rate. However in a competitive LFD assay it is the omission of a test line that denotes a positive result and conversely, a faster travel time across the test antigen capture line can enhance test sensitivity. Ultimately however it is a critical balance of the activity level of the antibody detection molecule and, assay time across the test line that will result in a meaningful test.

For both membrane types an antibody dilution of 1 : 150 resulted in an excess of detection antibody which, even at the highest spore numbers of 2000 per sample tested, retained activity on the test line capture antigen. By decreasing the activity of the detection antibody, detection sensitivity for onion downy mildew conidia increased *i.e.* omission of the test line. However this was limited to a dilution factor of 1 : 400 for the 135 HiFlow membrane where, at a dilution of 1 : 600 no test line formation was visible for any of the samples tested. This included a test sample with spores omitted. The flow rate of the 135 HiFlow membrane did not provide an assay time optimal for retention of low level free 'unbound' detector antibodies. In contrast the slower run time of the 240 HiFlow , enabled detection of the onion downy mildew conidia when at or above 500 spores per sample tested.

The LFD format using either a 135 or 240 HiFlow membrane provides a *c lfd* which currently has a detection threshold of approximately several hundred onion downy mildew conidia per sample. The intensity of the test line is similar for both providing the detection antibody activity is selected appropriately. Commercially the cost of the test will need to be examined ie reagent costs (detection antibody) and required run time by the end user. Further studies will be needed to reduce the detection threshold of the test.

5.0 EVALUATION OF MICROTITER WELL COATINGS FOR OPTIMISATION OF ONION DOWNY MILDEW TRAPPING

5.1 Microtiter immunospore trap (MTIST)

The spore trapping equipment used in this study (MTIST) is manufactured by Burkard Manufacturing Company (Rickmansworth, Herts, UK) (Plate 6). The MTIST spore trap uses a suction system to directly trap air-particulates by impaction in to microtiter wells. Air is drawn through the device and particulates in the airstream are impacted on to the base of each collection well of 4 microtiter strips (Figure 5). The collected impacted target particulates may, if appropriate antibodies are available, be immunoquantified by PTA ELISA.

Plate 6 Outdoor Microtiter Immunospore Trap (MTIST)

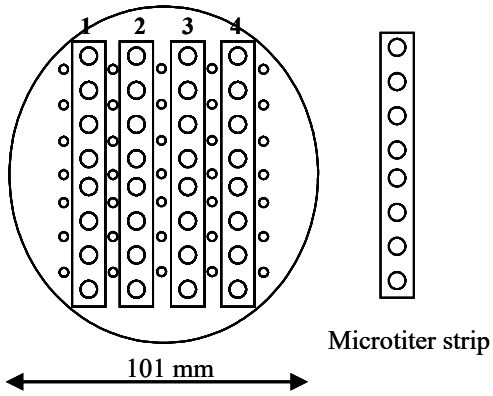


Figure 5 Schematic drawing of MTIST base plate and microtiter strips.

5.1.1 Preparation of MTIST microtiter strips for field exposure

Microtitre well coating solutions were examined for use in the collection and immunoquantification of MTIST trapped *P . destructor* inoculum Four types of well coating preparations were used: Poly-L-lysine (Sigma P-1524) diluted in distilled water to 0.1mg ml^{-1}, a 5:1 mixture of petroleum jelly (Vaseline) and paraffin wax which had been melted in a water bath and thoroughly mixed before being diluted with hexane (1 in 16), silicone which following melting was mixed with hexane as previously described and, a well coating of distilled water. One hundred µl of a single coating solution was applied to each well of 60 microtitre strips (catologue no. 469957, Nunc Immunodiagnostics, Life Technologies Ltd, Paisley, Scotland). This process was repeated for the remaining three coating preparations. After treatment the coated microtitre well strips were secured within ELISA multiframes (Catalogue No. 9503060, Life Technologies Ltd, Paisley, Scotland) and incubated at 20 ºC for 1 hour, after which any unbound material was removed by inverting the microtitre strips and tapping them down on to absorbent towelling. An inverted binocular microscope (Nikon model TMS) was used to check that the well coatings had been applied evenly. Prior to field exposure the microstrips were stored at 4ºC in a sealed container.

5.1.2 Monitoring downy mildew conidia in air samples in a over-wintered crop of bulb onions

The MTIST spore trap was placed in an over-wintered crop of bulb onions. Held within the base plate of the machine were four coated microtitre strips. Each strip contained a different well coating. The MTIST spore trap was operated for 12 H periods form 06:00 H to 18:00 daily. The coated microtitre strips were changed daily and, after 18:00 H. The collected microtitre strips were sealed after exposure and stored at -20ºC.

5.1.3 Enumeration of trapped spores in air samplers

Of each microtitre strip, wells 1,3,5 and 7 were viewed by microscopic examination (x 200) and, the total number of *P. destructor* conidia deposited on the base of each microtitre well was counted, by using a Nikon model TMS inverted binocular microscope (x 200). After which the wells were processed by PTA ELISA

5.1.4 Monitoring *P. destructor* presence by PTA ELISA

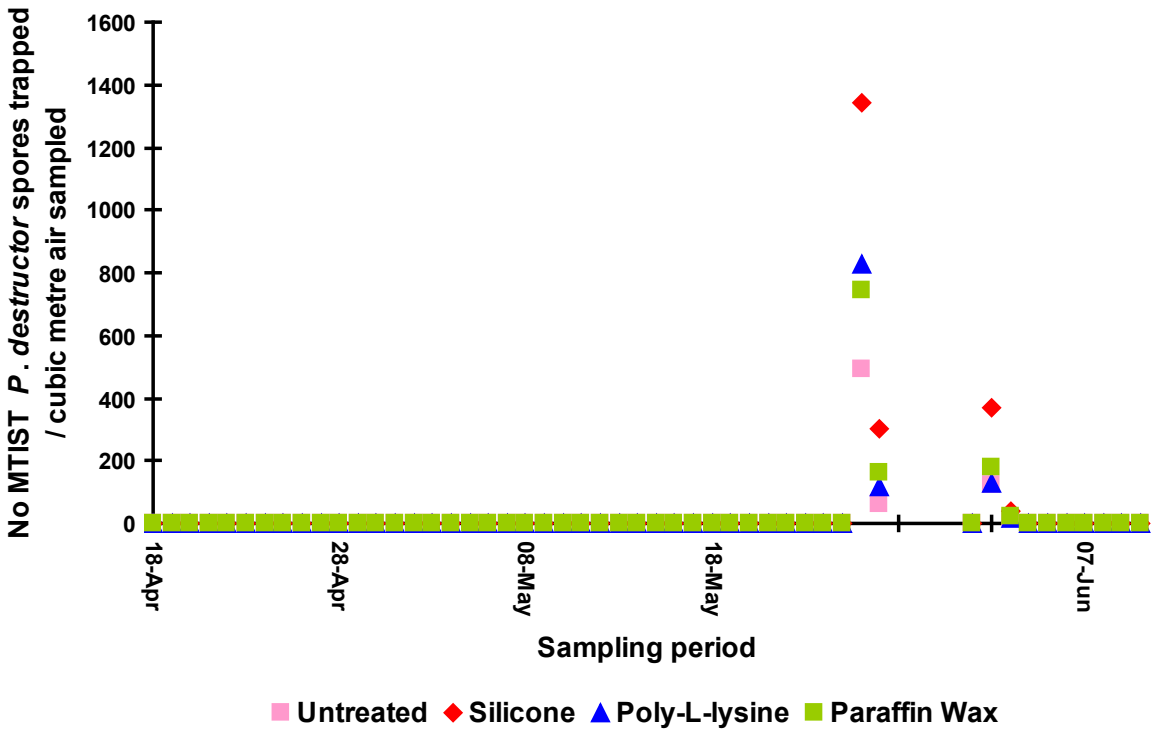
To each microtiter well 200 µl of 1 % casein buffer (1% {wt/vol} casein in PBS) was aliquoted, sealed and incubated at 37°C for a period of 30 mins. After which the residual blocking buffer was removed, and the wells were washed four times (1 min each) with 200 µl of PBS-Tinc TwC (phosphate buffered saline (pH 7.), 0.05% tincture of Merthiolate (1mg of thimerosal / ml and 1 mg of pararosanoline / ml ethanol), 0.05 % Tween 20, 0.1% casein buffer). After washing 100 µl of PBS-Tinc TwC and monoclonal antibodies EMA 242 and 243, both of which recognised conidial material of *Peronospora destructor* were aliquoted to four microtiter wells of each strip. To the remaining four wells PBS alone was added (100µl). Following incubation at 37°C for a period of 1H the wells were washed four times (1 min each) with 200 µl PBS-Tinc TwC. After which a DAKO duet amplification system was used (catalogue no. K0492; DAKO Ltd, Angel Drive, Ely, Cambridge,UK) to amplify the signal generated by the bound monoclonal antibodies. All wells received the DAKO duet amplification system and, the protocol was carried out according to the manufacturers instructions. After which the wells were washed as described above and to each well 100µl of 3,3',5,5'- tetramethylbenzidene substrate (catalogue no. T-3405 and P- 4922 Sigma) was then added. The reaction was stopped by adding 25µl of a 20% 1M H_2SO_4 solution to each well. Absorbance at 450 nm was determined with a Biohit BP800 ELISA plate reader (Alpha Laboratories, 40 Parham Drive, Eastleigh, Hampshire, UK).

5.2 Results

5.2.1 Enumeration of trapped spores in air samplers

Using a well coating solution, other than water, improved the collection efficiency of the MTIST spore trap for *P. destructor* conidia (Figure 6). In all instances the use of silicone proved superior, collecting 60 % more spores than the untreated and aprox. 50% more than both the Poly-L-Lysine or Paraffin wax coated wells.

Figure 6 Number of onion downy mildew conidia trapped per cubic metre of air in the field using the MTIST trap with varying micotitre well coatings.



5.2.2 Monitoring *P. destructor* presence by PTA ELISA

In the previous study silicone coated wells increased the total number *of P. destructor* spores trapped. However in the PTA ELISA assay there was little difference in absorbance signal strength between those wells treated with silicone and those with a paraffin wax derivative. However the relationship between the number of trapped *P. destructor* spores and the corresponding PTA ELISA figures generated proved optimal employing a paraffin wax well coating (Figure 7). Coating wells with distilled water derived a similar relationship between trapped P. *destructor* spore numbers and the PTA ELISA absorbance values as observed using silicone (Figures 8,9) however greater differentiation was observed between the upper and lower detection limit of the test employing the silicone coated wells (Figure 10). Poly-L-Lysine proved inhibitory to the PTA ELISA test and had a correlation with the PTA ELISA of $r^2 = 0.0465$.

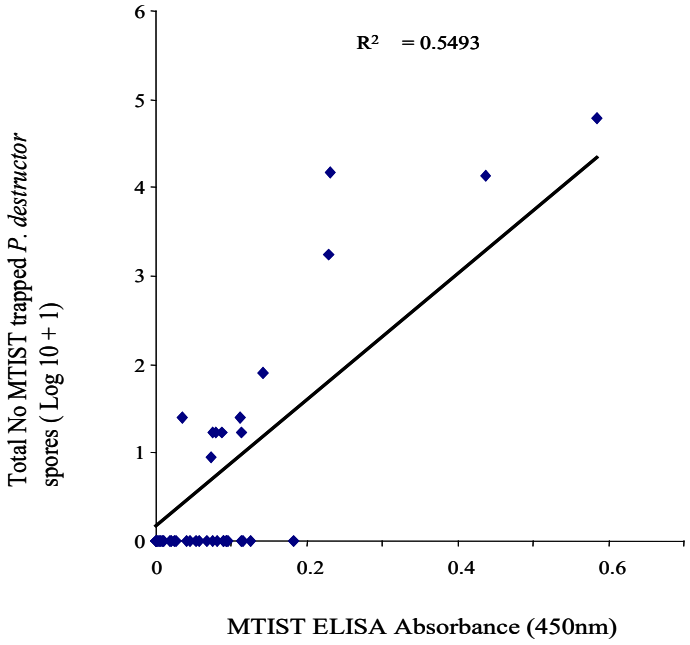


Figure 7 Relationship of MTIST trapped spores per m^3 of air sampled and the absorbance value generated post PTA ELISA using paraffin coated microtiter wells

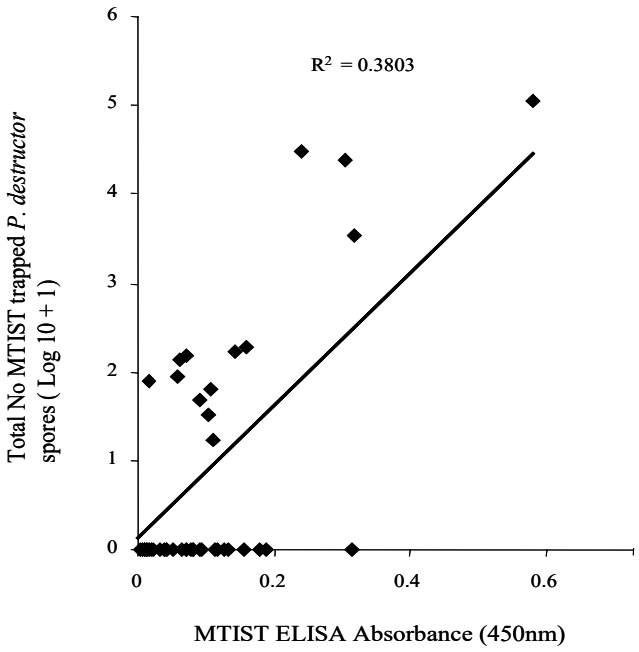


Figure 8 Relationship of MTIST trapped spores per m^3 of air sampled and the absorbance value generated post PTA ELISA using silicone coated microtiter wells

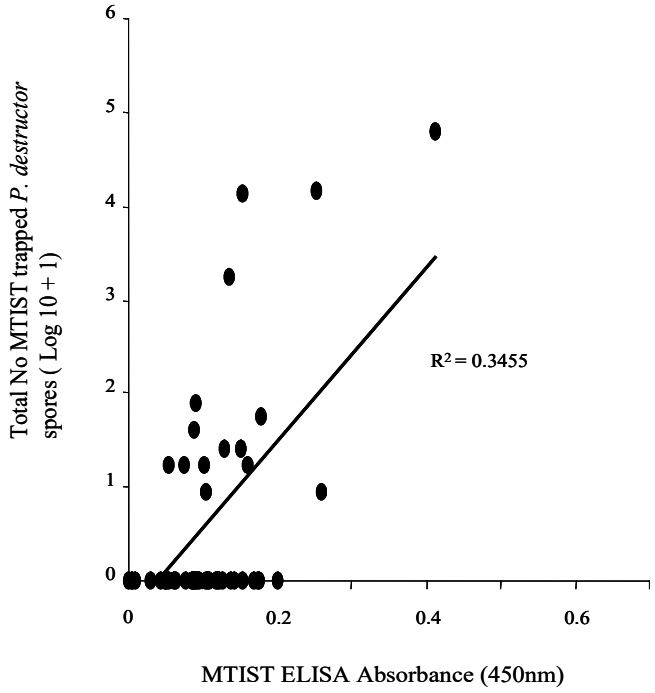


Figure 9 Relationship of MTIST trapped spores per m^3 of air sampled and the absorbance value generated post PTA ELISA using distilled water to coat microtiter wells

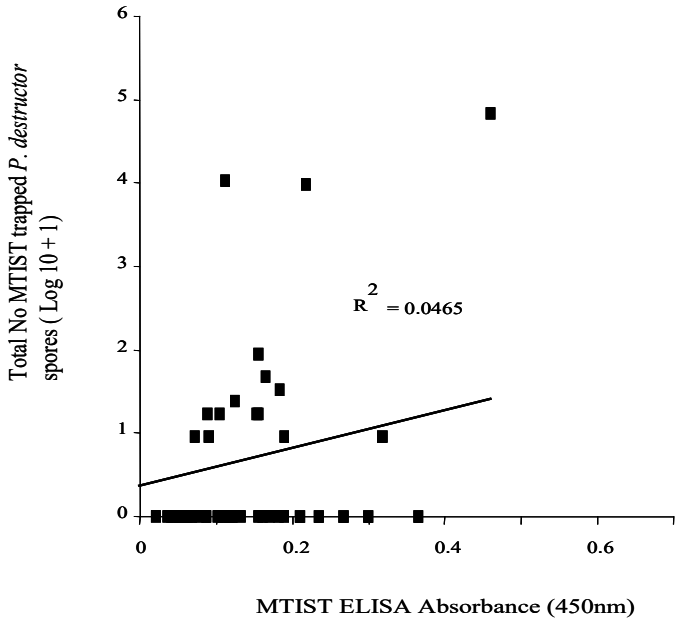


Figure 10 Relationship of MTIST trapped spores per m^3 of air sampled and the absorbance value generated post PTA ELISA using poly-L-lysine coated microtitre wells

5.3 Conclusions

The MTIST trap could be used to determine the effect of variation in coating of the trapping vessel on the collection and retention of onion downy mildew conidia. Due to the use of 4 microtitre strips with the trap 4 separate well coatings could be tested simultaneously. There is no variation in number of trapped fungal spores between strips (Kennedy *et al*., 2000). Tests were conducted under field conditions and over an extended trapping period. The results of these trials consistently showed that coatings comprising of paraffin wax or silicone gave consistently higher collection /retention of downy mildew conidia in comparison to an untreated control strip of wells and another coated with poly-lysine. It was important in these tests that the well coating used did not affect the ELISA. Correlations between PTA absorbance values and numbers of onion downy mildew conidia in microtitre well stripes varied significantly between paraffin wax ($r^2 = 0.5493$) and poly-l-lysine wells ($r^2 = 0.0465$) or untreated wells ($r^2 = 0.3455$). The results suggest that well coatings can be used to significantly enhance the number of downy mildew conidia trapped with air samplers. Further work would be required to determine if these well coatings could be used to increase the collection and retention of onion downy mildew conidia within cyclone samplers.

6.0 COMPARISON OF TRAPPING FORMATS AND WEATHER BASED DISEASE FORECASTS FOR ONION DOWNY MILDEW IN THE FIELD

6.1 Monitoring downy mildew conidia in air samples in a over-wintered crop of bulb onions

The available antibodies were evaluated for their ability to detect downy mildew in the air in an over-wintered crop of bulb onions at Warwick HRI, Wellesbourne. Three trapping formats were compared in this trial with disease development in the plot and disease development on trap plants exposed within the plot for 24 H periods .

6.1.1 Materials and Methods

A field experiment was conducted to compare different types of trapping systems for their accuracy in trapping onion downy mildew conidia.

6.1.1.1 Infected bulb onion crop production

A plot of bulb onion (cv.Armstrong) measuring 15 m x 15 m. were direct drilled in beds The trial was drilled in each year at a density of approximately 55 seeds/m^2. Plots measured 9 x 10 m with 1.8m wide beds. The spacings used between rows in each bed was 30, 35, 30 cm with 4 beds. The crop was produced during 2004 at Warwick HRI Wellesbourne. In June 2004 potted salad onion plants (two pots) infected with downy mildew was introduced into the centre of the plot. Downy mildew lesions were observed on plants close to the points of inoculation. Downy mildew infection of the plot was not extensive due to hot and dry conditions which occurred during July 2004. The crop was over-wintered to produce a seeding crop in spring 2005.

6.1.1.2 Air samplers used in the trial

Three types of air sampler were used in a field trial to compare different traps types in their accuracy in trapping onion downy mildew conidia. An MTIST sampler and 7 day cyclone sampler were operated for 12 H periods form 06:00 H to 18:00 daily. These were compared to a Burkard 24 H volumetric trap which ran continuously over each 24 H period. The MIST trap containing microtiter strips (catologue no. 469957, Nunc Immunodiagnostics, Life Technologies Ltd, Paisley, Scotland) was changed daily after 18:00H. The 7 day cyclone sampler contained epindorf tubes for which a fresh tube was used automatically for each days sampling. Sample tubes were changed at weekly interval. The Burkard 24 H volumetric trap used a glass slide which had been coated with silicone (Name and manufacturer). An air flow was directed onto the discreet areas of the slide which corresponded to different time intervals. Particulate matter from the airflow was directly impacted on to the glass slide. The glass slide

was replaced daily after 18:00 H. The slide, epindorf tube and microtitre strips were stored at – 20 C after their removal from each air sampler.

MTIST sampler

A detailed description of the MTIST device can be found in Kennedy et al., (2000). In the outdoor version air is drawn through a manifold consisting of a plastic tube with a right angle bend placed over the sampler inlet. The manifold samples air through a 9cm diameter vertical circular inlet and directs it into the sampler body that is held horizontally. For field use the sampler (including manifold) is mounted on a wind vane so that the manifold inlet faces into the wind (Kennedy et al., 2000). Within the sampler the airflow is channelled through 32 trumpet-shaped nozzles each directed at the base of a microtiter well. The sampler contains four microtiter strips (catologue no. 469957, Nunc Immunodiagnostics, Life Technologies Ltd, Paisley, Scotland) each containing eight wells which had been coated with a 5:1 mixture of petroleum jelly (Vaseline) and paraffin wax applied at 1:32. The petroleum jelly and paraffin wax were melted in a water bath and thoroughly mixed before being diluted with hexane until the mixture dissolved (British Aerobiology Federation, 1995). Air flow through the sampler was estimated in still air by measuring the air speed at different points across the inlet manifold using a hot film anemometer (Air velocity transducer model number 8460, TSI Incorporated, St Paul, MN, USA) and integrating over the area of the inlet. In the tests reported here, the volume flow rate through the device was measured at 57-litre min-1.

Burkard 24 H volumetric traps

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

Burkard 7 Day cyclone sampler

The characteristics of the spore cyclone sampler has been described by Ogawa & English (1995). Air is drawn through this sampler using a vacuum pump in the form of a cyclone. The characteristics of the trap namely the height of the cyclone, height of the air inlet, width of the air inlet, air exhaust diameter and the diameter of the cyclone with the length of the exhaust pipe influence the relative efficiency of the trap. These characteristics have been

drawn together and standardised within the Burkard cyclone sampler. The quantitative efficiency of this type of trap is high as the sample volumes can be much higher than other types of trap. This type of trap is suitable for use with "in field" detection kits. Cyclone samples were collected within plots results but could not be reported because of their use in detection of onion downy mildew with "in field" kits (not mass produced until year 3 of the project).

6.1.1.3 Enumeration of trapped spores in air samplers

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

6.1.1.4 Detection of onion downy mildew conidia in air samples using ELISA

Using polysorp microtitre well strips (Nunc, Roskilde, Denmark; Cat. No. 469957), 100µl of *P. destructor* soluble conidial washings in 0.01M Phosphate buffered saline, pH 7.4, were aliquoted in to each of 96 wells. The strips were then incubated overnight in an enclosed chamber at 18ºC. Unbound material was removed and the microtitre wells were washed once with 200 µl PBS. The microtitre wells were blocked with 200 µl of 1 % Casein buffer (1 % (w/v) casein PBS) and incubated at 37 º C for 45 min. Residual blocking buffer was removed and wells were washed four times for one min each with 200 µl PBS, 0.05 % Tween 20 and 0.1% Casein (PBSTw C). After which each well received 100 µl per of fusion hybridoma tissue culture supernatant mixed with PBS, 0.05 % Tween 20 and 0.1% Casein. Following incubation in a Wellwarm shaker incubater (30° C) for a period of 45 mins as above, wells were washed three times for one min each with 200 µl PBSTincTw. After which a DAKO duet amplification system was used (DAKO Ltd, Angel Drive, Ely, Cambridge,UK; Cat no. K0492) to amplify the signal generated by bound tissue culture supernatant antibodies. Wells were washed as described above and 100µl of 3,3',5,5'- tetramethylbenzidene substrate (Sigma, Poole, Dorset UK; Cat. No. T-3405 and P-4922) was then added to each well. The reaction was stopped by adding 25µl of a 20% 1M H_2S0_4 solution to each well. Absorbance at

450nm was determined with a Biohit BP800 ELISA plate reader (Alpha Laboratories, 40 Parham Drive, Eastleigh, Hampshire, UK).

6.1.1.5 Monitoring onion downy mildew in air samples in relation to plant infection

Plants were exposed in an over-wintered plot (15 x 15 m) of bulb onions previously heavily infected with onion downy mildew. For each of the sampling periods five pots bait plants (Bulb onion c.v. Renate, 5 true leaves), each containing five plants (one plant located in each corner with an additional plant positioned centrally in each pot) which had been grown in the absence of disease, were positioned adjacent to the spore traps. After each 24 H sampling period, the plants were removed from the field, and placed in an environment of 100% humidity for 48 hrs. This fulfilled the environmental requirements for infection by dark leaf spot. The plants were then removed, dried and retained in a glasshouse, at a temperature of 12 - 14ºC for 21 days. Plants were visually examined for expression of disease and number of leaves infected and uninfected with onion downy mildew counted.

6.1.1.6 Monitoring onion downy mildew incidence within the field plot

A total of 20 plants were assessed in each plot for disease incidence (presence or absence of *Peronospora destructor*) on a leaf basis. At each assessment time the numbers of lesions on each leaf of each plant was counted. Crops to be examined at 7 day intervals and disease recorded on all leaves (leaf one was regarded as the oldest leaf on the plant at first assessment). The presence or absence of disease was recorded at each assessment time.

6.1.1.7 Micro-climate measurements

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

6.1.1.8 Prediction of spore production in the field

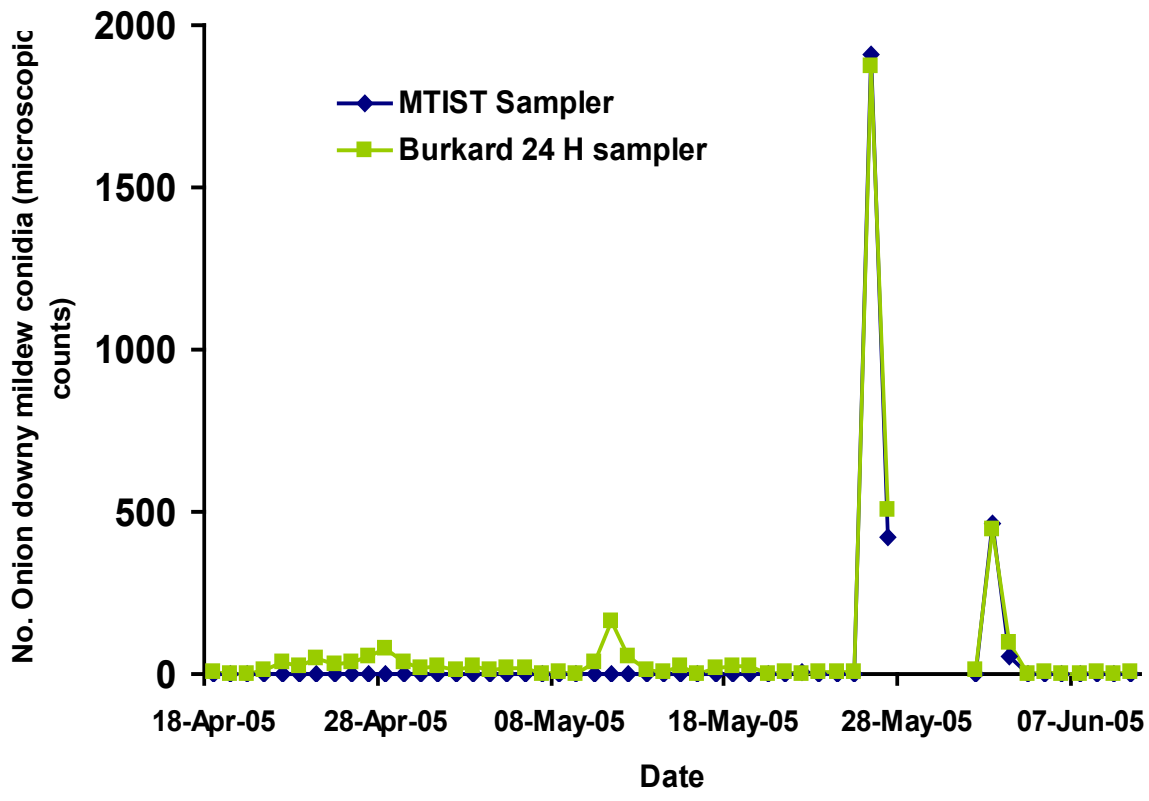
Disease forecasting models were used to predict when downy mildew conidia were produced under prevailing environmental conditions. The Warwick HRI onion downy mildew disease forecasting models (MILIONCAST) were used to predict the occurrence of infection, sporulation and time to symptom development. MILIONCAST uses 30 minute environmental summaries of temperature, humidity, leaf wetness duration and rainfall to calculate the rate and the occurrence of each life cycle stage. Environmental data was measured at 5 minute intervals using sensors positioned within the onion plot in the field. The Warwick HRI onion downy mildew models are summarised in Gilles Phelps, Clarkson & Kennedy, 2004.

6.1.2 Results

6.1.2.1 Comparison of onion downy mildew conidial counts using 24 H volumetric and MTIST samplers

The number of downy mildew conidia trapped using the Burkard 24 H glass slide sampler was compared to onion downy mildew counts taken within microtitre wells from the MTIST samplers using microscopic counts. Both air samplers were positioned in the crop at the same point and were air sampling over the same time period. The results show (Figure 11) that there was little difference in the numbers of onion downy mildew conidia trapped on most days regardless of the type of sampler used. However four distinct peaks in onion downy mildew conidial numbers were observed during the trial period (18 April to the 10 June 2005). These occurred on the 28 April 2005, 11 May 2005, 26 May 2005 and the 11 June 2005 using the Burkard 24 H sampler. The MTIST sampler showed peaks on the 26 May 2005 and the 11 June 2005. Significantly lower numbers of onion downy mildew conidia were observed during the early part of the trial period using the MTIST sampler in comparison to the Burkard 24 H sampler.

Figure 11 Comparison of the numbers of trapped onion downy mildew conidia in the field using a Burkard 24 H volumetric sampler and an MTIST sampler

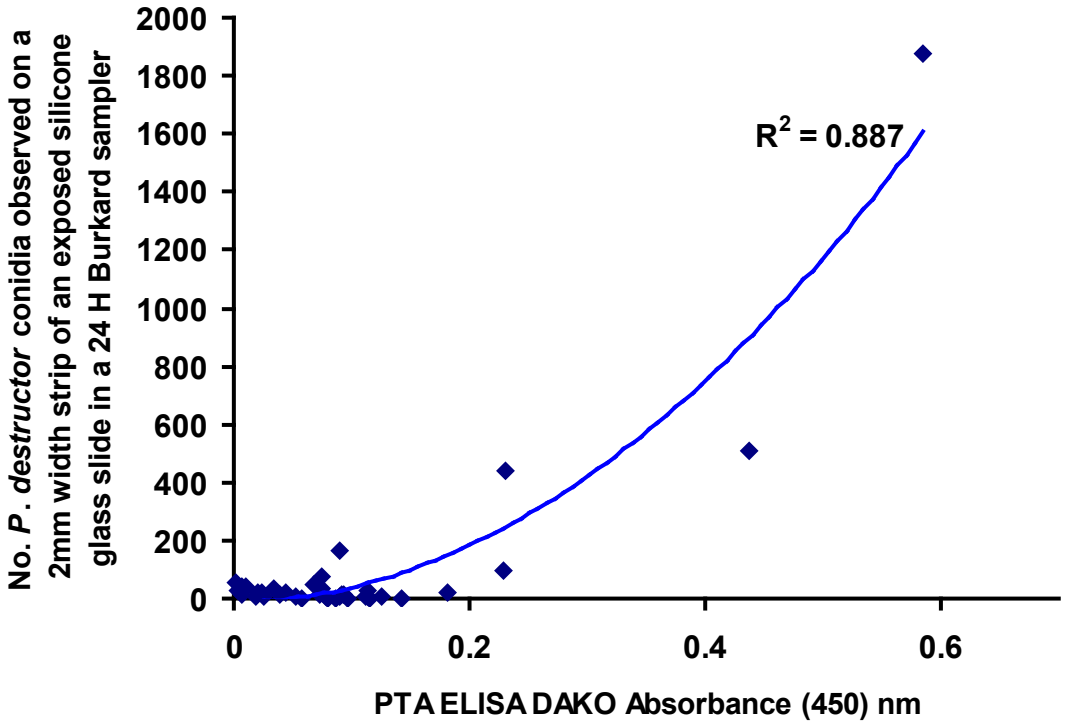


6.1.2.2 Comparison of onion downy mildew conidial counts using 24 H volumetric and MTIST samplers and MTIST microtitre well PTA ELISA

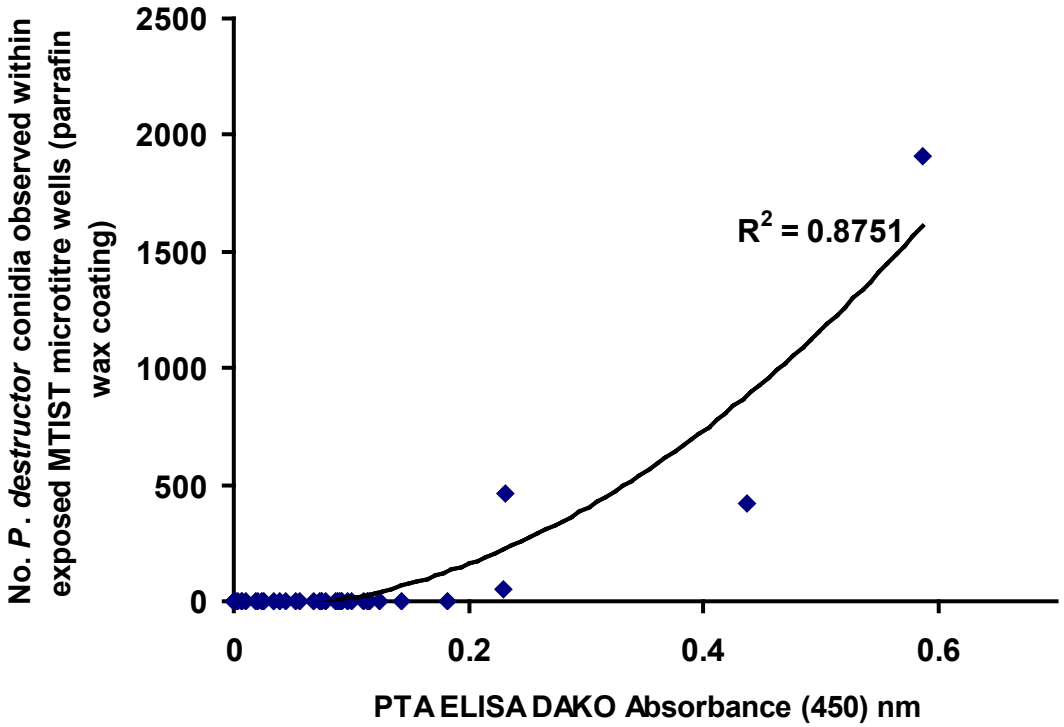
Comparison of the numbers of spores observed in each sampler from microscope counts against MTIST PTA ELISA of microtitre strips coated with paraffin wax hexane is shown in Figures 12a and 12b. Paraffin wax was used in these comparison because it proved to be the most effective well coating for retaining onion downy mildew conidia (see section 5.3) in comparison poly-l-lysine and silicone well coatings and an untreated control.

Figure 12 Relationship between PTA ELISA and number of onion downy mildew conidia trapped using (a) 24 H volumetric sampler (b) MTIST sampler

(a)



(b)

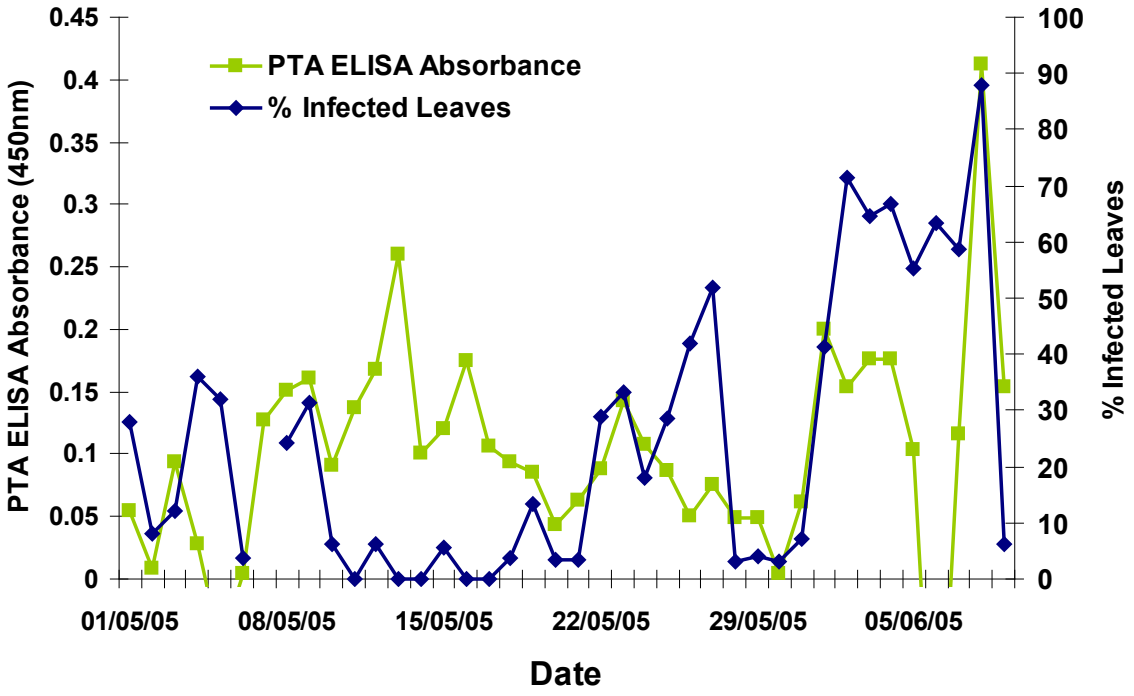


There was a good exponential relationship ($r^2 = 0.887$) between PTA ELISA absorbance and number of conidia of *P. destructor* trapped on a silicone coated glass slide using a Burkard 24 H volumetric air-sampler (Figure 12a). A similar relationship was observed ($r^2 = 0.875$) between PTA ELISA and number of conidia of *P. destructor* trapped within wells of a microtitre well coated with paraffin wax/hexane using an MTIST air-sampler.(Figure 12b). Absorbance values of 0.2 indicated that there were high number of onion downy mildew conidia present in the slide or in the microtitre well.

6.1.2.3 Comparison of onion downy mildew trap plant exposure and either PTA ELISA (MTIST) or onion downy mildew conidial counts (Burkard 24 H glass slide sampler)

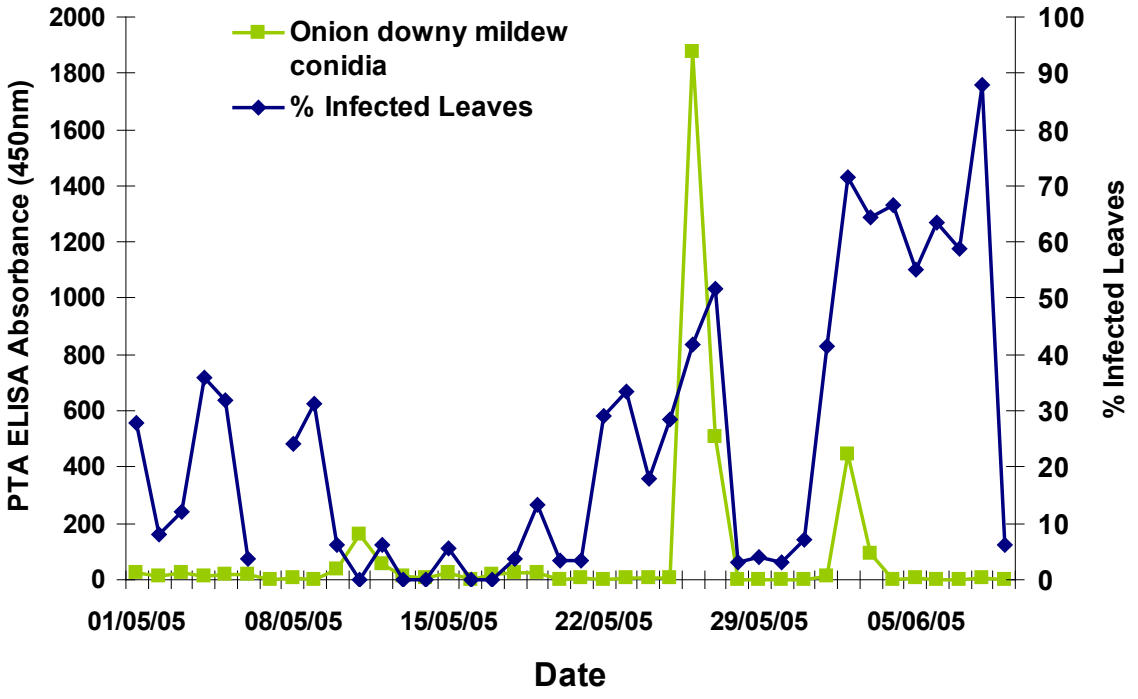
Plants were exposed for 24 H periods (which corresponded to the trapping period used for the air sampler) within the field plot prior to being given a 48 H wetting period within the glasshouse at approximately 16 C. Plants were then incubated for a 2 week period prior to being exposed to 24 H leaf wetting (within the glasshouse) and the presence or absence of sporulation recorded. Corresponding PTA ELISA values for each day could be compared to the % of leaves infected by onion downy mildew. The relationship between PTA ELISA and % leaf infection of plants exposed within the field plot is shown in Figure 13. During the trial most days that onion plants were exposed within the field plot resulted in some infection by onion downy mildew. However there was considerable variation in the degree of infection of exposed plants. Three periods of downy mildew activity were observed from exposed trap plants. These occurred between the 1 - 10 May 2005, 21 – 28 May 2005 and the 6 – 9 June 2005 (Figure 13)

Figure 13. Relationship between PTA ELISA and % Infected leaves of plants exposed for 24 H periods within the infected plot (before 48 H leaf wetness).



Comparisons between % infected leaf number and onion downy mildew conidial counts are shown in Figure 14. There was a poor relationship between onion downy mildew conidial counts and % leaf infection on exposed plants particularly during the early part of the trial period due to low conidial counts. Later peaks in conidial counts corresponded well with observations on trap plant infection (Figure 14). Infection on trap plants resulted from exposure to a 48 H wetting period in the glasshouse after the 24 H field exposure period. There were very few days during the trial period when rainfall occurred. Other forms of wetness although recorded were not significant (see section 6.1.2.4). For this reason the number of onion downy mildew required to initiate infection could not be ascertained.

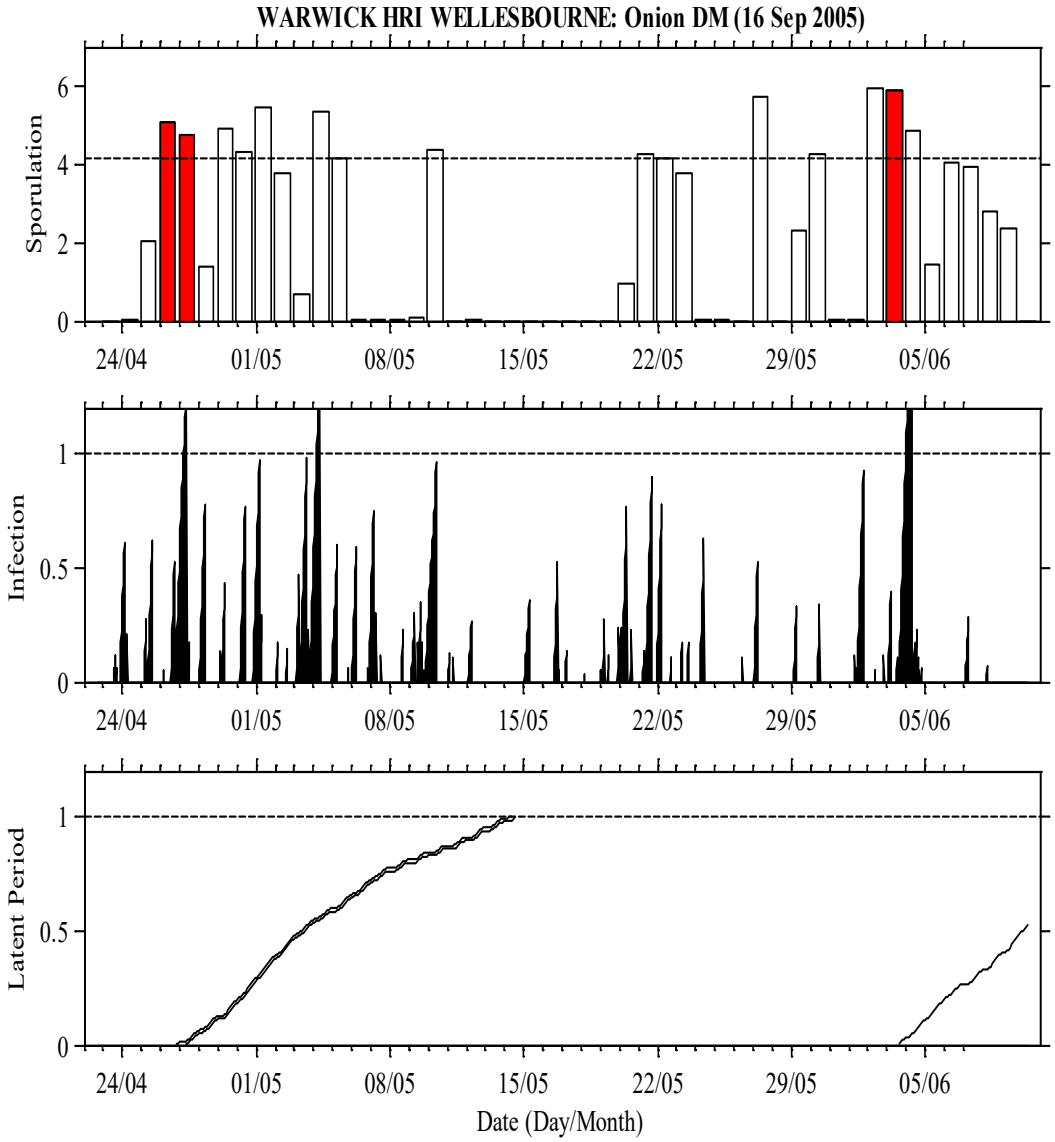
Figure 14. Relationship between % Infected leaves of plants exposed for 24 H periods within the infected plot (before 48 H leaf wetness) and onion downy mildew conidial counts (Burkard 24 H sampler).



6.1.2.4 Prediction of onion downy mildew conidial presence

The results of using the Warwick HRI onion downy mildew forecasting system are shown in Figure 16. There were many periods where onion downy mildew sporulation was predicted. Sporulation *P. destructor* was predicted if the bar representing each day (Figure 15) reached the threshold value of 4.15 as represented by the dotted line in Figure 15. According to the Warwick HRI forecasting system significant risk of downy mildew development occurred in the crop only when sporulation and infection conditions occur in combination. There were three periods (26, 27, April 2005 and the 3 June 2005) when significant risk of downy mildew development occurred in the crop. However sporulation was predicted as having occurred on the 26, 27, 29, 30, April 2005, 1, 4, 10, 21, 27, 30, May 2005 and the 2, 3, 4 June 2005. There was a close relationship between % infected leaves and predicted sporulation according to the Warwick HRI onion downy mildew forecasting system.

Figure 15. Forecasts of onion downy mildew sporulation, infection and time to symptom development during the trial period using the Warwick HRI onion downy mildew forecasting system

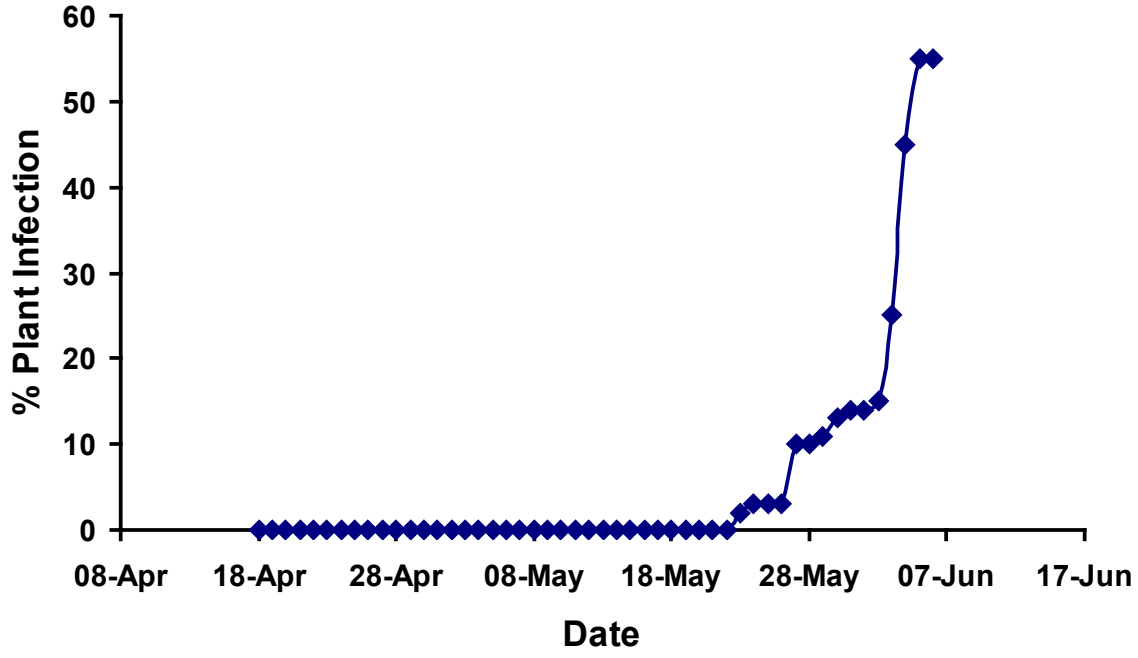


6.1.2.5 Onion downy mildew development within the field plot

The development of onion downy mildew, within a plot of over-wintered bulb onions (seed crop) was monitored during April, May and June 2005. Onion downy mildew trapping studies were also conducted within the same plot over this periods (see sections 6.1.2.2 and 6.1.2.3).

There was no onion downy mildew observed on plants within the plot until the 23 May 2005 Figure 16). Infected plants were observed at points on the outside rows of the plot initially. Onion downy mildew spread rapidly within the plot and many onions showed onion downy mildew symptoms during early June 2005 (Figure 16).

Figure 16. Onion downy mildew development (% plant infection) within an over-wintered crop of bulb onions at Warwick HRI in 2005.



6.1.3 Conclusion

Systems which can detect onion downy mildew conidia have been developed and used in the field within an over-wintered crop of bulb onions which grow during the spring to produce fresh susceptible growth and seeding heads. Over-wintered onion crops frequently carry onion downy mildew infection as latent infections which can occur within bulbs as oospores or infected tissues. Over-wintered bulb onions produce new growth in the spring which includes seeding heads. These are very susceptible to onion downy mildew infection. The crop however remains symptomless for long periods of time during its re-growth in the spring. The results of this trial demonstrate that onion downy mildew conidia could be detected within the crop for many weeks before symptoms were visible. Several peaks in onion downy mildew conidial numbers occurred during late April and early May well in advance of visible symptoms which were present at the end of May and early June 2005. Results show that peaks

of several hundred onion downy mildew conidia were necessary to initiate infection in the crop. This suggests that onion downy mildew exists within developing crops. The results suggest that there must be either atypical symptoms of onion downy mildew present which are unrecognised or a level of disease is present in many onion crops (especially those grown from onion sets) which is very difficult to detect visually. This also suggests that disease could also be initiated in many onion crops by threshold levels of inoculum from outside the crop. The results of the trial also confirmed the accuracy of the Warwick HRI onion downy mildew forecasting system. Cyclone samples collected during this trial will be used in conjunction with onion downy mildew lateral flow devices to determine suitable conidial thresholds for detection of onion downy mildew in the field. The results will be reported in year three of the project.

7.0 DISCUSSION

In the current project "in field" tests for conidia of onion downy mildew have been developed. Successfully developing these tests requires the development of specific antibodies, which can be used to visualise the presence of target inoculum (onion downy mildew conidia). The success of the system also depends on the ability of the trapping system to catch and retain the spores in sample tubes. These aspects of the work have been successfully carried out in year two of the work on FV189a.

7.1 Using air-borne spore numbers within disease forecasting systems

Airborne inoculum plays a vital role in the development of epidemics caused by *Botrytis* leaf blight on onion crops (Carisse, 2005). In this work a linear relationship was found between number of lesions on plants and the air-borne *Botrytis* conidial concentration. Air-borne conidia concentrations of 25 to 35 conidia m $^{-3}$ of air were associated with 2.5 lesions per leaf. When this system was used under field conditions to control *Botrytis* it led to a reduction in fungicide usage of 75 and 56 % in 2002 and 2003. It is likely that a similar relationship exists for onion downy mildew. One of the objectives of the work reported in this project is to determine the relationship between air-borne conidial concentration of onion downy mildew and disease appearance on plants. This information could then be used as the forecast criteria required which leads to disease in the field. Developing accurate tests which can be used to determine when and if growers are above or below this critical level would be a measure of the success of the project. The information could be used to reduce the number of badly timed fungicide applications reducing the possibility of the build up of fungicide resistance. Additionally it would allow greater reliance on protective sprays as fungicides would be applied in response to inoculum levels. This might mean that sprays could be applied in the absence of conditions which promoted infection thus improving the efficiency of fungicidal activity. It might also mean that using the system could improve the activity of weaker fungicide products or biological control agents. All of these outcomes would produce better control, at lower costs and help guarantee economic yields.

One of the disadvantages of the work on *Botrytis* was that the conidial counts could only be taken using microscopes. Therefore vessels from the traps used (rotarods) had to be sent back to the laboratory for processing which is costly and time consuming. By developing tests which growers and consultants use themselves this disadvantage could be removed. One form of "in field" test which could be used in this way is the lateral flow. An objective of the current work in year two was to develop the lateral flow format for detecting conidia of onion downy mildew.

7.2 Producing "in field" test kits for detecting onion downy mildew

An "in field" test kit for onion downy mildew conidia has been successfully developed in year two of this project. The competitive lateral flow format enabled the rapid detection of *P. destructor* spores within a sample however the other assays tested (double antibody sandwich format) were unable to detect onion downy mildew. Many fungal assays are based on antigen-trapped assays (competitive LFD format) where antibodies are used to label antigen rather than attach it to a solid phase. In the development of a successful test format the antigen concentration at the test line and activity of the specific antibody used is critical to optimal development of the test. The type and capillary flow rate of the membrane is also of importance. For the competitive LFD test a Millipore 135 HiFlow™ cellulose ester membrane direct cast on to 2ml Mylar backing proved most successful. The antigen line concentration of *P. destructor* spores in the range of 125 to 250µg protein ml^{-1} gave the most optimal test line when a gold conjugated antibody dilution range of between 1 : 160 to 1 : 320 was used. The next phase of the work requires that the tests are mass produced and some further work on these aspects will be required. For example the antibody will need to be conjugated with gold to form an immunogold complex. This will be required so that the test can be standardised to a specific cost as without this step the conjugate would have to be manufactured each time a batch of tests were required which might lead to variability. Prototypes with the final format will be available for year three testing in the field. Additionally cyclone samples taken during the trial in year two have been collected daily and stored at – 20 C. These can also be used in tests where the sensitivity of the LFD test can be determined as the number of onion downy mildew conidia detected.

7.3 Optimising the air sampling trapping format for onion downy mildew conidia

In the experiments described in this report spores were trapped in a range of collection vessels from slides within 24 H Burkard volumetric samplers to eppindorf tubes within cyclone spore traps. An important aspect of the work is to develop reliable methods whereby spores trapped within collection vessels within traps can be removed for measurement in tests. Additionally it would be important to know when it is best to trap conidia of onion downy mildew in the field. The ability of specific well coatings to improve the collection and retention of onion downy mildew conidia was tested using an MTIST sampler. In these studies either paraffin wax of silicone proved to be superior coatings for trapping and retaining spores in comparison to untreated wells (controls) and those treated with poly lysine. Downy mildew conidia are relatively large and it is therefore important that a trap vessel coating is used if spore samples are to accurately reflect those found in larger air volumes. Positioning of the trap is another consideration determining accuracy however this aspect could not be investigated within these trials. This aspect could be investigated by positioning more than one sampler with the same characteristics in the plot and comparing

the results. This aspect will also be investigated during the year three trials. Variability of trap readings within larger cropping systems would also need to be investigated.

7.4 Disease forecasting criteria based on inoculum detection

Using these techniques the critical date for applying fungicide applications to the crop can be identified. Disease development can also 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. This information would be required for larger cropping areas however it is unclear how this information can be applied to large onion crops.

One possible route might be to establish networks of traps (3- 4 traps) which could be applied to larger cropping areas. With high sampling rates these traps if positioned to reflect prevailing wind patterns could be used to designate to onset of disease risk in different areas and pinpoint specific transmission events affecting different crops and areas. 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.

Detecting onion downy mildew conidia would be particularly useful early in the season as a method of preventing disease transfer between over wintered salad onion crops and bulb onions grown as sets or as seeded crops. The approach is already being successfully used for controlling *Botrytis* on onion crops. Onion crops where thresholds of 15 - 20 conidia/m^3 could be used to reduce fungicide application by up to 50 – 75 % (Carisse *et al.,* 2005). The use of weekly inoculum estimates worked well in practice. However, weekly estimates were used because of the practical difficulties of determining numbers of onion downy mildew conidia using microscopes. This would not be problematical where rapid test formats existed for detecting onion downy mildew from traps in the field. Tests which, can be conducted in the field are necessary if information on air-borne inoculum concentration is to be of more practical value. As tests for pathogenic inoculum can be carried out in the field (by using lateral flow devices) the system meets the criteria necessary for its uptake by the onion industry.

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